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I am submitting herewith a dissertation written by James Edward Bond entitled "Transformation as a tool to study the genetics of nodulation in *Glycine max*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

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Transformation as a tool to study the genetics of nodulation in *Glycine max*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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James Edward Bond

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ABSTRACT

Transformation has proven a powerful tool to elucidate the function of specific genes in animal, plant and bacterial systems. Nodulation of *Glycine max* is the result of a complex symbiosis between the bacterium *Bradyrhizobium japonicum* and the plant. The infection of root hairs by nitrogen fixing bacteria and the subsequent nodule development is regulated by numerous genes in both the plant and the bacteria. Little is known about the underlying biochemistry of this symbiosis. However, this symbiosis offers an excellent model for studying the various mechanisms which control plant cell division and development. Plant transformation will help to elucidate the role of these symbiotic plant genes.

In this study methods were developed for the efficient transformation of a range of soybean nodulation mutants. The introduction of specific gene sequences has shown the ability to fundamentally alter nodule structure. Analysis of this alteration suggests changes in the nodule development are stimulated by extended cell divisions in specific areas of the nodule. This is the first time that transgenic soybean nodules have been produced or genetically altered.

Abnormal maintenance and normal integration of transgenic sequences was observed in the soybean plants. Transgenic sequences appeared both autonomously maintained and integrated into the genome. These events were routinely observed in the same plant depending on the DNA sequence transferred. The non-classical transformation events were typified by autonomously replicating plasmids in the plant cell. This is the first time that such an effect has been observed in soybeans; however this observation mirrors autonomous plasmid maintenance in the animal kingdom.

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INTRODUCTION

This dissertation is composed of seven discrete chapters. Each chapter has separate sections entitled introduction, methods, results, conclusion and references. This format is designed to make each chapter a publishable unit. Thus some repetition of references and methods is unavoidable.

Chapter 1 is an introduction to this particular area of research. It reviews the current information on the soybean-*Bradyrhizobium japonicum* symbiosis, plant transformation and the application of transformation to elucidating the plant genes involved in the symbiosis. Chapter 2 covers the development of regeneration protocols for the soybean nodulation mutants used in this study. Chapter 3 investigates the feasibility of using an *Agrobacterium tumefaciens* based transformation system to transform the soybean mutants. Chapter 4 investigates the feasibility of constructing and using a micro-projectile gene gun to transform the soybean nodulation mutants. The embryogenic suspension culture regeneration system developed in chapter 3 is used for transformation studies in this chapter. Chapter 5 investigates the use of *Agrobacterium rhizogenes* induced roots to introduce transgenic sequences into roots and nodules. The efficient production of transgenic roots and nodules and the rare altered nodule phenotype induced by the transfer of specific DNA sequences is studied. Chapter 6 investigates the role of chalcone synthase (CHS) expression on the nodule function and phenotype using the transformation procedure developed in chapter 5. The effect of CHS expression on the nodulation process is investigated by the introduction of anti-sense CHS constructs into the plant. Chapter 7 concludes the results from each chapter and discusses achievements, pit falls, and ideas for future experiments resulting from this study.

CHAPTER 1

TRANSFORMATION AS A TOOL FOR UNDERSTANDING THE GENETICS OF NODULATION IN *GLYCINE MAX*

INTRODUCTION

Nodulation of leguminous plants is the result of a complex symbiosis between *Rhizobium* and the plant. Infection of root hairs by nitrogen fixing bacteria and subsequent nodule development is regulated by numerous genes in both the plant and the bacteria. Little is known about the underlying biochemistry of this interaction (Caetano-Anollés and Gresshoff 1991). However, this symbiosis offers an excellent model for studying the various mechanisms which control plant cell division and development.

One of the ways to study this process is to genetically modify either the plant or the bacteria. More than fifty bacterial genes involved in nodulation have been identified and characterized in detail (see next section). Many of these genes were identified by genetic modification of the bacteria (e.g: transposon tagging, suicide plasmids and *lacZ* fusions) and subsequent observation of the mutated phenotype (Simon *et al.*, 1983a, 1983b). The ability to genetically modify the microsymbiont produced a 'boom' in the number of nodulation and nitrogen-fixation genes isolated and the knowledge of the genetic mechanisms of nodulation in the bacteria. To date only a few plant genes have been characterized to the same degree (see following section; Nap and Bisseling, 1990). There are many reasons for the limited progress in elucidating the plant genetics in this association. Plant are genetically and biochemically more complex. They have much larger genomes than bacteria. Plant cell walls pose technical difficulties for efficient DNA transfer into the plant cell. Unlike bacteria, plants are highly differentiated and have relatively long developmental periods between reproduction stages. This makes DNA transfer to germline cells difficult. Development of new and efficient techniques which allow genetic manipulation (transformation) of these leguminous plants have enabled us to study the genetics of nodulation at a new level.

This thesis covers research in the development of new transformation systems in the commercially important legume *G. max*, and the application of these transformation systems to the genetics of nodulation.

Legume nodulation

Physiological events leading to nodule formation

In general, each bacterial symbiont is only able to nodulate a limited range of host plants. Likewise, each leguminous plant is only nodulated by a limited range of bacterial microsymbionts. The limitation of host range is thought to be regulated at a number of levels in both the plant and bacteria. The best understood regulatory stage involves chemical signal molecules. The legume root exudes chemotaxants such as the flavonoids: luteolin, genestein, naringenin and daidzein into the rhizosphere. These chemotaxants stimulate specific host range genes in the nearby bacterial microsymbiont (Banfalvi *et al.*, 1988; Göttfert *et al.*, 1988; Kosslak *et al.*, 1987). If the signal molecule is correct then common nodulation genes are activated in the bacteria and infection of the root is initiated. For example, the isoflavone diadzein is exuded by the soybean root to attract the microsymbiont *Bradyrhizobium japonicum* (Kosslak *et al.*, 1987).

Bacteria in the Rhizobiaceae family possess flagella which propel them towards the plant root surface. Infection starts with attachment of the symbiotic bacteria to a root hair. Initial attachment of the bacterium is not specific. Host specificity may be determined later by the specific binding via lectins and exopolysaccharides (Bohlool and Schmidt 1974). Root hair curling is stimulated after bacterial attachment. An infection thread from the bacteria then grows through the root hair into the surrounding plant cells. Around the time of root hair curling, plant cells in the root are stimulated to divide (Goodchild and Bergerson, 1966; Turgeon and Bauer, 1982; Rolfe and Gresshoff, 1988). In determinate nodule types such as soybean, these cell divisions and meristem formation are initiated in the hypodermal cell layers (Calvert *et al.*, 1984). In indeterminate nodule types such as alfalfa, cell divisons are initiated in the root inner cortex (Dudley *et al.*, 1987; Libbenga *et al.*, 1973). Once the infection thread reaches an area of dividing cells individual bacteria are released from the infection thread into the cytoplasm of a cortical cell. These bacteria are enveloped by a plant-derived peribacteroid membrane (PBM) and differentiate into nitrogen fixing bacteroids. Cell division, differentiation and expansion continue until a fully differentiated nodule is formed.

The plant regulates the number, size and morphology of the resultant nodule structures. Some plant mutants are either unable to nodulate or arrest the process at the root hair curling or the first cell divisional stage (Carroll *et al.*, 1986). Other plant mutants are

unable to regulate the number of nodules formed on the root system (Carroll *et al.*, 1985a, b).

Different plant species show different nodule morphology. Plants such as soybean have a spherical or determinate type of nodule with no active meristem. Other plants such as alfalfa and clover have an indeterminate nodule. Indeterminate nodules appear elongated and have an active meristem in the mature nodule (Figure 1)(Noel *et al.*, 1986).

Bacterial genes involved in nodulation

Bacterial genes involved in the nodulation process can be broadly separated into genes involved in nodule formation (*nod* genes) and those genes which are involved in the nitrogen fixation process (*fix* and *nif* genes). The *nod* genes can be further divided into two major groups. Common *nod* genes (*nodD*, *A*, *B*, and *C*) which appear to be functionally and structurally conserved across the spectrum of *Rhizobium* and *Bradyrhizobium* species and host specific *nod* genes which confer specificity to the microsymbiont-host combinations. The common *nod* genes are transcribed as two transcriptional units; *nodD* and *nodABC*. The *nodD* genes are involved in host specific activation of the other common *nod* genes and are present in more than one allelic form. Products from the inducible *nodA*, *B*, *C* genes are responsible for root hair curling and cortical cell divisions in the hypdermal layers (soybean) or middle cortex (alfalfa, clover). The locations of these early developmental stages suggest that determinate nodules have a different ontogeny. A signal molecule released from *Rhizobium meliloti*, *Nod*Rm-1; which causes root hair curling and cortical cell divisions in alfalfa has been identified as a sulphated and acylated glucosamine oligosaccharide (Lerouge *et al.*, 1990). It is proposed that the common *nod* genes *nodABC* produce a common signal molecule which undergoes host specific modification (for example by *nodH* and *Q* in *R. meliloti*) in different microsymbionts (Faucher *et al.*, 1988).

Host specificity nodulation (hsn) genes *nodF*, *E*, *G* and *H* determine the specificity of the host-microsymbiont combination. These genes determine specificity for root hair curling (*nodH*), infection thread formation and the initiation of nodules (*nodF*, *E* and *G*). Mutations in these genes can either increase or decrease the host range of the microsymbiont (Long 1988).

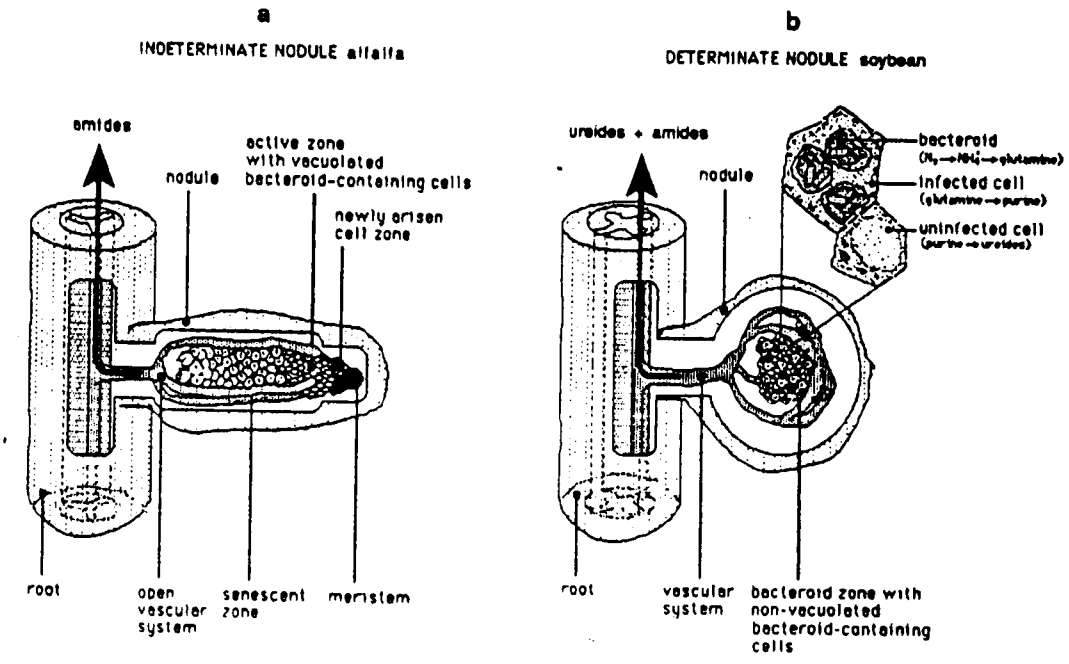


Figure 1. Examples of indeterminate and determinate nodule types. Indeterminate nodules such as those found on alfalfa (a) are characterized by a persistent active meristem and cylindrically shaped nodules. Cells produced from the meristematic region are colonized by nitrogen fixing bacteria. These cells develop into mature nitrogen fixing cells. The older nitrogen fixing cells eventually senesce. In general, the main export molecules from indeterminate nodules are amides. Determinate nodules such as those found on soybean roots (b) are spherical due to the early cessation of meristematic activity. Cells within the nitrogen fixing zone persist throughout the lifetime of the nodule without senescing. The main export molecules of determinate nodules are ureides and amides. (reproduced from Gerahty, 1990)

The final set of bacterial genes to be 'turned on' during nodule development are the nitrogen-fixation (*nif*) genes. At this stage the bacteria switch from a carbon metabolism mode to a symbiotic mode in which they receive carbon from the plant. Nitrogenase is the most important enzyme encoded by the *nif* genes and is responsible for converting free nitrogen into ammonia

Plant nodulation mutants

Mutagenesis of plants has proved to be a useful way of defining important genetic loci in the legume-*Rhizobium* symbiosis. A range of non-nodulating and supernodulating soybean mutants have been isolated by ethyl methane sulfonate (EMS) mutagenesis (Carroll *et al.*, 1985a, b, 1986; Buzzell *et al.*, 1990; Gremaud and Harper, 1989). The germplasm (M2 family selection) produced from such mutants has been used for physiological and developmental studies of nodulation. Genetic analysis of these mutants has shown that there are several plant loci involved in controlling nodulation. All nodulation mutants derived to date are controlled by single recessive Mendelian genes that affect different stages of the symbiosis (for review see, Caetano-Anollés and Gresshoff, 1991).

Several non-nodulating mutants have been isolated through mutagenesis or discovered as naturally occuring mutations. The first non-nodulating mutant rj1 was discovered by Williams and Lynch (1954). Non-nodulating mutants nod49, nod772 and nod139 were produced by chemical mutagenesis of seed (Carroll *et al.*, 1986). The block in infection in nod139 is at an early stage. In this mutant no infection threads or subepidermal cell divisions are observed in the inoculated root. Upon inoculation, the non-nodulating mutants nod49, nod772 and rj1 show subepidermal cell divisions. However, no bacterial infection threads are seen. This suggests that the block in nodulation in nod49, nod772 and rj1 is later than that seen in nod139. By complementation analysis nod 49, nod772 and rj1 were shown to be located at the same locus, whereas nod 139 was shown to belong to a new complementation group (Mathews *et al.*, 1989a). In experiments where wild-type shoot and root systems are grafted to non-nodulating mutant shoot and root systems, it has been shown that the non-nodulating phenotype is regulated by the root stock from which it originated and is not altered by a wild-type shoot (Delves *et al.*,1986, 1987a).

The supernodulating nitrate tolerant soybean mutants have the ability to form large numbers of nodules, up to 3-40 times that of the parent. This supernodulating phenotype is even seen in the presence of nitrate levels which would inhibit nodulation in the wild-type parent (Carroll *et al.*, 1985a, b). Thus the supernodulating mutants have been termed nitrate tolerant symbionts (nts). The nts mutation is recessive and unlinked to the non-nodulating nod49 and nod139 loci. If a plant is made homozygous recessive for the non-nodulating mutants and the supernodulating mutant (a double mutant) the supernodulating phenotype is epistatically suppressed. The resulting phenotype is that of a non-nodulating plant (Mathews *et al.*, 1990).

Experiments were conducted where the wild-type shoot and root systems were grafted to the supernodulating mutant root and shoot systems respectively. It was shown that the supernodulating or wild-type nodulation phenotype was regulated by the shoot. A supernodulating shoot grafted to a wild-type root produced a supernodulating root system (Delves *et al.*, 1987a, b).

When roots of an intact wild-type plant were separated into two compartments and one compartment inoculated, nodulation of that root branch strongly inhibits future nodulation of the other root branch. This inhibition of nodulation in the split root experiment is not seen in the supernodulating mutant even in the presence of nitrate (Olsson *et al.*, 1989).

Excision of nodules from a root system of a wild-type plant allows the development of replacement nodules in the mature root region (Nutman 1952). This indicated that the nodules induced a feed-back regulation in the plant. These results suggest that there is a shoot derived signal molecule (SDI) which regulates nodule formation systemically, (autoregulation) and that SDI is missing in the supernodulating soybean mutants.

The supernodulating or *nts* locus has been shown by RFLP analysis to be less than 1cM from the molecular marker pA132 (Landau-Ellis *et al.*, 1991). This is the first step towards isolation of the supernodulation gene by a reverse genetics approach. Any sequence that is found to be homologous to the *nts* locus will have to be tested via plant transformation. If a plant with a supernodulation phenotype is corrected to the wild-type phenotype by the addition of a specific DNA sequence from the wild-type plant, the function of that specific DNA sequence will have been proven. This type of experiment is equivalent to the classical complementation experiments performed in bacteria and yeast.

Plant genes involved in nodulation

Bacterial nodulation genetics is much better understood than plant nodulation genetics. Only a few genes that control nodulation and nitrogen fixation in legumes have been studied so far (Nap and Bisseling 1990; Sánchez *et al.*, 1991: Figure 2). Genes that are expressed either specifically or preferentially in nodule tissue are termed nodulins (*nod*). It is clear that many genes now termed nodulins have other functions elsewhere in the plant.

The nodulin genes are separated into early (*Enod*) and late nodulins (*nod*) depending upon where they are expressed in terms of nodule development. One of the most notable early nodulins in soybean is *Enod2* (N-75) which encodes two hydroxyproline-rich glycoproteins (HRGPs; Franssen *et al.*, 1987). This nodulin has been found in the nodule tissue of several legumes, but its sequence suggests that it is part of a large family of HRGPs expressed in various plant tissues (Nap and Bisseling 1990). The *in situ* hybridization of Enod2 transcripts shows the highest level of expression in the inner cortex of the nodule. It is also found in empty nodules produced by exopolysaccaride defficient bacterial mutants, pseudonodules induced by auxin transport inhibitors (Hirsch *et al.*, 1989; Van de Wiel *et al.*, 1990) and spontaneous nodules formed in the absence of *Rhizobium* spp (Truchet *et al.*, 1989).

Other HRGPs produced during infection thread development are Enod5 and Enod12. Enod5 is a hydroxyproline rich cell wall protein and is only expressed in cells with actively growing infection threads. Enod12 is an arabinogalactan-related protein and is expressed in all cells of the nodule primordium. Application of purified extra cellular factor NodRm1, from *R. leguminosarum* induced expression of Enod 12 in pea root hairs (Nap and Bisseling 1990).

Four other soybean nodulins (N-75, N44, N41 and Enod40) are also expressed early during the formation of the nodule primordia (first cell divisions). Their expression is thought to be related to nodular meristematic activity.

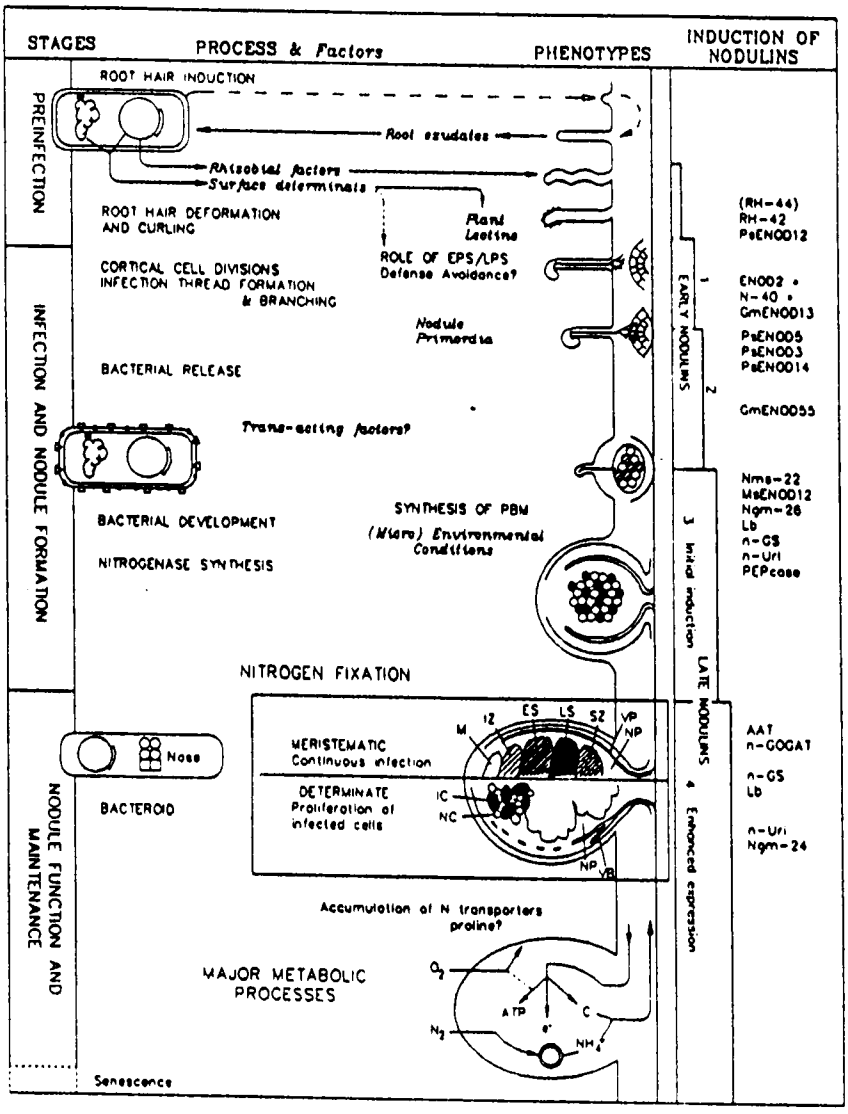


Figure 2. Schematic representation of developmental and metabolic events associated with nodulin expression. Distinct stages, processes and factors (*left* and *center*) are shown in relation to nodule developmental phenotypes. The figure also depicts the sequence of expression of nodulins and nodule-enhanced activities (right) in an arbitary time scale. At least four steps in the nodulin induction are indicated (1-4), with representitive examples of several species (* = ubiquitous nodule type). Nodule types are represented in the middle (MERISTEMATIC: M = meristem; IZ = infection, ES = early sybiotic, LS = late symbiotic and SZ = senescing zones; VP = vascular bundle; NP = nodule parenchyma. DETERMINATE: IC= infected and NC = uninfected cell; VB = vascular bundle; NP = nodule parenchyma/inner cortex.) Major metabolic processes (bottom) are described as linked pathways of carbon, nitrogen, and oxygen metabolism, in which several nodulins participate. Figure and legend produced from Sánchez *et al.* (1991)

Many nodulins expressed late in the infection process are key polypeptides in the soybean-*Rhizobium* symbiosis. Nodulins found in mature nodules include uricase (Nguyen *et al.*, 1985), leghemoglobin (Hyldig-Nielsen *et al.*, 1982), sucrose synthetase (Thummler and Verma, 1987), glutamine synthase (Sengupta-Gopalan and Pitas, 1986) and proteins of the peribacteroid membrane (Katinakis and Verma 1985).

Other genes not specifically considered nodulins are thought to have a role in nodule development. Phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) are key enzymes in the phenylpropanoid pathway. Both genes are multi-gene families and the compounds synthesized in this pathway have various roles in plant growth and development. These include lignins which provide structural integrity; flavonoid pigments for flower and leaf color and UV protection, and phytoalexins which are anti-micriobial substances used for plant defense. Phenylpropanoid compounds such as the flavonoids, luteolin, genestein, naringenin and daidzein act as signals molecules (chemotaxants) to *Rhizobium* spp during the infection/nodulation process in leguminous plants (Kosslak *et al.*, 1987; Caetano-Anollés *et al.*, 1988).

Estabrook and Sengupta-Gopalan (1991) studied the differential expression of PAL and CHS during nodule development. They demonstrated that subsets of both gene families are induced after specific infection by *Bradyrhizobium japonicum*. This subset (PAL and CHS), induced early in the symbiosis, are different from gene family members induced in response to stress or pathogen attack. CHS and PAL expression was shown to increase in the one to four day post infection period. Over this period the supernodulating mutant nts382 (Carroll *et al.*, 1985a, b) showed a greater increase in CHS and PAL levels than did its wild-type parent cv Bragg.

It appears that the enzymes in the flavonoid pathway are more active post-inoculation in the supernodulating mutant nts382 than in Bragg. Because of the complexity of this pathway this could mean the involvement of one or more compounds. Kapulnik *et al.* (1987) showed a direct correlation between the amount of flavonoids exuded from the roots of alfalfa and the number of nodules subsequently formed. They also showed that direct application of luteolin (the flavonoid inducer of *R. meliloti nod* genes) to the rhizosphere of alfalfa resulted in increased nodulation. These results indicate that flavonoid biosynthesis may be a component of autoregulation in legumes.

However, Sutherland *et al.* (1990) studied the flavonoid content of the root and root exudates of nts382 and Bragg. No significant variation in the ability to induce a nod box-*lacZ* fusion was observed between inoculated and uninoculated roots of cv. Bragg and the supernodulating mutant nts382. Mathews *et al.* (1989b) showed that 3 day old seedlings of the soybean cv. Williams possesed a 10 times greater ability to induce *Bradyrhizobium* nod genes than 3-day old seedlings of cv. Bragg. However, mature plants of both cultivars had the same nodulation frequency.

Van Brussel *et al* (1990) proposed a model for the *V. sativa-R. leguminosarum* symbiosis. In this model bacterial nod genes are initially induced by the plant. Upon infection of the root, bacteria produce factors which signal the plant to make more flavonoids. Estabrook and Sengupta-Gopalan (1991) postulate that the additional flavonoids are involved in maintaining nod gene expression while the infection progresses.

Flavonoids are known to perform other functions in plants. Jacobs and Rubery (1988) proposed that flavonoids act as natural auxin transport inhibitors. Auxin transport inhibitors have been shown to elicit nodule-like structures on alfalfa roots (Hirsch *et al.*, 1989). Some phenylpropanoid derivatives have been shown to have cytokinin-like activities (Binns *et al.*, 1987). Cytokinins have also been implicated in nodule initiation (Long and Cooper 1989). Thus it is possible that the secondary flavonoids produced as a result of infection function by affecting hormone distribution and are involved in cortical cell proliferation.

An additional level of regulation may exist in the phenylpropanoid pathway. Phytoalexin (antimicrobial) compounds are also produced in this pathway. The important phytoalexin in soybean is glyceollin (I, II and III; Ribereau-Gayon 1972). Work by Parniske *et al.* (1991a, b) has shown that the glyceollin I is important in limiting the expansion of bacterial infection zone during nodule development. It is clear that the phenylpropanoid pathway is important in the Legume-*Rhizobium* symbiosis but to date we are a long way from understanding the role of these genes or their biochemical functions.

Transformation of soybean

Gene transfer for the improvement of soybean

Soybean (*Glycine max*) is one of the world's most important agronomic crops. As such it has been the focus of extensive efforts towards genetic improvement both by conventional breeding techniques and genetic engineering. Conventional breeding has yielded impressive improvements in overall yield, oil composition, protein content, and resistance to disease. However, as in many other highly bred crop plants, modern soybean cultivars are based on a very limited gene pool. North American cultivars are derived from only 11 introductions (Allen and Bhardwaj, 1987). The vulnerability of crops to genetic uniformity was demonstrated in 1970 during the Southern corn leaf blight. In the near future the majority of genetic variation available for continued progress in soybean breeding (especially yield increase) will come from collections maintained in gene pools and cultivars in current production (Harlan, 1975; Hawkes, 1983). However, only limited variation exists in areas such as disease and pest resistance as well as drought and salt tolerance.

Biotechnology promises other routes for genetic improvement in these areas. Somaclonal variation from tissue culture of plants can be one useful source of genetic variation (Larkin *et al.*, 1984). The culture procedure offers an efficient selection system for traits such as disease and herbicide resistance. A second route involves the use of tissue culture coupled with transformation of plants to introduce new genetic material. Transformation overcomes sexual barriers for the introduction of new genes from different plant genera or other kingdoms. The theoretical increase in genetic diversity of a crop plant is limitless. Transformation is not only useful in the addition of new genes but also in the manipulation of endogenous genes, and characterization of unknown genes. These methods will lead to better genetic, molecular and biochemical characterization of crop plants, enabling more precise manipulation and overall genetic improvement.

Progress in soybean transformation

Soybean has historically been a difficult crop to transform. Early attempts to transform soybean were confounded by difficulties in tissue culture as well as the limited susceptibility of cultivars to the classical plant transformation agent, *Agrobacterium tumefaciens*. Transformation via electroporation of naked DNA into protoplasts was also

hindered by the inability to regenerate whole plants. Soybean transformation has only become possible after a long and difficult commitment from many research groups attempting to find suitable culture and gene transfer techniques. This review will describe the early tissue culture work that was so important in developing successful gene transfer procedures, and the transformation methods and applications currently in use.

Tissue Culture of Soybean

In the past few years various tissue culture systems have been developed for soybean. These include shoot morphogenesis from primary leaves, cotyledonary nodes and epicotyls (Barwale *et al.*, 1986; Wright *et al.*, 1986, 1987a,b), or somatic embryogenesis from immature zygotic embryos (cotyledons or embryo axis) (Christianson *et al.*, 1983; Ranch *et al.*, 1985). The cotyledonary node regeneration system was used by Hinchee *et al.* (1988) to transform soybean plants using *Agrobacterium tumefaciens*. However, this system was hampered by low efficiency due to the infrequent transformation of regenerable cell types. The application of protoplast culture to the field of soybean transformation was slowed by the inability to regenerate whole plants. Lin *et al.* (1987) and Christou *et al.* (1988) demonstrated transformation of soybean protoplasts and the production of transgenic calli but were unable to regenerate plants. Dhir *et al.* (1991) since reported plant regeneration from protoplasts of soybean, but the frequency of regeneration was low. It was not until 1992 that Dhir *et al.* regenerated whole transgenic plants from protoplasts. This system has limitations due to the inefficiency of the regeneration system and its genotypic dependency.

Initiation of embryogenic suspension cultures (cv. 'Fayette') and subsequent regeneration of whole plants has been achieved from immature zygotic embryo axes (Christianson *et al.*, 1983) and immature cotyledons (Finer and Nagasawa, 1988). These suspension cultures were initiated by culturing immature cotyledons to produce embryogenic cell clumps capable of producing small secondary embryoids. This tissue was used to initiate embryogenic suspension cultures termed 'highly embryogenic ontogenetic stage' ('early staged') suspensions. However, it has been reported that the first transgenic plants regenerated from these cultures were sterile. Regeneration systems such as the embryogenic supension (Finer and Nagasawa, 1988) and protoplast regeneration systems (Dhir *et al.*, 1991) are prone to produce regenerant plants showing somaclonal variation. Larkin *et al.* saw somaclonal variation in tissue culture derived plants as early as 1984.

This is thought to be a problem associated with tissue in long term culture. Freshly initiated suspensions of cv. Fayette gave fertile plants (pers. comm. J. Finer, Ohio State University).

This study has initiated embryogenic suspension cultures of cv. Bragg and its derived supernodulating mutant nts1007. Whole fertile plants have been regenerated from these cultures. Bailey and Parrott (1992) also reported initiation of embryogenic suspension cultures and regeneration of fertile plants from cv. Century, Davis, Lee, Hutcheson, Peking and line PI 417138.

Embryogenic suspension cultures have proven to be efficient regeneration systems when coupled with particle gun transformation (Finer and McMullen, 1991). The reasons for the success with embryogenic suspension cultures is twofold. First, embryo development is initiated from single surface cells of older embryos which are readily accessible for particle gun bombardment. Second, the suspension conditions and the descent of subsequent embryos allows efficient selection of transgenic material via antibiotic selection. This is in contrast to the mixed results reported with bombardment of meristem tissue. McCabe (1988) reported regeneration of whole chimeric transgenic plants. Buising *et al.* (1990) reported that their extensive efforts bombarding meristematic tissue did not yield transgenic plants. These mixed results highlight the difficulty of transforming the meristematic cells responsible for regeneration and germ line transfer.

Agrobacterium tumefaciens transformation

Agrobacterium tumefaciens has been used successfully to transform a wide range of dicotyledonous plants (Figure 3). This has been achieved by utilizing disarmed vectors to insert foreign genes (Bevin, 1984). However, susceptibility of different species and even cultivars to *Agrobacterium* varies greatly. Soybean is not very susceptible to *Agrobacterium* (DeCleene and DeLey, 1976).

The compatibility or virulence of a specific *Agrobacterium* is dependent on the genetic background of both host and pathogen. Thus much of the work in the early and mid-eighties concentrated on finding compatible *Agrobacterium*/plant associations. DeCleene and DeLey (1976), Pedersen *et al.* (1983), Owens and Cress (1985), and Bryne *et al.* (1987) all showed that induction of tumors in soybean tissue was possible, but showed significant variation between cultivars.

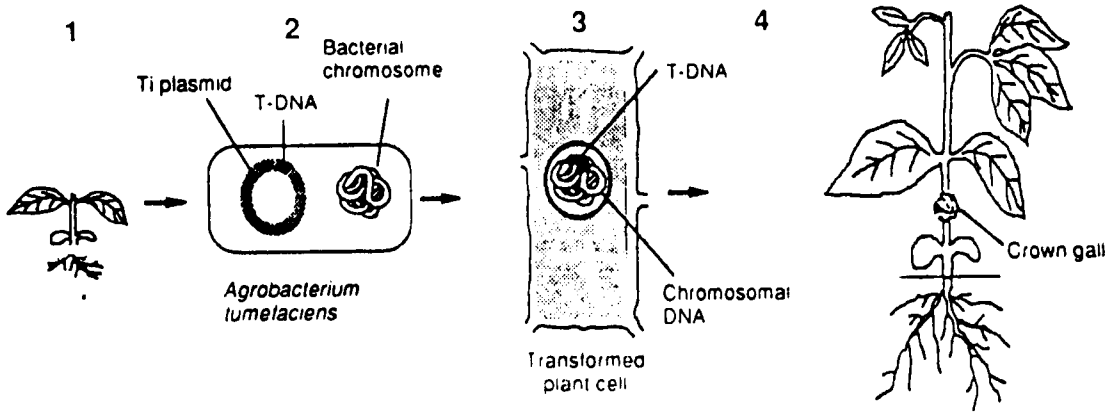


Figure 3. Transformation of plant tissue by the plant pathogen *Agrobacterium tumefaciens*. A virulent bacterium enters the plant at a wound site(1). The *Agrobacterium* harbors a Ti plasmid in addition to the bacterial chromosomal(2). A region of the Ti plasmid called the T-DNA is transferred into the plant cell. This becomes integrated into the plant chromosome(3). This transferred DNA encodes genes for the production of cytokinins and opines. The cytokinins induce cell divisions which eventually form a gall or tumor. In *Agrobacterium rhizogenes* auxin genes or genes which make the plant more susceptable to auxins are transfered. These genes stimulate the production of roots instead of galls. The opines serve as metabolites for the *Agrobacterium* living in the intercellular spaces of the gall.

Facciotti *et al.* (1985) were able to introduce a light inducible chimeric gene into soybean tissue but could not regenerate plants.

Byrne *et al.* (1987) screened a large number of soybean genotypes and found cultivar Peking highly susceptible to the *Agrobacterium tumefaciens* nopaline strain A208. The soybean research group at Monsanto engineered a disarmed A208 strain which could be modified to harbor marker and selection genes either integrated into the Ti plasmid or located on a separate binary plasmid (Hinchee *et al.*, 1988). This vector was used to inoculate soybean petioles which were induced to produce multiple shoots as in the Barwale *et al.* (1986) and Wright *et al.* (1986a, b) culture procedures. It was soon found that the disarmed engineered A208 strain had much lower virulence than the non-disarmed strain making transformation *in vitro* and regeneration of whole transgenic plants difficult. An extensive tissue culture effort was required to overcome this problem This was compounded by the rarity of transformation and regeneration events occurring in the same cell types.

Eventually, successful transformation of whole plants was reported with the susceptible soybean cultivar Peking (Hinchee *et al.*,1988). Low transformation frequency (4% of plants regenerated through selection) and difficulties with reproducibility in other research groups coupled with the limited application to cultivars other than cv. Peking have suppressed the use of this system. Nevertheless, Townsend *et al.* (1992) also reported successful transformation of soybean using this system.

Chee *et al.* (1989) reported transformation of soybean cv. A0949 by infecting germinating seeds with *Agrobacterium tumefaciens* strain C58Z707. However, inheritance of the neomycin phosphotransferase (NPT II) gene was extremely infrequent (0.07% of infected seedlings showed inheritance to the R1 generation). Zhou and Atherly (1990) reported transformation of the maize controlling element (Ac) into soybean plants but inheritance to the progeny was not demonstrated. This work was repeated by Muhawish and Shoemaker (1992; including molecular evidence for inheritance).

Problems associated with Agrobacterium transformation systems

Agrobacterium naturally invades plant tissue and persists in the intercellular spaces and vascular tissues of the plant. This can complicate the detection of true transformation events when using this pathogen for gene transfer. Most commonly used marker and

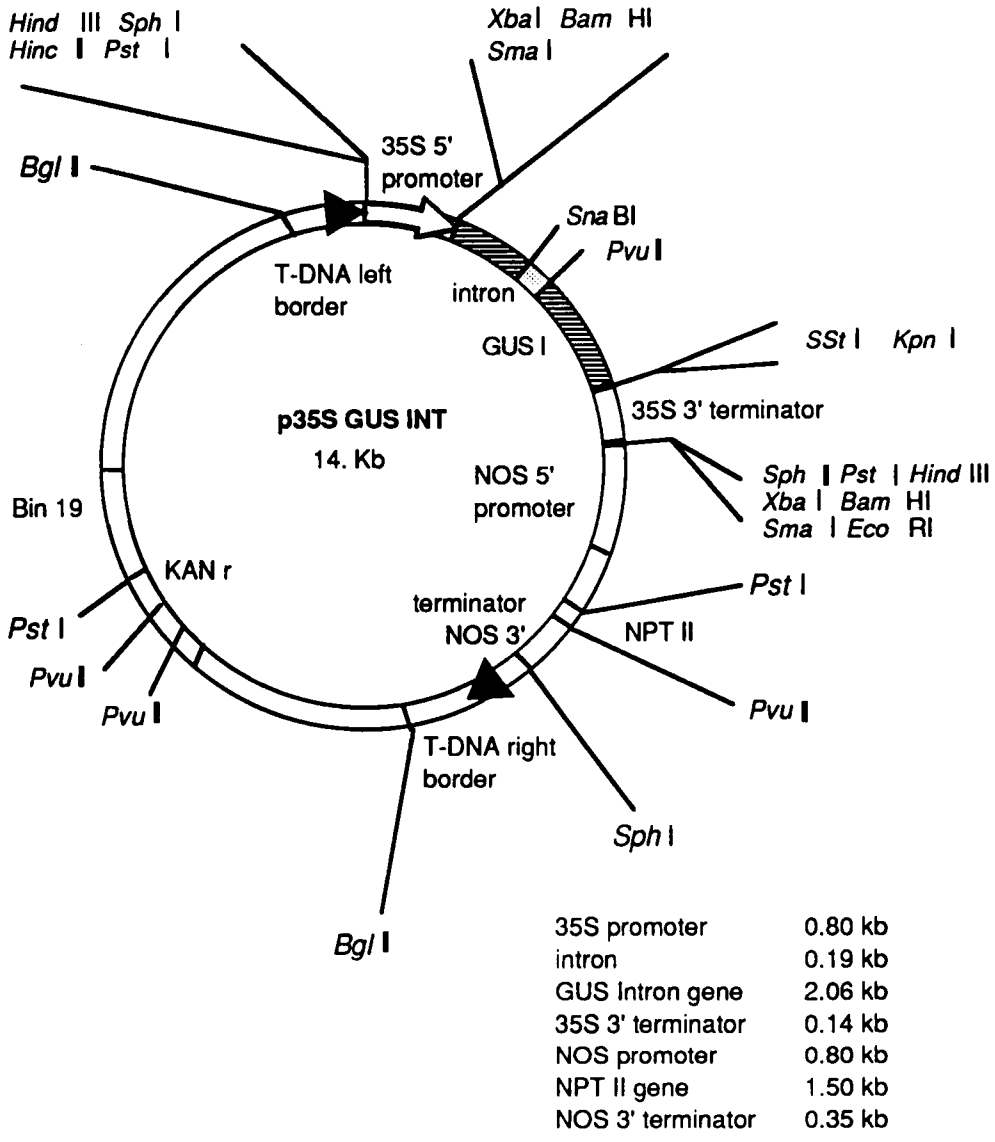
selection genes (GUS: ß-Glucuronidase; CAT: Chlorophenicol acetyltransferase; NPT II: Neomycin phosphotransferase II, HYGRO: Hygomycin phosphotransferase) are positioned within the T-DNA borders and driven by eukaryotic promoters. Initially it was believed that the genes within these borders were not transcribed or translated in the *Agrobacterium*. Also it was assumed that the eukaryotic promoters did not function in the prokaryote. Both of these postulations were later found to be false (Vancanneyt *et al.*, 1990). *Agrobacterium* has clearly been shown to express most commonly used marker and selection genes giving false positives in most tests and assays for transgenic tissue. With this in mind, any report of transformation should be reviewed critically.

The problems associated with assays that could not differentiate between bacterial contamination and true transformation events were overcome in two ways. First, many research groups turned towards particle gun gene transfer. Second, other groups looked for ways to modify marker or selection genes to eliminate bacterial expression. One such gene that has been modified is the ß-glucuronidase gene (GUS). GUS has widely been used as a reporter gene in transformation systems (Jefferson 1987a, b), but it has also suffered from the ability of *Agrobacterium* to transcribe and translate the gene (Vancanneyt *et al.*, 1990).

Vancanneyt *et al.* (1990) introduced a plant intron in the coding region of the GUS gene (Figure 4). *Agrobacterium* lacks the RNA splicing apparatus required to remove this intron and produce an RNA transcript that can be translated. Thus the bacteria are not able to express GUS activity. Only when the T-DNA harboring the GUS gene is transferred to the eukaryotic cell will the GUS gene be expressed. This construct is now used extensively to test for transformation. To date there have been no published reports that show bacterial splicing of the gene and expression of GUS activity.

Southern hybridization of transgenic material

Southern hybridization is an important technique to demonstrate the integration of foreign DNA in transgenic plant tissue (Southern, 1975). However its use in *Agrobacterium tumefaciens* transformation must be viewed critically. Many reports show Southern blots where the plant genomic DNA is restricted with enzymes that cut twice inside the T-DNA borders of the inserted DNA. When probing the plant DNA with sequences internal to the T-DNA region a single or double band identical to one obtained from a plasmid digest will be obtained. This shows that the sequence is present but does not indicate whether



35S promoter	0.80 kb
intron	0.19 kb
GUS Intron gene	2.06 kb
35S 3' terminator	0.14 kb
NOS promoter	0.80 kb
NPT II gene	1.50 kb
NOS 3' terminator	0.35 kb

Figure 4. p35S GUS intron plasmid: a disarmed binary vector for use in soybean transformation. Restriction mapping performed by Natasha Taranenko (PMG, UT. Knoxville).

the introduced DNA is integrated, non-integrated, or is due to bacterial contamination. The correct procedure for Southern analysis is to probe the Southern blot with a border fragment. This means using a sequence that crosses a T-DNA border (preferably the right border as this border is essential for T-DNA transfer).

A restriction enzyme must be chosen which cuts once inside the T-DNA border and once outside the border. The genomic DNA should be cut with the same enzyme or enzymes. For example in the p35S GUS I plasmid (Figure 1), this can be achieved by probing the blot with the *Bgl*II *Pvu*II fragment which contains part of the DNA from outside the T-DNA border as well as the 35S promoter and the GUS I gene. The genomic plant DNA should be digested with the same enzymes (*Bgl*II and *Pvu*II). If hybridization is seen only at 1.5 kb this shows either that there is bacterial contamination of the cultures or the plasmid is present in the plant cell but is not integrated. If hybridization is seen at different sized bands ie. any larger or smaller than the expected 1.5 kb band, integration of the T-DNA into the plant genomic DNA would be proven. This is because the *Bgl*II site just outside the T-DNA should not be transfered but other *Bgl*II sites in the genomic plant DNA would be variable depending upon the transgene integration site in the plant DNA.

Because many plants including soybeans show low susceptibility to *Agrobacterium* research has been undertaken on the induction of the virulence genes of *Agrobacterium* with specific media (Alt-Mörbe *et al.*, 1989., Ankenbauer, 1990) as well as isolating, duplicating and increasing expression of virulence genes (Jin *et al.*, 1987).

Agrobacterium rhizogenes transformation

Agrobacterium rhizogenes is similar to *Agrobacterium tumefaciens*, but instead of being the causal crown gall disease it is the causal agent of hairy root disease. This bacterium is virulent on a wide range of dicotyledonous plants (De Cleene and De Ley, 1981). It transforms plants by insertion of a region of DNA called T-DNA (transfer DNA) from the root-inducing (Ri) plasmid into the plant genome (Chilton *et al.*, 1982; for review of the molecular biology of the Ri plasmid see Sinkar *et al.*, 1987).

The *A. rhizogenes* strains are classified by the opines which are synthesized by genes in the Ri T-DNA. Opines which are conjugated amino acids and carboxylic acids are synthesized by these genes and serve as metabolites for the *Agrobacterium*. (Petit *et al.*,

1983). To date four different opine types have been found in *A. rhizogenes* induced roots. These are agropine, mannopine, cucumopine and mikimopine (Davioud *et al.*, 1988; Filetici *et al.*, 1987; Isogai *et al.*, 1988; Petit *et al.*, 1985).

In strains of *A. rhizogenes* that produce agropine the T-DNA is split into two regions T_L and T_R, each approximately 15-20 kb in size. Both T-DNA regions are integrated separately into the plant genome (Phelep *et al.*, 1991). Agropine strains have both the agropine synthesis and auxin indole acetic acid (IAA) synthesis genes referred to as *aux1* and *aux2* or tms1 and *tms2* on the T_R segment. Cucumopine and mannopine *A. rhizogenes* strains have only one T-DNA region between 34 to 42 kb in size.

The function of rol genes in Agrobacterium rhizogenes transformation

The T_L segment of the agropine strain contains 18 open reading frames consisting of 255 nucleotides or more (Slightom *et al.*, 1986). Transposon mutagenesis of this region has revealed the presence of at least four genetic loci (*rolA, rolB, rolC* and *rolD*) which also affect root induction. The *rol* (rooty loci) and *aux* genes are responsible for root induction either individually or together depending on the plant species or type of tissue (White *et al.*, 1985).

Interestingly cucumopine and mannopine *A. rhizogenes* strains do not have *aux* genes only *rol* genes and yet they still produce a hairy root phenotype. Also, although *aux* genes have been identified in agropine *A. rhizogenes* strains, it has been shown that their presence is not required for hairy root induction in tobacco (Vilaine *et al.*, 1987). In fact it has also been shown that the entire T_R fragment is not required for hairy root production. Transgenic lines elicited by the agropine-type *A. rhizogenes* strains do not always synthesize agropine (Petit *et al.*, 1983). In these cases it seems that sufficient auxin is supplied by the plant and that the *aux* genes may play a role in supplementing auxin only when it exists in limiting amounts (Grierson and Covey 1988).

The *rolA, rolB, rolC* and *rolD* loci affect virulence and hairy root formation. Originally it was thought that the *rol* genes synthesized auxins causing the characteristic hairy roots similar to the way that *Agrobacterium tumefaciens* initiate callus production by transforming cytokinin synthesizing genes (Zambryski *et al.*, 1989). It now appears that the mechanism is much more complex. Shen *et al.* (1988) reported that transformed roots of this type contained less auxin but were 10 to 100 times more sensitive to the presence

of auxins than normal roots. Because these roots did not show altered, auxin-independent proton excretion, altered ethylene sensitivity or increased auxin uptake, they suggested that the transformed genes affected the auxin receptor-transduction system. Shen *et al.* (1988) proposed that increased auxin sensitivity induced root induction. This result is supported by the work of Maurel *et al.* (1991) with tobacco protoplasts. They reported that sensitivity to auxin by transformation of single *rol* genes could be increased 30 fold by the addition of all the *rol* genes on the T_L-DNA segment of an agropine strain.

Roots are known to be initiated by endogenous concentrations of auxins. However, Croes *et al* (1989) detected no differential auxin sensitivity between normal and transformed root cultures. Quattrochio *et al.* (1986) showed that anti-auxins had an inhibitory effect on the growth of transformed potato roots. Cardarelli (1987) and Spena *et al.* (1987) have demonstrated that when *rolA*, *rolB* and *rolC* genes were expressed individually, transgenic roots were still produced but at an attenuated level. The effects of each *rol* gene on root initiation and growth are different (Schmülling *et al.*, 1988, 1989). The *rolB* and *rolC* genes seem to be involved in root branching, but the level and region of their expression are different. Expression of *rolB* is in the root cap whereas *rolC* is expressed in the phloem area (Schmülling *et al.*, 1988). Mutations in the *rolA* gene (*rolA*$^-$) result in the formation of long straight roots. A mutation in *rolB* eliminates both callus and root formation at the wound site. *The rolC* and *rolD* mutations are more subtle. Root growth is retarded with a *rolC* mutation, whereas a *rolD* mutation gives rise to *Agrobacterium tumefaciens* like tumors (White *et al.*, 1985). The primary determinate in the severely wrinkled leaf phenotype seen in *A. rhizogenes* transformed plants is the *rolA* gene (Sinkar *et al.*, 1987).

The rol gene proteins

Estruch *et al.* (1991) studied the *rolC* peptide and found that it was a non-transported 20kDa cytosolic protein. Plants expressing this protein showed dwarfing and reduced apical dominance. Chimeric plants for *rol*C expression showed leaves with pale green sectors with sharp borders, indicating lack of diffusion or transport of this protein. This is compatible with the hypothesis that plant growth and morphogenesis is altered by the *rol* gene proteins which modify endogenous plant hormones (Estruch *et al.*, 1991a).

Estruch *et al.* (1991b) found that the rolC protein releases cytokinins from glucoside conjugates. Also the rolB protien was found to hydrolyses indole glucosides thus

releasing auxins from its conjugate (Estruch *et al.*, 1991c). The combined action of these two genes is thought to modulate the intracellular concentration of auxins and cytokinins to produce 'rhizogenes' roots. Plant developmental and physiological processes including nodulation can be influenced by enzymatic systems leading to conjugation and deconjugation of auxin and cytokinin phytohormones.

Production of transgenic plants from Agrobacterium rhizogenes transformed roots

A. rhizogenes induced hairy roots of approximately 50 different plant species have been cultured. Transgenic plants have been regenerated from these roots either by spontaneous shoot formation or through a callus stage initiated on a medium supplemented with auxins and cytokinins (Potter, 1991).

Desired sequences can be transfered into plants using *A. rhizogenes* mediated transfer. This can be achieved either by inserting the DNA sequence into an *A. tumefaciens* derived binary plasmid (Simpson *et al.*, 1986) or by integrating the sequence into the pRi TL-DNA segment of the Ri plasmid (Stougaard *et al.*, 1987a).

Phelep *et al.* (1991) used the *A. rhizogenes* strain K599 as well as the agropine strain A4 to transform the woody non-legume *Allocasuarina.* Their study was characterized by careful interpretation of Southern hybridization data which showed covalent integration of *Agrobacterium* sequences into plant DNA. Limited success, however, has been reported in the *Glycine* genus. Only in the wild soybean *Glycine canescens* have whole plants been regenerated from transgenic roots (Rech *et al.*, 1988).

Susceptibility of soybean to Agrobacterium rhizogenes

To date there are no reports of whole plants regenerated from *A. rhizogenes*-induced roots of soybean. Even production of these roots has proven difficult. Owens and Cress (1985) tested the response of 26 *G. max* genotypes to *A. rhizogenes* strain A136 harboring the pRiA4b (Ri) plasmid. Seven out of 26 of these genotypes, produced roots at the infection site. Attempts to culture these roots failed and assays for 'hairy root' markers (opine content) were not performed. Simpson *et al.* (1986) used *A. rhizogenes* roots to introduce transgenic sequences from *A. tumefaciens* via a binary plasmid conferring kanamycin resistance. This plasmid functioned in *A. rhizogenes* (agropine strain A4) and produced roots which had the transgenic sequence integrated and expressed in tomato, tobacco and

alfalfa. Although Simpson *et al.* (1986) demonstrated a relatively high frequency of transgenic roots in alfalfa (33% nopaline positive, 4% kanamycin resistant), tomato (33% nopaline positive, 19% kanamycin resistant) and tobacco (19% kanamycin resistant, nopaline not determined), in soybean only 2% of the roots were nopaline positive and none were kanamycin resistant. In *G. max* a high percentage of non-transgenic roots were produced from the inoculation/wound site (Simpson *et al.*, 1986). The ability of soybean to show intrinsic nopaline activity (Christou *et al., 1986*), coupled with the fact that none of the roots showed kanamycin resistance seriously limited the use of *A. rhizogenes* to produce transgenic roots in soybean.

It was not until 1990 that Savka *et al.* found an *A. rhizogenes* strain (cucumopine strain K599/pRi2659) capable of inducing 'rhizogenes' roots at a high frequency in *G. max* (37% of the cotyledons produced roots on the 10 genotypes tested). Root cultures derived from these infection events were positive for cucumopine production and grew rapidly in culture. Savka *et al.* (1990) used these cultures to propagate soybean cyst nematodes *in vitro* but did not evaluate their nodulation phenotype.

Application of micro-projectile 'gene ' gun technology to transformation of soybeans

In recent years, plant transformation strategies have come to rely on micro-projectile gun delivery of DNA to plant cells (Figure 6). This process also called the 'biolistic' method was developed to overcome the transformation barriers of species and cell types (Klien *et al.*, 1987). The technology uses micron-sized DNA-coated tungsten (or gold) particles accelerated to high velocities for penetration into plant cells and DNA delivery. Yamashita *et al.* (1991) presented evidence that only the cells where the DNA coated tungsten particles enter the nucleus express the transgene activity.

This approach was used to obtain and examine gene expression of both transient (i.e. short-term) and stable (i.e. inheritable) transformations of plant cells. In some cases whole transformed plants have been regenerated (see Mendel, 1990 for a review). More commonly, transformations have involved tissue-cultured cells transiently expressing reporter genes such as those encoding chloramphenicol acetyltransferase (CAT) (Klein *et al.* 1988*a*) or ß-glucuronidase (GUS) (Jefferson, 1987a, b).

Christou *et al.* (1988) were first to transform soybean tissue by particle bombardment of immature embryos, which gave rise to kanamycin-resistant protoplasts and subsequent

callus. This break-through was followed by stable transformation and regeneration of soybean plants from immature embryo axes bombarded with a GUS construct (McCabe *et al.*, 1988; Christou *et al.*, 1989). Since then Finer and McMullen (1991) have transformed soybean by particle bombardment of embryogenic suspension cultures. Finer and McMullen's (1991) work has been shown to be reproducible in other laboratories. Parrott *et al.* (1992) introduced the *Bacillus thuringiensis* (BT) gene into soybean using embryogenic supension cultures and particle bombardment.

Future applications of soybean transformation

Soybean transformation is being used commercially to introduce genes conferring herbicide tolerance, improved protein quality and composition as well as disease and pest resistance. The Monsanto company has already produced soybean (cv. Peking) plants resistant to the herbicide glyphosate (Round Up^{TM}). The BT gene has been introduced into soybean and conferred increased resistance to velvet bean caterpillar, a common soybean pest (Parrot *et al.*, 1992). Also, the methionine rich seed storage protein (bex) from brazil nut has been transformed into soybean to improve the seed protein quality (Townsend *et al.*, 1992). Soybean mosaic virus resistance will be conferred to commercial cultivars by the transformation and expression of the viral coat protein in the plant. Similar commercial applications in superior soybean cultivars will be seen in the future.

From the academic point of view there are many fundamental questions which can be answered using gene transfer techniques (transient and stable transformation systems). Modifications of plants by gene addition or manipulation of endogenous genes will help elucidate the action of specific sequences. The analysis of promoter sequences by using reporter gene fusions will help elucidate the timing and tissue specificity of gene expression. Understanding what these sequences do and when and where they express will lead to better genetic, molecular and biochemical characterization of soybean. This will enable precise manipulation and genetic improvement of this valuable crop plant. Some of the techniques that will be used for this include site-directed mutagenesis in the form of transposon tagging of genes, ribozyme and antisense technology. Transposons introduced through transformation can jump into genes and delete their function. Transformation could also help to find the genes mutated in plant mutants by classical complementation.

Application of gene transfer techniques to the genetics of nodulation in legumes

The isolation of several nodulation cDNA clones and the comparison of sequence homology and amino acid composition with other genes has defined the biochemical function of only a few nodulins (Nap and Bisseling 1990). Many like Enod 2 elude precise assignment, although similarity to other proteins exist. The nodulin Enod 2 was found to be similar to hydroxyproline rich cell wall proteins. Plant transformation will help to elucidate the role of these symbiotic plant genes (Delauney *et al.*, 1988).

Stougaard *et al.* (1987b) were the first researchers to use transgenic plants to study the genetics of nodulation. They demonstrated nodule specific expression of a complete soybean leghemoglobin gene in the root nodules of transgenic *Lotus corniculatus*. Later work focused on using the 5' promoter sequence from the leghemoglobin gene fused to the CAT reporter gene in order to show tissue specific expression in transgenic nodules of *Lotus corniculatus* (de Bruijn *et al.*,1989; de Bruijn *et al.*,1990; Jensen *et al.*, 1988; Szabados *et al.*, 1990). Similar work was carried out using the *Parasponia* hemoglobin promoter and GUS reporter gene fusions in transgenic *Lotus* and tobacco (Bogusz *et al.*, 1990). Miao *et al.* (1992: submitted) used the same promoter reporter gene fusion technique to study the tissue specific expression of nodulin 26. Nodulin 26 is a major protein of the peribacteroid membrane. GUS expression was seen at emerging nodules and root primordia. Roots expressing anti-sense N-26 showed retarded hairy root growth (*A. rhizogenes*) in *Vigna aconitifolia*. Anti-sense studies were also conducted with nodulin 35 (N-35). Soybean nodulin-35 encodes a subunit of uricase localized in the peroxisomes of uninfected nodule cells (Nguyen *et al.*, 1985). Lee *et al.* (1992: in press) introduced an anti-sense cDNA clone into *Vigna* behind a CaMV-35S promoter. Nodules formed on the resultant transgenic roots were smaller in size and plants exhibited a yellow leaf phenotype. Uricase activity was reduced by 50-60% as shown by enzymatic assays. Electron microscopy also showed that the size of the peroxisomes was reduced.

Application of gene transfer techniques to the genetics of nodulation in soybean

To date no transformation system has been used for the study of symbiotic nitrogen fixation in soybean. In part this is because no suitable transformation system exists for such a study in soybean.

Research in our laboratory has focused on the plant genes involved in the nodulation process. The production of a series of soybean nodulation mutants (supernodulating nts382 and non nodulating nod49 and nod139 lines) via EMS mutagenesis (Carroll *et al.*, 1985a, b, 1986; Gresshoff and Delves 1986) has led to research aimed at finding the genes responsible for this changed phenotype. Any candidate sequences found will have to be tested via plant transformation to prove functionality of these genes. This will be performed as in classical complementation experiments. The candidate wild-type sequence will be transformed into the mutant plant to determine if the wild-type nodulation phenotype is restored.

Our interest also extends to how cloned and characterized plant genes such as chalcone synthase, nodulin 26, and cell cycle genes (e.g. CDC 2) are involved in the nodulation process. Transformation would enable us to study these genes at a different level. Such genes can be regulated by antisense or ribozyme technology. This will enable us to see if the alteration of the expression of specific nodule proteins (or associated proteins) is critical to the symbiosis or nodule phenotype. Promoter sequences can also be studied to determine exactly when and where (in time and space) these genes are turned on in the nodulation process. In this respect transformation offers a novel way of looking at developmental processes in plants.

Dissertation goal

Much work has gone into the study of physiology, biochemistry, classical and molecular genetics of the plant-*Rhizobium* symbiosis. However, these areas of research cannot be directly coupled without the application of plant transformation. Transformation has proven to be a very useful way of identifying important bacterial nodulation genes. Gene transfer should also prove to be useful in identifying nodulation genes in plants. Without the information obtainable from genetic manipulation of plants we can only guess at roles for proteins. Transformation will enable us to determine exactly when and where these proteins are expressed in the developing plant.

In this dissertation the writer has explored the possible routes for transformation of soybean (*Glycine max*). These include various regeneration systems. The ultimate goal of this research is the development of a transformation system suitable for the analysis of nodulation in soybean. Such a system would enable and the analysis of nodulation by the by genetic modification of plants.

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CHAPTER 2

SUPERNODULATION AND NON-NODULATION PHENOTYPES OF *GLYCINE MAX* (SOYBEAN) ARE STABLE THROUGH ORGANOGENIC AND EMBRYOGENIC REGENERATION SYSTEMS

ABSTRACT

Conditions for the regeneration of soybean *Glycine max* cultivar Bragg and its derived nodulation mutants nts382, nod49, and nod139 (Carroll *et al.*, 1985a; b; 1986) via organogenic and embryogenic regeneration systems were investigated. Very high benzyl amino purine (BAP) levels (100 μM) were required for optimal shoot organogenesis from cotyledonary nodes of Bragg and the nodulation mutants when compared to cv. Peking (5 μM). Liquid embryogenic suspension cultures were initiated of Bragg and the nodulation mutants nts382, nts1007 nod49 and nod139. Initiation of embryogenic tissue from these genotypes required significantly longer culture periods (approx two months longer) than for Fayette, the model cultivar for suspension work. Initiation of this embryogenic tissue was improved when the medium was modified with purified agar, purified 2,4-D and adjusted to pH 7 (instead of pH 5.5). The size of immature seed picked also affected success in culture. The best immature seeds were collected from 5 cm pods (5 mm seeds) produced from the first flowers set on the plants. Whole plants were regenerated from suspension cultures of the supernodulating mutant nts1007. All of the plants regenerated from embryogenic suspension cultures and shoot organogenesis of the cotyledonary node were fertile and showed nodulation phenotypes identical to the parental material from which they were derived.

INTRODUCTION

The nodulation mutants of Bragg used in this study were created by ethyl methyl sulfonate (EMS) mutagenesis of seeds (Carroll *et al.*, 1985a; b). The plants produced from these seeds were screened for nodulation phenotype. Non-nodulating mutants nod139 and nod49 failed to nodulate (Carroll *et al.*,1986; Mathews *et al.*; 1989a) whereas the nitrate tolerant symbiosis (nts) allelic mutants nts382 and nts1007 (Carroll *et al.*, 1985a, b) had a supernodulating phenotype (up to 40 times the nodule number of the wild-type Bragg parent).

Research in our laboratory has focused on the plant genes involved in the nodulation process. The production of these nodulation mutants has lead to research aimed at finding the genes responsible for these changed phenotypes. A molecular marker has already been found that is closely linked to the *nts* locus of the nts382 supernodulating mutant (Landau-Ellis *et al.*, 1991). Any candidate sequences discovered will have to be tested via plant transformation to prove functionality to these genes. This will be performed as in classical complementation experiments. The candidate wild-type sequence will be transformed into the mutant plant to determine if the nodulation phenotype is thereby corrected. The transformation proceedure used will most likely require a tissue culture regeneration stage. Thus research was conducted to develope and evaluate suitable regeneration proceedures for the soybean nodulation mutants.

Regeneration of *G. max* by shoot organogenesis from the cotyledonary node has been demonstrated in a wide range of genotypes from all maturity groups (Cheng *et al.*,1980; Saka *et al.*, 1980; Wright *et al.*, 1986a, b; Delzer *et al.*,1990). Hinchee *et al.* (1988) used this regeneration system in conjunction with *Agrobacterium tumefaciens* infection to regenerate transgenic plants of Peking. These shoots were produced on a Gamborg's B_5 based medium (B_5; Gamborg *et al.*,1968) supplemented with 5 mM BAP (Hinchee *et al.*, 1988). Soybean genotypes differ widely in regeneration potential (Delzer *et al.*,1990). Wright *et al.* (1986b) demonstrated shoot organogenesis in a wide range of commercial cultivars (10 cultivars) using a Murashige and Skoog (MS) based medium (Murashige and Skoog., 1962) supplemented with 5 mM BAP. Saka *et al.* (1980) recognized a marked difference in the cytokinin requirements of two cultivars (Amsoy and Wayne) when cultured on Gamborg's B_5 medium or MS. Multiple shoot organogenesis was obtained in the 1 to 5 μM BAP range when cotyledonary nodes were plated on MS medium, but 1 to 50 μM was required when the explants were plated on B_5 medium (Saka *et al.*, 1980). As we were interested in developing a transformation system similar to that published by Hinchee *et al.* (1988) we used B_5 medium for our experiments. It was found that Peking would produce multiple shoots on B_5 medium at 5 μM BAP but Bragg and its derived nodulation mutants would not undergo shoot organogenesis at this level. Our screening of these plants showed that they required an optimal 100 μM BAP to stimulate organogenesis on B_5 medium. This finding correlates with the results of Saka *et al.* (1980).

Christianson *et al.* (1983) initiated regenerable embryogenic suspension cultures from immature zygotic embryo axes (cv. Fayette). Five years later Finer and Nagasawa,

(1988). initiated regenerable embryogenic supension cultures from immature cotyledons of cv. Fayette. Embryogenic tissue capable of producing secondary embryos upon embryos was produced by culturing the immature cotyledons for several months at a very high level of 2,4-D (40 mg/l). This tissue was then used to initiate embryogenic suspension cultures termed 'highly embryogenic ontogenetic and early staged' suspensions. Ranch *et al.*. (1985) published the production of regenerable embryogenic cultures in soybean. These embryos were initiated at a lower level of 2,4-D (10 mg/l) and did not produce secondary embryos. Thus the system was not suitable for the production of suspension cultures but only for the direct production of plants.

In 1991 Finer and McMullen reported the regeneration of transgenic plants from embryogenic suspension cultures. However, these first transgenic plants were sterile (John Finer; Ohio State University, pers. comm). This was thought to be a problem associated with long term culture as freshly initiated suspensions of cv. Fayette gave fertile plants. Bailey and Parrott (1992) also reported initiation of embryogenic suspension cultures and regeneration of fertile plants from cultivars Century, Davis, Lee, Hutcheson, Peking and PI 417138.

Embryogenic suspension cultures of cv. Bragg and its derived nodulation mutants nts382, nts1007 and nod49 were initiated. To date only nts1007 plants have been regenerated. The nts1007 regenerants were fertile and maintained a supernodulating phenotype.

The effect of high hormone levels (2,4 D and BAP) on induction and culture in both the organogenic and embryogenic suspension culture systems was examined with respect to nodulation phenotype of the regenerant plants. Neither culture procedure interfered with the nodulation phenotype of the regenerant plants. This suggests that the production of transgenic plants utilizing these regeneration procedures could be used to study the genetics of nodulation. Cotyledonary petioles of cv. Peking (inoculated with *Agrobacterium tumefaciens)* have been used as the regeneration system by Hinchee *et al.* (1988) to produce transgenic plants. Embryogenic suspension cultures have also proven to be efficient regeneration systems when used for particle gun transformation of soybean (Finer and McMullen, 1991).

MATERIALS AND METHODS

a) Embryogenic regeneration system

Selection of plant tissue

Soybean plants *G. max* (L.) Merrill, cv. Peking, cv. Bragg and its nodulation mutants nts382, nod49 and nod139 were greenhouse grown in soil supplemented with Osmocote^{TM} (14:14:14) fertilizer. The nodule phenotype was checked 4 weeks after sowing and inoculation with *Bradyrhizobium* USDA 110. Between November and March plants were grown with the aid of supplemental lighting from metal halide lights (16/8 hr light /dark photo-period). Plants with immature pods of a suitable size were obtained 2-3 months after sowing. Pods 30-40 mm long containing immature seeds approximately 4-5 mm in length were harvested. The seed size was checked by holding the pod up to the light. Only the pods set from the first flowers on each plant were used (as recommended by J. Finer, Ohio State University, Columbus, Ohio. pers. comm.)

Initiation of embryogenic callus

Initiation and culture of the embryogenic suspensions were performed as described by Finer and Nagasawa (1988) with a few modifications. Immature pods were surface-sterilized for 20 minutes in a 20% solution of commercial bleach containing 0.05% Tween-20, then rinsed four times in sterile distilled water. The sterile pods were cut open aseptically and the immature seed dissected out. The cotyledons were separated and the embryo and seed coat removed. The cotyledons were then placed abaxial side down on a medium composed of MS salts (Murashige and Skoog., 1962), Gamborg's B_5 vitamins (Gamborg *et al.*, 1968), 6% sucrose, 40 mg/l 2,4-D and 0.8% purified agar (Sigma). The pH for culture was modified from 5.6 to 7 according to Komatsuda, (1988). All media were autoclaved for 20 mins at 121°C, 15 psi. The cultures were maintained at 26°C in an illuminated incubator (40 $\mu Em^{-2}s^{-1}$, 16/8 hr light/dark photo-period). The tissue was subcultured every 4 weeks onto the same medium for a period of between 2 to 6 months until embryogenic tissue formed on the callused tissue.

Initiation of embryogenic suspension cultures

The embryogenic tissue formed was dissected from the explant callus and analyzed under the dissecting microscope to determine its embryonic stage. If small secondary embryoids were seen initiating from the primary embryos the tissue could be used to initiate suspension cultures. Approximately 8 mm^3 pieces of this embryogenic tissue were placed in sterile 125 ml DeLong flasks containing 35 ml of suspension medium. This medium (Finer and Nagasawa 1988) consisted of MS salts modified by the replacement of the MS nitrogen with 10 mM NH_4NO_3 and 30 mM KNO_3, B5 vitamins (Gamborg *et al* .,1968), 6% sucrose, 5 mg/l, 2,4-D and 15 mM glutamine (pH 5.7). The flasks were capped with stainless steel closures and sealed with Para-film^{TM}. The suspensions were then placed at 24°C on a shaker agitated at 150 rpm and continuously illuminated with a light intensity of approximately 60 $\mu Em^{-2}s^{-1}$. The suspensions were subcultured every 3 to 4 weeks at very low inoculum density (two 8 mm^3 clumps per flask). If the medium became cloudy the suspensions were subcultured early.

Regeneration of plants from suspension cultures

Small clumps (8 mm^3) of green embryogenic suspension were removed from suspension and placed on a medium composed of MS salts, Gamborg's B_5 vitamins, 6% maltose, 0.2% Gel-rite (Phytagel, Sigma) and 0.5% activated charcoal (J. Bailey, Georgia State University, pers. comm.), pH 5.7. These embryos were incubated under the same conditions as for embryo induction. After 4-8 weeks of growth the embryos gradually turned yellow, indicating that they had reached physiological arrest and were ready for desiccation. Yellow embryos of a minimum size of 5 mm in length were desiccated in the dark at room temperature for 3-7 days. Approximately 10-15 embryos were desiccated in a wrapped (Para-film^{TM}) 15x100 mm Petri dish. It was important to only desiccate the embryos to a 'flaccid' and not a 'crisp' stage. After desiccation the embryos were again placed on the embryo development medium which was only modified by the addition of 3% sucrose instead of maltose. Three to four weeks were required to allow normal root and shoot development. During this stage the Petri dishes were wrapped in Scotch^{TM} pressure sensitive tape (vent tape) and opened in the sterile laminar flow hood every 7 days for 10 minutes. This prevented the build-up of ethylene which could arrest germination. Once a normal root system and shoot had developed the plants were transferred for two weeks to Magenta^{TM} jars and grown in 20 ml of liquid medium consisting of Monniers salts (1976) and Morel and Wetmore vitamins (1951; Mon-Mor

medium). This step stimulated rapid root and shoot growth. The resultant plantlets were transferred to 50% Vermiculite^{TM} 50% Faffard^{TM} soil-less compost and cultured under a plastic bag for 1-2 weeks to acclimatize the plants to greenhouse conditions. They were then inoculated with 10 mls of $1x10^6$ ml^{-1} *Bradyrhizobium japonicum* USDA 110 both upon transfer to soil and once more 2 weeks later. The plants were checked to see if they displayed normal nodulation profiles 6 weeks after transfer to soil. They were then grown to maturity and checked for nodule phenotype, fertile flowers and seed set.

b) Organogenic cotyledonary node regeneration system

Seeds of cultivars Peking, Essex, Bragg and nodulation mutants nts382, nod49, nod139 were hand-harvested from greenhouse grown plants. Their nodulation phenotypes were verified upon harvest. The seeds were surface sterilized by the following procedure; a 5 minute wash in a soapy aqueous solution of the fungicides Captan^{TM} and Banrot^{TM}, followed by a 10 min wash in 40% Chlorox, containing 0.1% Tween 20, then 5 rinses in sterile distilled water. Vigorous agitation was employed at each stage. The seeds were allowed to soak for one hour in an aqueous solution of Captan^{TM} and Banrot^{TM} before plating on a medium (1/10th $B_5$0) composed of 1/10th $B_5$0 salts, 20g/l sucrose, 8g/l purified agar (Sigma) at pH 5.7. $B_5$0 medium was identical to 1/10th $B_5$0 medium except that full strength Gamborgs B_5 salts were used. The culture procedure for inducing shoot organogenesis from the cotyledonary node was basically as described by Wright *et al.* (1986a,b) and Hinchee *et al.* (1988) with some media modifications. After 6-10 days of culture under continuous light (60 $\mu Em^{-2}s^{-1}$) at 27°C the green cotyledons were removed from the seedling with approximately 5 mm of the petiole. The cotyledons were separated by a longitudinal cut through the adjoining hypocotyl tissue. The seedling meristem was carefully removed and the cotyledons placed adaxial side down on a regeneration medium ($B_5$0+5 μM BAP) consisting of B_5 salts, 20 g/l sucrose, 8 g/l purified agar (Sigma) and 5 μM BAP, pH 5.6. Further experiments were performed with BAP levels of 0 to 300 and 0 to 800 μM in the regeneration medium. Dissected cotyledons of all of the aforementioned cultivars were cultured on these media ($B_5$0+various BAP levels) for 4-8 weeks. The rate of shoot organogenesis determined the amount of time the explants were left on the media. Sub-cultures were generally performed every 4 weeks. The explants were placed on $B_5$0 medium for 4 weeks for shoot elongation, then 2 weeks on 1/10th $B_5$0 medium for rooting. Shoots could be divided from the explant at this rooting stage. The resultant plantlets were transferred to Vermiculite^{TM} and cultured under a clear plastic bag for 1-2 weeks to acclimatize the plants to greenhouse conditions. The

regenerant plants were then either placed in plastic pouches (Vaughan's Seed Co., Downers Grove, Ill.) or in 50% Vermiculite^{TM} 50% Faffard^{TM} soil-less compost and inoculated with *Bradyrhizobium japonicum* USDA 110.

Analysis of nodulation : plastic growth pouches

One tissue culture regenerated plant and one 5 day old seedling of the same mutant genotype were placed in a plastic growth pouch, watered with 1/4 Herridge's solution (Delves *et al.*, 1986) and cultured for 3 days in a growth chamber. The culture conditions were 500 $\mu Em^{-2}s^{-1}$ illumination with 70% humidity and a 16:8 hr day cycle set at 25°C. On the third day the plants were inoculated with 100 µl of $1x10^6$ per/ml *Bradyrhizobium japonicum* USDA 110. The plants were cultured as before in an environmental chamber and watered every day with 1/4 strength Herridge's solution. After 14 days culture the roots were screened for nodule number.

Analysis of nodulation : soil screening

Regenerant shoots were placed in 50% Vermiculite^{TM} 50% Faffard^{TM} soil-less compost in six inch pots. These plants and 5 day old seedlings of the same mutant genotype were inoculated with 10 ml of $1x10^6$ per/ml *Bradyrhizobium japonicum* USDA 110 on transfer. The nodule phenotype was screened six weeks after transfer to soil.

RESULTS

a) Embryogenic regeneration system

Initiation of embryogenic callus

The first attempt to initiate embryogenic tissue from cv. Peking, Bragg and nodulation mutants nts382, nts1007, nod49 and nod139 used Difco Bacto Agar obtained from Difco Laboratories, Detroit, MN, and 2,4 D from Sigma Chemicals St Louis MO. This attempt failed to produce embryogenic tissue. After personal communication with John Finer, the following modifications to the culture conditions were made. Only the pods set from the first flowers on each plant were used (Figure 1, Plate 1). The immature cotyledons (Figure 2) were initially plated on Gelrite^{TM} instead of Difco Bacto Agar.

Plate 1. Production of embryogenic tissue from immature cotyledons.

Figure 1. Selection of immature cotyledons by the developmental size of the soybean pod. Flat (cotyledons not filled) pods 30-40 mm in length were selected.

Figure 2. After dissection an immature cotyledon was placed on the embryo induction medium. The optimal size for cotyledon culture was 4-5 mm.

Figure 3. Embryogenic tissue forming from immature cotyledons of Bragg derived mutant nts1007. These cotyledons were cultured on a medium with very high levels of 2,4-D (40 mg/l). Four months of culture on this medium killed most of the immature cotyledonary tissue and stimulated the initiation of small areas of embryogenic tissue covered with pro-embryos. This tissue was used to initiate suspension cultures in liquid medium.


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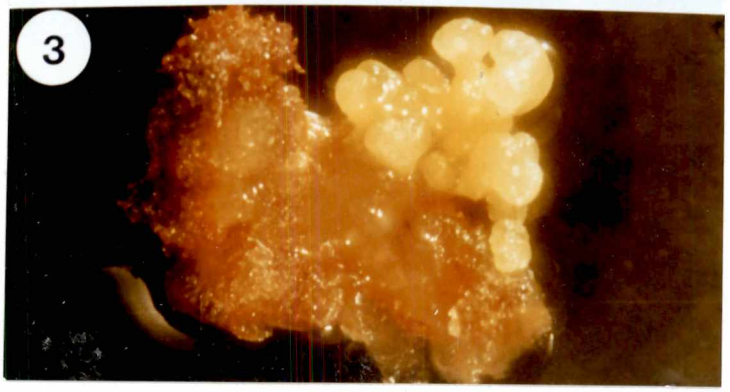
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Tissue culture purified agar from Sigma Chemicals was used for all subsequent culture stages on solid media. The 2,4-D was also purified by dissolving the hormone in 100% ethanol then precipitating it. Precipitation was performed by the addition of one volume of water then filtering and drying the salt in the fume hood. The pH of the initiation medium was changed from pH 5.7 to pH 7 according to Komatsuda *et al.* (1988). After these modifications it was possible to routinely initiate embryogenic tissue from the cultures (Figure 3). This suggests that embryogenesis was possible only under stringent conditions with the purest chemicals. No experiments were performed to clarify the nature of the impurities, or whether impurities in the agar or the 2,4-D inhibited embryogenesis. Size of the immature seed plated proved significant. Immature seeds smaller than 2 mm in length died and any seeds larger than 6 mm in length failed to produce rapidly growing callus or initiate embryogenic tissue. The optimum size for plating appeared to be 4 mm. There was significant variation in time taken to initiate embryogenic tissue and the number of embryogenic events occuring in the different soybean genotypes (Table 1).

Table 1. Time in culture before initiation of embryogenic tissue from cv. Peking, Bragg and nodulation mutants nts382, nts1007, nod49 and nod139. The embryogenic tissue was produced by culturing immature cotyledons (10 plates, 10 cotyledons per plate) on a medium containing very high levels of 2,4-D (40 mg/l). It appears that most embryogenic tissue was produced in the two to five month period after the initiation of culture. The production of embryogenic tissue is also very rare. At best 9% of the cotyledons produced embryogenic tissue, nod139 produced none.

	# of cotyledons which developed embryogenic tissue								
	Months in culture								**Genotype**
Genotype	1	2	3	4	5	6	7	8	**total**
Peking	0	0	0	0	1	0	0	0	1
Bragg	0	1	3	2	2	1	0	0	9
nts1007	0	0	0	1	0	0	0	0	1
nts382	0	0	0	3	0	0	0	0	3
nod 49	0	0	0	1	0	0	0	0	1
nod139	0	0	0	0	0	0	0	0	0
Fayette	0	3	3	1	0	0	0	0	7
Total events/month	0	4	6	8	3	1	0	0	0

Bragg and Fayette produced embryogenic callus after two months in culture and produced approximately the same number of embryo induction events. All of the nodulation mutants showed less induction events and required more time in culture to do so. The nodulation mutant nod139 did not produce any embryo induction events. Because of the low number of total initiation events it was not clear if there was any significance to these observations. This observation may be due to the reduced fitness of these mutants in culture. Often the explant would appear dead or darkened before embryogenic tissue was induced (Figure 3).

Growth in suspension culture

The embryogenic suspension also varied in their viability in culture. Bragg and nts1007 produced healthy early stage embryogenic suspension cultures (Figure 4, Plate 2) similar to those initiated by Dr. John Finer with cv. Fayette. Peking and nod49 produced embryogenic callus but it did not thrive in the liquid suspension medium. The supernodulating mutant nts382 embryogenic suspension cultures seemed to consist of larger green 'later staged' embryogenic clumps. This stage of embryonic tissue was not ideal for liquid culture. Increasing the 2,4-D level did not improve the cultures. Maintaining the cultures at a very low inoculum was of advantage.

There are two possible explanations for the variations in these cultures. First, the embryogenic explant tissue used to initiate the suspensions may have been at different embryogenic stages. Alternatively, different responses may have arisen from pleiotropic effects in the different mutants. A larger experiment would provide statistical evidence for one of these hypotheses.

Embryo germination capacity varied between the mutant types. Bragg and nts1007 germinated relatively easily, whereas nts382 embryos did not develop and germinate normally. When placed on the embryo development media the nodulation mutant nts1007 produced a mixture of funnel-shaped and normal embryos (Figure 5). A proportion of these funnel embryos did not possess normal meristems. The germination rate from these funnel-shaped embryos could be increased by culturing with ABA. We used 10 μM ABA in the embryo development medium for 2 weeks prior to desiccation (data not shown). The ABA helped 'normalize' the embryos increasing the total number of embryos with two cotyledons and an active meristem.

Plate 2. Regeneration of plants from embryogenic suspension cultures.

Figure 4. Embryogenic suspension cultures of Bragg in liquid suspension medium. Low inoculation density, high light (60 μM $E.m^2 s^{-1}$) conditions, and frequent sub-cultures (every 3 to 4 weeks) helped to produce healthy suspension cultures.

Figure 5. Embryos (nts1007) from embryogenic suspension clumps after 2 weeks on an embryo development medium. Embryos with a normal phenotype would grow with two separate cotyledons. A proportion of the embryos would grow with continuous or funnel-shaped cotyledons.

Figure 6. Physiologically arrested embryos. Once the embryos had grown to a sufficient size (5 mm) and had reached physiological arrest (indicated by the yellow color of the embryo), they were ready for desiccation.

Figure 7. Germination of a Bragg embryo 3 weeks post-desiccation. This embryo had funnel-shaped fused cotyledons

Figure 8. A regenerant nts1007 mutant derived from embryogenic suspension cultures. This plant was fertile and had a supernodulating phenotype.

Figure 9. A regenerant nts1007 mutant derived from embryogenic suspension cultures exhibiting a supernodulation phenotype (approx 200 nodules on this plant).


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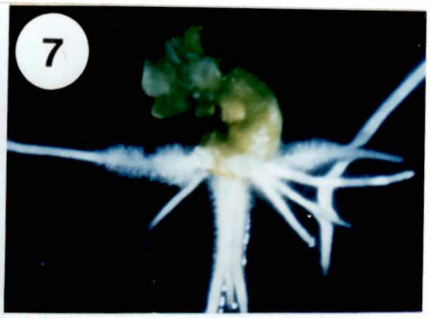
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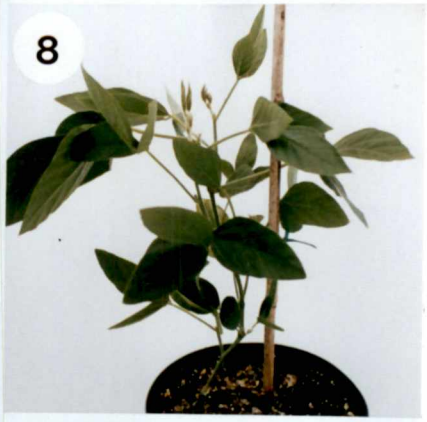
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Regeneration of plants from embryogenic suspension cultures

To date, only nts1007 plants have been regenerated. It was extremely important to wait until the embryos reach physiological arrest before transferring them to the desiccation stage (Figure 6). Physiological arrest was indicated by the embryos turning from green to yellow. Early in the procedure many green embryos were wasted in the desiccation stage. Germination of the somatic embryos took 8 to 15 days. Approximately 2-5 days were required for root formation and 7-10 days for the shoot formation (Figure 7). After transfer to soil and a period of slow growth due to tissue culture carry-over, the nts1007 plants developed into normal looking healthy fertile plants with a supernodulating phenotype (Figure 8). The nts1007 supernodulation phenotype was not subject to somaclonal variation (Larkin *et al.*,1984) in the embryogenic regeneration system (Figure 9). Thus, this regeneration system would be a suitable for the production of transgenic plants of nts1007 to analyze the genetics of this nodulation mutant.

b) cotyledonary node organogenic regeneration

Initial experiments in this area attempted to repeat the results of Wright *et al* .(1986a,b) and Hinchee *et al.* (1988) with organogenesis of shoots from cotyledonary petioles of the nodulation mutants using 5 μM BAP. The Bragg derived soybean mutants did not produce any adventitious shoots at this hormone level (Table 2). Cultivar Peking however displayed multiple shoot organogenesis with 5 μM BAP, confirming the results by Wright *et al* . (1986a,b) and Hinchee *et al.* (1988).

Table 2. Initiation and regeneration of adventitious shoots from Peking, Bragg and nodulation mutants on a B_5 medium containing 5 μM BAP. Fifty cotyledons were plated for each cultivar (5 cotyledons per Petri plate).

Genotype	cotyledons producing adventitious shoots	plants regenerated per petiole (average)
Peking	30/50	10
Bragg	none (roots only)	none
nod139	none (roots only)	none
nod49	none (roots only)	none
nts382	none (roots only)	none

Shoot organogensesis from the cotyledonary nodes of the Bragg derived nodulation mutants

If no BAP was included in the culture medium of Bragg and the derived nodulation mutants the axillary shoots (if present) and roots grew out from the cotyledonary petiole (Figure 10, Plate 3). With 5 μM BAP in the culture medium rooting was reduced but only the axillary shoots (if present) grew out of the petiole. No shoot organogenesis occurred with Bragg and the nodulation mutants at 5 μM BAP (Figure 11). As the explants produced root growth at 5μM BAP this indicated that the cytokinin level was too low to initiate shoot organogenesis. Further experiments were initiated with elevated BAP levels to stimulate shoot organogenesis. The experiments were repeated with two different ranges of BAP in each case 0 to 300 μM BAP (Figures 12, 13, 14, 15, 16 and 17) and 0 to 800 μM BAP (Table 3). In the latter experiment both Bragg seed from USDA (Huntsville) and Essex seed were compared to our Bragg seed (derived from Australian seed stock) and the nodulation mutants. This comparison acted as an 'internal' control to evaluate if there were any differences in our seed used for these experiments.

Table 3. Mean number of adventitious shoots initiated from Peking, Essex, Bragg and the nodulation mutants on a B_5 medium containing a range of BAP concentrations (± standard error). Ten cotyledons per cultivar were plated with two cotyledons per Petri dish.

	BAP concentration (μM)							
Genotype	0	5	25	50	100	200	400	800
Peking	0	6.7±1.1	8.8±1.4	17.6±1.2	16.8±.2.7	33.5±2.8	9.7±2.5	8.4±1.6
Bragg (PMG)	0	0	5.5±0.8	24.8±2.8	33.0±4.7	6.2±2.3	3.5±1.8	0
Bragg (USDA)	0	0	4.1±0.5	12.3±0.7	24.4±3.1	7.7±2.1	1.4±0.8	1.0±1.0
nod139	0	0	5.0±1.0	24.0±1.2	10.7±2.7	2.9±1.0	1.3±0.7	1.7±1.2
nod49	0	0	0	6.3±0.8	13.4±1.9	10.3±1.3	6.2±1.8	0
nts382	0	0	10.0±2.4	13.5±3.0	22.0±2.5	7.9±2.0	3.3±1.5	0
Essex	0	0	4±1.2	15.1±0.9	6.5±1.6	7.4±2.0	4.3±1.2	1.1±0.8

With cv. Peking we were able to produce multiple shoots from the cotyledonary node over a wide range of BAP concentrations (5 to 800μM). In contrast, the level required for shoot initiation in Bragg, nts382, nod49 (Figures 18 and 19) and nod139 was at least 25 μM BAP.

Plate 3. Regeneration of plants from cotyledonary petioles.

Figure 10. Axillary shoots and root growing out from Bragg cotyledonary petioles after plating on B_5 medium without phytohormones for 4 weeks. No organogenesis of adventitious shoots was seen.

Figure 11. Axillary shoots and root growing out from Bragg cotyledonary petioles after plating on B_5 medium supplemented with 5 μM BAP for 4 weeks. No organogenesis of adventitious shoots was seen.

Figure 12. Axillary shoots growing out from Bragg cotyledonary petioles after plating on B_5 medium supplemented with 50 μM BAP for 4 weeks. No organogenesis of adventitious shoots was seen.

Figure 13. Axillary shoots and the initiation of adventitious shoot organogenesis in Bragg cotyledonary petioles after plating on B_5 medium supplemented with 100 μM BAP for 4 weeks.

Figure 14. Adventitious shoot organogenesis in Bragg cotyledonary petioles after plating on B_5 medium supplemented with 150 μM BAP for 4 weeks. Axillary shoot production is suppressed.

Figure 15. Adventitious shoot organogenesis in Bragg cotyledonary petioles after plating on B_5 medium supplemented with 200 μM BAP for 4 weeks.

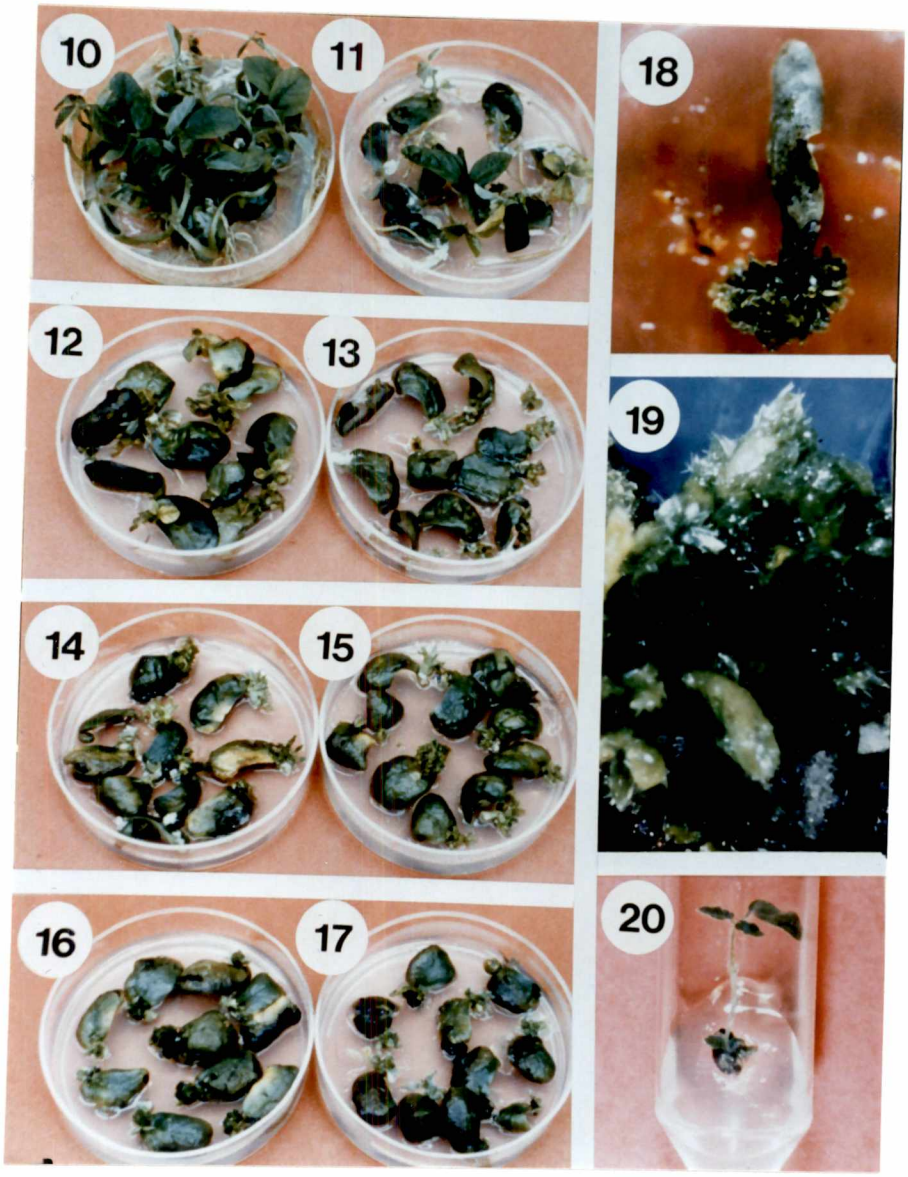
Figure 16. Adventitious shoot organogenesis in Bragg cotyledonary petioles after plating on B_5 medium supplemented with 250 μM BAP for 4 weeks.

Figure 17. Adventitious shoot organogenesis in Bragg cotyledonary petioles after plating on B_5 medium supplemented with 300 μM BAP for 4 weeks.

Figure 18. Multiple shoots growing out from the cotyledonary node of nodulation mutant nod49. This cotyledon has been plated on 200 μM BAP for 8 weeks.

Figure 19.A close view of multiple shoot organogenesis from a cotyledonary petiole of the nodulation mutant nts382. This petiole was plated on 100 μM BAP for 4 weeks.

Figure 20. An adventitious shoot derived from cv. Peking cotyledonary petiole tissue. Rooting and elongation of shoots was achieved in 50 ml sterile polycarbonate tubes containing culture medium (B_5 medium) devoid of hormones.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The optimum level for regeneration of shoots was 100 μM BAP (Figure 19), except nod139 which exhibited optimum regeneration at 50. μM BAP. The frequency of shoot production in these genotypes was reduced at 400 μM BAP. Regeneration of plantlets from shoots that were induced on such high levels of BAP was not difficult (Figure 20) and these plants were fertile and of normal phenotype.

Nod*ulation phenotype of cotyledonary node derived shoots*

Plants regenerated from tissue-culture showed stable nodulation phenotypes. These tissue-culture derived plants when compared to seed-derived plants exhibited slower root and shoot growth. This was evident in pouch analysis of nodulation phenotype (Table 4) where the nodule number of the tissue-cultured plants was approximately half that of the seedlings. *Rhizobium* infects only actively growing roots.

Thus, slow root growth limited the frequency of nodules formation. It generally took one month before the tissue-cultured plants to begin growing at a normal rate in the greenhouse.

Table 4. Mean nodule number (± standard error) from seedling and tissue culture derived plants grown in plastic pouches. The nodule numbers were compared 14 days after each root was inoculated with 100 μl of *Bradyrhizobium japonicum* USDA110 ($1x10^6$ ml^{-1}). Plants were grown in controlled environment cabinets and watered with 1/4 strength Herridge's solution. One tissue-cultured and one seed derived plant were placed in each pouch. Ten pouches of each cultivar were maintained.

Cultivar	seed derived plants	tissue culture derived plants
Peking	18.3±3.2	9.7±1.5
Bragg	18.0±2.6	8.1±1.3
nod139	0	0
nod49	0	0
nts382	60.5±7.9	39.2±7.75

Nodule number was subsequently screened on larger plants grown in soil for 6 weeks after inoculation and their transfer from culture. Control plants were screened 6 weeks after germination and inoculation. There was no significant difference in the mean

number of nodules produced from tissue culture derived and seedling derived plants (Table 5).

Table 5. Mean nodule number (± standard error) of seedling and tissue culture derived plants grown in soil for six weeks. The nodule numbers of seed and tissue culture derived plants were compared six weeks after inoculation with 10 ml of *Bradyrhizobium japonicum* USDA110 ($1x10^6$ ml^{-1}).

Cultivar	seed derived plants	tissue culture derived plants
Peking	28.6±3.2	29.4±4.2
Bragg	28.0±4.1	32.2±4.7
nod139	0	0
nod 49	0	0
nts 382	313.6±40.3	316.0±32.5

By screening the nodulation phenotype 6 weeks after inoculation the plant growth habits and nodule numbers of the tissue culture and seed-derived plants were almost directly comparable. The pouch-grown plants were slow growing and nodulated poorly (Figure 21, Plate 4). In contrast plants grown in the soil nodulated at a normal level (Figure 22) and grew into vigorous fertile plants (Figures 23 and 24). This demonstrated that the nodulation phenotype of these tissue culture regenerant plants was not affected by tissue culture induced variation (Larkin *et al.*, 1984).

CONCLUSION

Conditions were elucidated for the regeneration of Bragg and its derived nodulation mutants nts382, nod49, and nod139 (Carroll *et al.*, 1985a, 1986) in organogenic and embryogenic regeneration systems. The shoot organogenesis system proved very efficient in regenerating whole plants relatively rapidly (3-4 months). Multiple shoots could be produced and rooted from one cotyledonary petiole. Initiation of healthy embryogenic suspension cultures however, took up to five months. In addition regeneration from embryogenic suspension cultures took up to three months. The regeneration procedure was reproducible for nts1007, but only approximately 10% of the desiccated embryos germinated. This compares to 10 to 30% in other laboratories (Georgia State, Ohio State and the University of Kentucky)

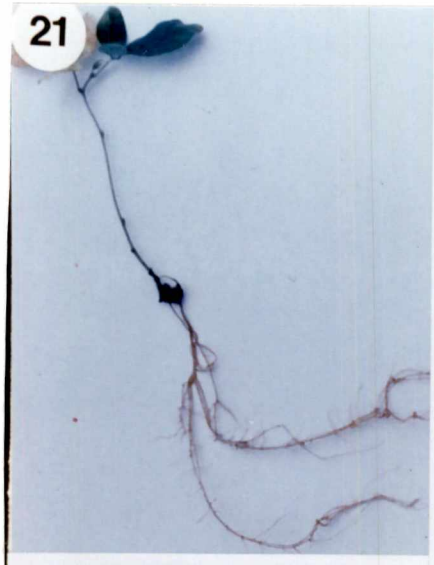
Plate 4. Nodulation of tissue culture derived plants.

Figure 21. A tissue culture derived Bragg plant obtained from shoot organogenesis. This plant exhibited nodules after inoculation with *Bradyrhizobium japonicum* USDA110 and culture in pouches. The nodule number on such plants was limited by the slow growth of the plants.

Figure 22. A regenerant nts382 plant produced from an adventitious shoot induced from a cotyledonary petiole grown on B_5 medium at 150 μM BAP. This plant was subsequently grown and inoculated in soil. Its nodulation frequency was not significantly different to seedling-derived plants grown under the same conditions.

Figure 23. All of the plants regenerated from adventitious shoots of the cotyledonary petiole were fertile and normal in appearance.

Figure 24. The stunted growth common with tissue cultured plants did not persist. The regenerant plants reached normal proportions at maturity.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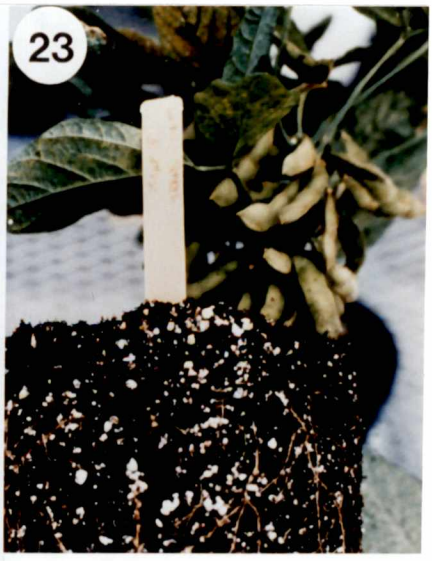
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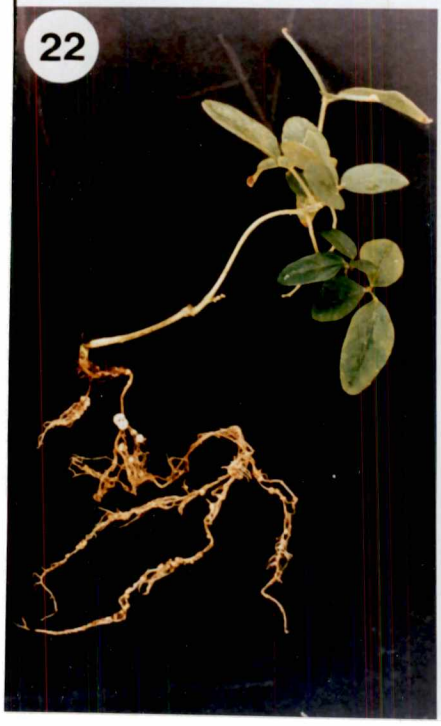
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Very high benzyl amino purine (BAP) levels (100 μM) were required compared to cv. Peking (5 μM) for optimal shoot organogenesis from the cotyledonary nodes in Bragg and the nodulation mutants. It is not clear whether cv. Essex Bragg and the derived nodulation mutants require unusually high levels of BAP or that Peking is unusually sensitive to BAP. These results could also reflect a higher hormone requirement for shoot organogenesis on B_5 media as compared to MS media. This effect was seen by both Cheng *et al.* (1980) and Saka *et al.* (1980). In these experiments, however, we did not test the regeneration potential of the nodulation mutants on MS medium.

This result raises the question as to what components in the two media make up the difference in cytokinin sensitivity. Gamborg *et al.* (1968) developed the B_5 medium which would induce and maintain soybean callus and suspension cultures. This was achieved by designing a medium with a high nitrate to ammonia ratio (B_5 medium) when compared to MS medium.

It is possible that the nitrogen balance in the medium would alter the regenerative potential of soybean (Gresshoff and Mohapatra, 1981). Indeed the embryogenic supension culture medium used in this study was modified by the replacement of the MS nitrogen with 10 mM NH_4NO_3 and 30 mM KNO_3. With this modification the cultures reportedly grow at a faster rate (Finer and Nagasawa, 1988).

Suspension cultures were initiated of cv. Bragg and nodulation mutants nts382, nts1007 nod49 and nod139. Initiation of embryogenic cultures from these cultivars took significantly longer when compared to cv. Fayette, the model cultivar for suspension work. Improvements in initiation of embryogenic tissue were observed when the medium was modified with purified agar, purified 2,4-D and pH 7. The size of immature seed harvested also affected success in culture. To date only the supernodulating mutant nts1007 has been regenerated from suspension culture. All of the plants regenerated from embryogenic suspension cultures and shoot organogenesis of the cotyledonary node were fertile and showed nodulation phenotypes faithful to the parental material from which they were derived.

The regeneration systems developed here could be used as transformation routes to study the genetics of nodulation in these mutants; without concern that the nodulation phenotype had not undergone tissue culture induced variation (Larkin *et al.*, 1984).

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CHAPTER 3

THE SUSCEPTIBILITY OF NODULATION MUTANTS OF *GLYCINE MAX* TO *AGROBACTERIUM TUMEFACIENS*

ABSTRACT

Soybean (*Glycine max* (L.) Merrill) cultivars Peking, Bragg and its derived nodulation mutants, nitrate tolerant (nts) supernodulating nts382 and non-nodulating nod49 and nod139 were screened for their susceptibility to 'wild-type' (non-disarmed) *Agrobacterium tumefaciens* strains A208 and A281. All plant types tested showed a wide variation in susceptibility and gall size with both strains. *A. tumefaciens* strain A208 showed the highest virulence. All of the nodulation mutants showed significantly lower susceptibility than the susceptible control cv. Peking. The supernodulating mutant nts382 showed the lowest susceptibility to either bacterial strains indicating that susceptability to *Rhizobium* infection was not correlated to *Agrobacterium* infection. Co-culturing the bacteria with a simple induction medium significantly increased gall size, fresh and dry weight in cv. Peking but not Bragg and its nodulation mutants. Duplication of virulence genes *virB* and *virG* from A281 in the 'wild type' A208 and A281strains failed to increase susceptibility to galling or increase gall size in the cultivars tested.

INTRODUCTION

Identification of plant nodulation genes by transformation

The nodulation mutants of Bragg used in this study were created by ethyl methyl sulfonate (EMS) mutagenesis of seeds (Carroll *et al.*, 1985a; b, 1986). The plants produced from these seeds were screened for their nodulation phenotypes. Non-nodulating mutants nod139 and nod49 failed to nodulate (Carroll *et al.*,1986; Mathews *et al..*, 1989a), whereas the nitrate tolerant symbiosis (nts) allelic mutants nts382 and nts1007 (Carroll *et al.*, 1985a, b) had a supernodulating phenotype which resulted in up to 40 times the nodule number of the wild-type Bragg parent.

The nodulation mutatants nts382, nod49 and nod139 are recessive and unlinked to each other. In a plant homozygous recessive for the non-nodulating locus and the

supernodulating locus (a double mutant), the supernodulating phenotype was epistatically suppressed (Mathews *et al.*, 1990).

In experiments where wild-type shoot and root systems were grafted to non-nodulating mutant shoot and root systems, the non-nodulating phenotype was regulated by the root stock from which it originated and was not altered by a wild-type shoot (Delves *et al.*,1986, 1987a,). In experiments where the wild-type shoot and root systems were grafted to the supernodulating mutant root and shoot systems respectively, the supernodulating or wild type nodulation phenotype was regulated by the shoot. A supernodulating shoot grafted to a wild-type root produced a supernodulating root system (Delves *et al.*, 1987a, b).

When roots of an intact wild-type plant were separated into two compartments and one compartment inoculated, nodulation of that root branch strongly inhibited future nodulation of the other root branch. This inhibition of nodulation in the split root experiment was not seen in the supernodulating mutant even in the presence of nitrate (Olsson *et al.*, 1989).

Research in our laboratory has focused on the plant genes involved in the nodulation process. The production of these nodulation mutants has led to research aimed at finding the genes responsible for these changed phenotypes. Through restriction fragment length polymorphism (RFLP) segregation analysis, a molecular marker has already been found that is closely linked to the *nts* locus of the nts382 supernodulating mutant (Landau-Ellis *et al.*, 1991). Work is now focusing on discovering the sequence of this mutated locus. Any putative sequences discovered will have to be tested via plant transformation to prove functionality to these genes. This will be performed as in classical complementation experiments. The putative wild-type sequence will have to be transformed into the mutant plant to determine if the nodulation phenotype is thereby corrected.

Plant transformation by Agrobacterium tumefaciens

Agrobacterium tumefaciens has successfully been used to genetically engineer a wide range of dicotyledonous plants (for a review: Chilton, 1983; Petit *et al.*, 1985; Zambryski *et al.*, 1989; Figure 1).

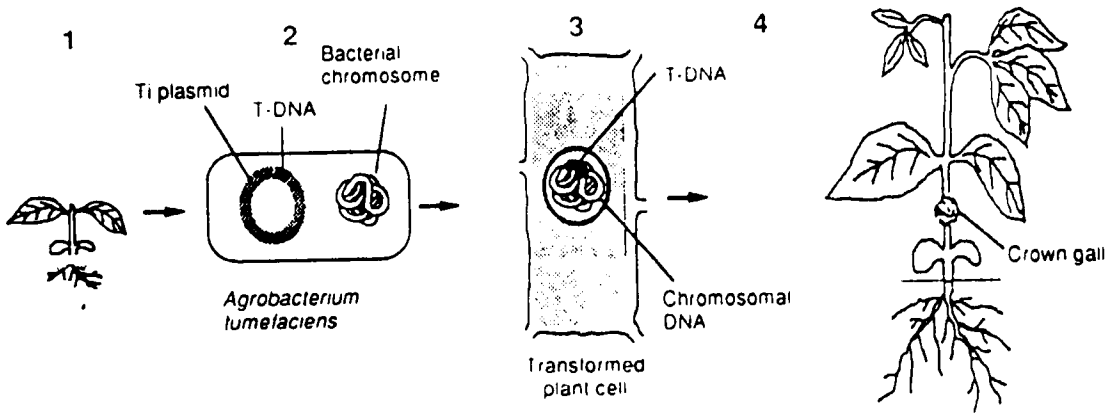


Figure 1. Transformation of plant tissue by the plant pathogen *Agrobacterium tumefaciens*. (1) A virulent bacterium enters the plant at a wound site. (2) The *Agrobacterium* harbors a Ti plasmid in addition to its bacterial chromosome. A region of the Ti plasmid called the T-DNA is transferred into the plant cell. (3) This becomes integrated into the plant chromosome. This T-DNA encodes genes for the production of cytokinins and opines. The cytokinins induce cell divisions which eventually to gall or tumor formation. The opines serve as metabolites for the *Agrobacterium* living in the intercellular spaces of the gall.

This has been achieved by utilizing disarmed vectors to insert foreign genes into plants (Bevin, 1984). However, the susceptibility of different plant species and cultivars to *Agrobacterium* varies greatly.

Susceptability of soybean to Agrobacterium

Soybean is not very susceptible to *Agrobacterium* (DeCleene and DeLey, 1976). The compatibility or virulence of specific *Agrobacterium* strains is dependent on the genetic background of both the host and the pathogen. Genes in the bacterial chromosome and the Ti plasmid determine the bacteria's virulence (Gresshoff *et al.*, 1978). Bryne *et al* (1987) screened seventeen soybean cultivars including cv. Bragg for their susceptibility to 'non-disarmed' *A. tumefaciens* strains A208 (nopaline) and A281 (octopine), and *A. rhizogenes* strains agropine (A4). They found that the nopaline strain A208 was the most virulent on all cultivars tested but showed a very large range of susceptibility between cultivars. Only 3% of the inoculation sites in Bragg produced galls, whereas 100% of the

Peking inoculation sites produced galls. The octopine strain A281 produced galls on only three of the seventeen cultivars.

Both Agrobacterial strains A208 and A281 are termed 'wild-type' by the fact that they are 'non-disarmed' and thus produce the crown gall disease. However strictly speaking the A281 and A208 strains are novel Ti plasmid/bacterial combinations. The 'super virulent' A281 strain was produced by the introduction of the non-disarmed pBo542 Ti plasmid into a cured C58 *Agrobacterium* background (Hood *et al.*, 1983, 1986). The A208 strain was produced by the introduction of the non-disarmed pT37 Ti plasmid into a cured C58 *Agrobacterium* background(Sciaky *et al.*, 1978)

Genes have successfully been transferred at a low frequency into the susceptible soybean cultivar Peking (Hinchee *et al.*,1988). This report used a disarmed A208 *Agrobacterium* coupled to a cotyledonary node regeneration system developed by Wright *et al.* (1986a,b). Zhou and Atherly (1990) reported soybean transformation using cv. Peking and the same regeneration system as above but with a non-disarmed A281 strain.

Agrobacterium virulence genes

There are a number of genes in *Agrobacterium* that are responsible for virulence (for reviews see Zambryski *et al.*, 1988, 1989; Stachel *et al.*, 1986a, 1986b). Most of these genes *virA, B, C, D, E* and *F* are located on the Ti plasmid (Stachel and Nester, 1986). Two virulence genes *chvA* and *B* are located on the bacterial chromosome. The VirA protein is a non-inducible trans-membrane chemo-receptor. The VirB protein is also thought to be a trans-membrane complex. The VirC protein is thought to determine host specificity. The VirD protein is thought to be an endonuclease which "clips" the T strand at the T-DNA border sequence (Wang *et al.*, 1987; Ward *et al.*, 1988). The VirE protein binds to the single stranded T-DNA strand possibly stabilizing the T-DNA during transfer into the plant cell. The VirF protein production is inducible and possibly involved in the host-pathogen interaction. Mutations in the *VirA, B, G,* and *D* loci abolish virulence where as mutations in *VirC* and *E* attenuate virulence (Klee *et al.*, 1983). The products from the chvA and B genes are thought to be involved in bacterial attachment to the plant cell. Mutations in the chvA and B genes produces a loss in virulence and bacterial mobility. Manipulation of these genes could possibly increase the virulence or host specificity of an *Agrobacterium* strain.

Purpose of study

The purpose of the study was to test the feasibility of transforming these soybean nodulation mutants with an *Agrobacterium* based system. This was undertaken in mind that transformation could be used to identify the the sequences mutated in the nodulation mutants by functional complementation.

It was decided to screen the Bragg derived nodulation mutants for their susceptibility to 'wild-type' A208 and A281 *in vitro* and *in vitro*. Much work has been undertaken on either inducing the virulence genes of *Agrobacterium* with specific media (Alt-Mörbe *et al.*, 1989., Ankenbauer and Nester, 1990) or the isolation and addition of virulence genes (Jin *et al.*,1987). Change in susceptiblity of the soybean mutants was attempted by stimulating the bacterial virulence genes with specific media or increasing the virulence of the *Agrobacteria* by gene addition.

One of the goals was to find out if nodulation mutations in these soybean plants had an effect on *Agrobacterium* susceptability. *Rhizobium* and *Agrobacterium* are members of the Rhizobiaceae family. As these bacteria are closely related it was feasible that non-nodulating soybeans could be non-susceptible to *Agrobacterium* and supernodulating soybeans could be ultra-susceptable to *Agrobacterium*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Initial experiments were conducted without the addition of binary plasmids to the wild-type *Agrobacterium tumefaciens* strains A208 and A281. In these wild-type bacteria the oncogenes, or cytokinin producing genes, responsible for gall formation were located on a large 200kb Ti (tumor inducing) plasmid (Watson *et al.*, 1975). Later experiments utilized expression markers on binary plasmids to make detection of the transformation events more accurate and easier. These plasmids were introduced by triparental mating (Van Haute *et al.*, 1983). Nopaline strain A208 and agropine strain A281 were grown for 4 days on Bergersen's minimal (BMM) (Bergersen, 1969) solid medium at 27°C with no selection prior to inoculation. In later experiments the plasmid p35S GUS INT (Vancanneyt *et al* ., 1990) was introduced into the wild-type *Agrobacterium* strains A208 and A281 by triparental mating using the helper plasmid pK2013. The strains harboring

this plasmid were cultured and maintained on BMM medium containing 100 mg/l kanamycin. In further experiments the plasmid pToK47 (kindly provided by Dr. Eugene Nester, University of Washington, Seattle) encoding the virulence regions of A281 (Jin *et al.*, 1987) was introduced again by triparental mating into both A208 and A281 already harboring the p35S GUS INT plasmid (Figure 2). The bacteria containing this plasmid was selected on spectinomycin 100 mg/l. All *Agrobacterium* matings were checked by isolation of plasmid DNA from the *Agrobacterium* and back-transformation of these plasmids back into *Escherichia coli* strain JM83. Restriction digest analysis was used to confirm the identity of the plasmid.

Nopaline assay

Nopaline was assayed by paper chromatography according to Otten and Schilperpoort (1978).

Stimulation of Agrobacterium virB and virG genes

Agrobacterium were stimulated by pipetting 1ml of one of the following solutions onto 4 day old bacterial plates and mixing the cells with a sterile loop: acetosyringone, simple induction medium (SIM) or sterile water (as a control) five hours before inoculation. Acetosyringone was added to the bacterial cells as a 200 µM aqueous solution. The SIM media described by Alt-Morbe *et al.* (1989) to induce the *virG* and *virD* genes consists of 2% sucrose, 200 µM acetosyringone and 20 mM sodium citrate, pH 5.5.

Plant growth and inoculation conditions

Soybean plants were grown from seed in the greenhouse under natural light (16/8 hour light/dark photo-period) in Faffard™ soilless compost supplemented with osmocote. Nine day old seedlings were used for inoculation. Four sites per plant were inoculated. The inoculations were made on the hypocotyl, epicotyl and both cotyledonary petioles (Figure 3) by stabbing the stem with a sterile wooden tooth picks swabbed with *Agrobacterium* grown on solid BMM medium containing the suitable antibiotics. Lanolin was used to cover each inoculation site to prevent desiccation.

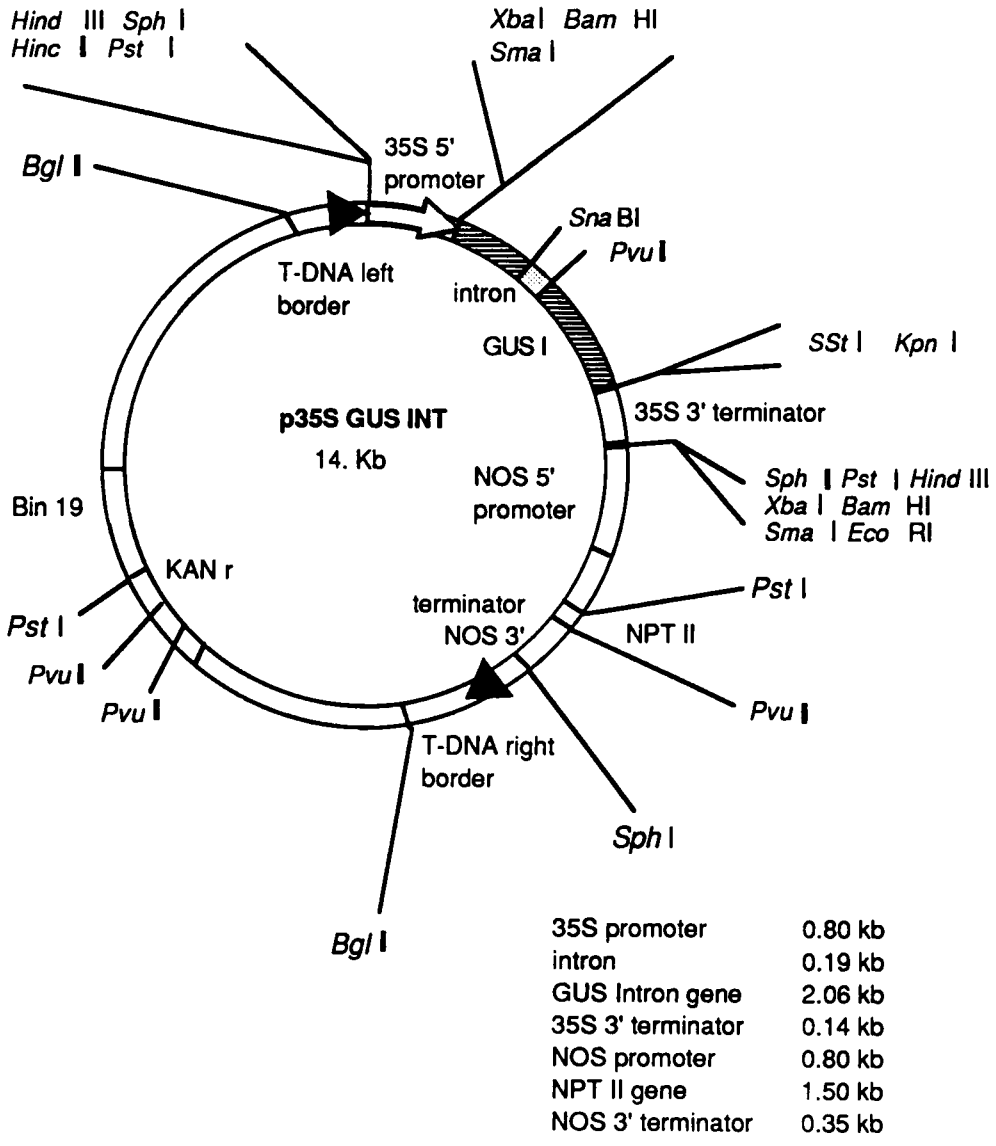


Figure 2. The p35S GUS Intron plasmid: a disarmed binary vector for use in soybean transformation. Restriction analysis by Natasha Taranenko (Plant Molecular Genetics, UT, Knoxville).

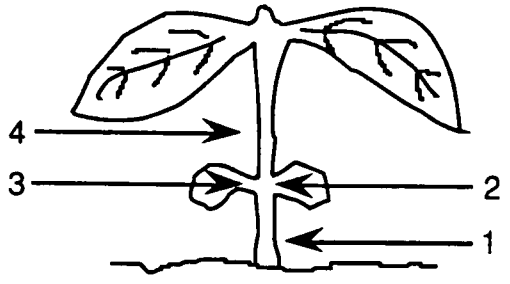


Figure 3. Sites of inoculation of *Agrobacterium tumefaciens* on nine day old soybean plants.

GUS assay of gall tissue

The gall tissue was assayed for ß-glucuronidase (GUS) activity by incubation for 12 hours in 5-bromo, 4-chloro, 3-indolyl ß-D glucuronic acid (X-Gluc) at 37°C as described by Jefferson (1987). The appearance of a dark blue color was taken as a indication of GUS activity. The assayed tissue was cleared and preserved in 70% ethanol.

Susceptibility of the nodulation *mutants to Agrobacterium tumefaciens A208 in vivo.*

Sixteen plants per genotype were tested at four inoculation sites per plant. Four plants per cultivar were used as controls by wounding with a sterile wooden tooth pick free of *Agrobacterium*. Gall formation was verified visually thirty five days after inoculation. Nopaline assays were performed on the galls according to Otten and Schilperoort, (1978). The total number of galls produced per cultivar was recorded.

Susceptibility of the nodulation mutants in vitro to Agrobacterium tumefaciens A208 infection.

Seeds were surface sterilized by the following procedure; 5 minute wash in a soapy aqueous solution of the fungicides Captan™ and Banrot™, followed by a 10 min wash in 40% Chlorox containing 0.01% Tween 20, and then rinsed five times in sterile distilled water. At each stage these solutions were vigorously agitated on a shaker at 50 rpm. The seeds were then allowed to soak for one hour in an aqueous solution of Captan™ and Banrot™ before plating on a medium composed of B_5 salts and minimal organics (Gamborg *et al.*., 1968), 30 g/l sucrose, 8 g/l sigma purified agar at pH 5.6.

After 5 days of culture under continuous light 40 $\mu Em^{-2}s^{-1}$ at 27°C, the cotyledons and small hypocotyl segments (3-5 mm) were dissected from the seedlings. Inoculation of *Agrobacterium* A208 was performed on each cut surface of the hypocotyl segment and the cotyledonary petiole. Twenty cotyledons (ten per plate) and twenty hypocotyl segments (twenty per plate) were inoculated. The tissue segments were then co-cultured for two days under the same growth conditions on a medium containing 1/10th B_5 salts, 30 g/l sucrose and 200 µM acetosyringone, pH 5.6. The tissue was then transferred to a medium composed of B_5 salts, 30 g/l sucrose, 8 g/l agar, 100 mg/l cefotaxime and 500 mg/l carbenicillin, pH 5.6 and further cultured for 35 days in an illuminated incubator 40

$\mu Em^{-2}s^{-1}$at 27°C. The subsequent tumor tissue formed was assayed for nopaline activity (Otten and Schilperpoort, 1978) as an indicator of transformed tissue.

Stimulation of gall formation by the culture of Agrobacterium with different media prior to inoculation

Stimulation of gall size and frequency by the culture of *Agrobacterium* with different media prior to inoculation was investigated. The stimulatory media (acetosyringone or SIM) were reported to increase the bacterial virulence by stimulating *VirB* and *G* gene expression (Alt-Mörbe *et al.*, 1989., Ankenbauer and Nester, 1990). The bacteria used in this experiment harbored the binary p35S GUS intron plasmid (Figure 2). This plasmid was included as marker for transformation. Incorporation of plasmids and stimulation of the bacteria were as described previously. Plant growth and inoculation were as previously described except that four plants with four inoculation sites were used per treatment (16 wound sites). As a control, tobacco plants cv. Xanthi were also inoculated. A visual assay of gall size and number was performed thirty five days after inoculation. A sub-sample of these plants showing gall formation were assayed for GUS activity. The average gall size per treatment was graded in the following way: very large > 10 mm(++++); large 5-10 mm (+++); medium 3-5 mm (++); small < 3 mm (+); none existent (-); (scale adapted from Bryne *et al.*, 1987). The gall fresh and dry weights were measured where the galls were large enough to excise.

Simulation of gall formation by the inoculation of Agrobacterium harboring a plasmid duplicating the virulence genes virB and virG region from A281

Inclusion of a plasmid harboring a duplication of the *virB* and *virG* region from A281 on the plasmid pToK47 into strain A281 was shown to increase the transformation efficiency (virulence) of *Agrobacterium tumefaciens* A281 to tobacco (Jin *et al.*, 1987). An attempt was made to duplicate the results *in vivo* with soybean. Bacteria and plants were prepared as described in previous sections. Sixteen plants were inoculated at four sites per plant (32 wound sites) and cultured for thirty five days prior to a visual assay of the total number of galls formed. Fresh and dry gall weights were collected only from cv. Peking as the galls from the other plant genotypes were too small.

RESULTS

In vivo gall formation

The susceptible cultivar Peking produced large galls at all of the inoculation sites on all the plants (Figure 4). The *in vivo* susceptibility of Bragg (Figure 5, Plate 5) and the derived nodulation mutants nod49 (Figure 6), nod139 (Figure 7) and nts382 (Figure 8) to *Agrobacterium* A208 was significantly lower than for Peking (Table 1). Wounded but uninoculated plants were used as controls to test for tissue callusing of the wound area, none was observed (Figure 9). *Kalanchoe daigremontiana,* a known susceptible plant species to *Agrobacterium tumefaciens,* was used as a positive control for gall formation (Figure 10). Nopaline assays were performed on a subset of the gall tissues to confirm transformation (Figure 11 and Table 2).

Table 1. Gall formation and nopaline assays on *in vivo* grown plants after inoculation with *Agrobacterium tumefaciens* A208.

Cultivar	frequency of gall formation	%	frequency of galls positive nopaline	%
Peking	64/64	100	8/8	100
Bragg	35/64	55	35/35	100
nod139	32/66	48	27/32	84
nod49	18/64	28	17/18	94
nts382	17/66	25	13/17	76

In vitro gall formation on cotyledonary petioles and hypocotyl segments inoculated with A208.

Soybean tissues from all the genotypes tested showed slight callusing *in vitro* (Figure 12, Plate 6). Cotyledonary petioles and hypocotyls from cultivar Peking galled extensively *in vitro* (Figures 13 and 14) Thus to differentiate between callus and gall formation nopaline assays were essential.

Plate 5. Susceptibility soybean nodulation mutants to *Agrobacterium tumefaciens in vivo.*

Figure 4. Gall formation on soybean cv. Bragg after inoculation with *Agrobacterium tumefaciens* A208.

Figure 5. Gall formation on a non-nodulating soybean nod139 thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

Figure 6. Gall formation on a non-nodulating soybean nod49 thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

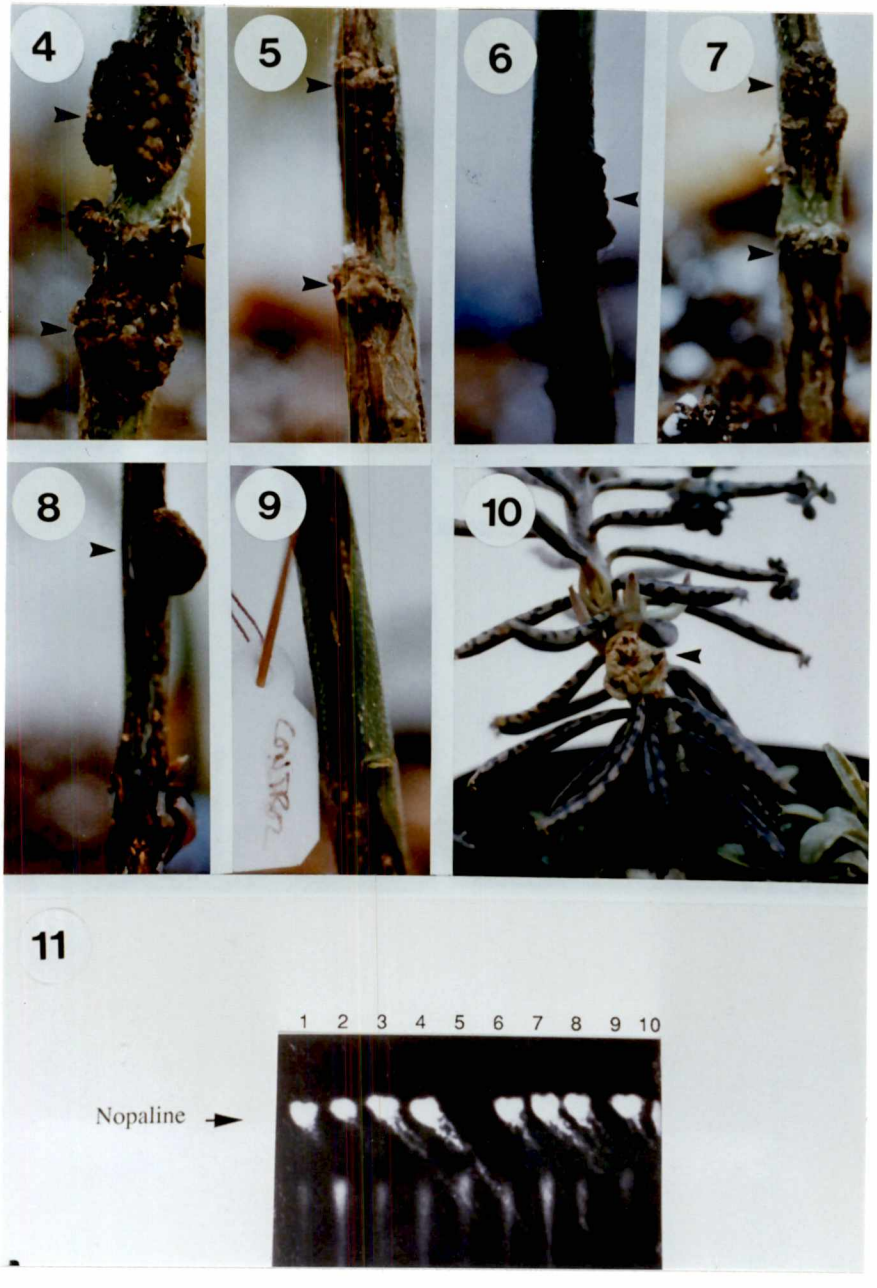
Figure 7. Gall formation on a supernodulating soybean nts382, thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

Figure 8. Gall formation on a plant cv. Peking thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

Figure 9. A control plant cv. Peking wounded but not inoculated shows that there is no callusing due to the wounding. This plant was one of the negative controls in the experiment.

Figure 10. Gall formation on a *Kalanchoe daigremontiana* plant thirty five days after inoculation with *Agrobacterium tumefaciens* A208. This plant was known to be highly susceptible to *Agrobacterium tumefaciens* and was a positive control.

Figure 11. Nopaline assays on the gall tissues from cv. Peking plants. Tissue was loaded on the paper and migrated up the paper (to the negative electrode). Nopaline could be visualized as a spot by its fluorescence under a UV light after spraying with a basic (OH^-) phenanthrenequinone solution. Other fluorescing plant compounds could be seen beneath this spot. Lanes 1 to 4 and 6 to 9 were tissue samples from individual Peking galls. Lane 5 was non-transgenic stem tissue from a Peking plant. Lane 10 was gall tissue from a Kalanchoe plant. This assay was used as an indicator of transformed tissue.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Nopaline

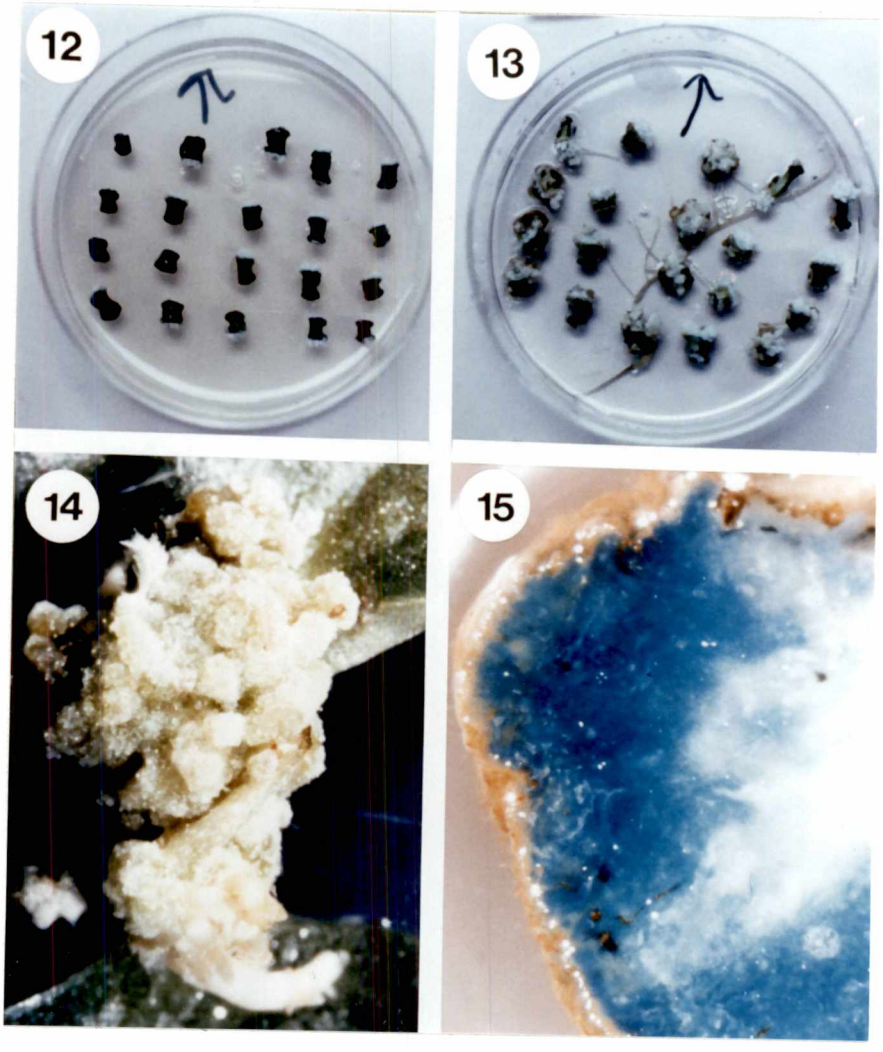
Plate 6. Susceptibility of soybean to *Agrobacterium tumefaciens in vitro.*

Figure 12. Hypocotyl segments from soybean non-nodulating mutant nod49 thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

Figure 13. Gall formation on hypocotyl segments from soybean cv. Peking thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

Figure 14. Gall/callus formation on a soybean cv Peking cotyledonary petiole thirty five days after inoculation with *Agrobacterium tumefaciens* A208.

Figure 15. An example of a cv. Peking gall expressing GUS activity. The *Agrobacterium* inoculum harbored a binary plasmid encoding the GUS intron gene. *Agrobacterium* is unable to express GUS activity because of the intron, and the GUS gene product is only found when it is properly modified in the plant cell.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Cultivar Peking showed the highest level of nopaline positive cotyledons with 59% testing positive compared to 10% for Bragg, and 0% for nts382, nod49 and nod139. The hypocotyl segments gave even lower levels of transformation with Peking exhibiting 20%, nod49 exhibited 5% and nts382, nod139 and Bragg exhibiting 0% nopaline positive (Table 2). This demonstrated that transformation levels for all the lines tested are lower *in vitro* and that stimulation of the *Agrobacteriium* with acetosyringone did not significantly increase the transformation level of an already non-susceptible cultivar.

Table 2. Nopaline assays on gall tissues grown *in vitro* after inoculation with *Agrobacterium* A208.

	nopaline positive		nopaline positive	
Genotype	hypocotyl culture	%	cotyledon culture	%
Peking	4/20	20	10/17	59
Bragg	0/40	0	5/50	10
nod139	0/20	0	0/20	0
nod49	1/20	5	0/20	0
nts382	0/20	0	0/50	0

GUS assays on transgenic galls

Agrobacterium strains containing the p35S GUS intron plasmid were used for all subsequent experiments to assay for transformed gall tissue. An example of a Peking gall expressing GUS activity can be seen in Figure 15. This assay for transgenic galls held the added advantage that the assay was quicker and simpler and clearer to read than the nopaline assay.

Stimulation of Agrobacterium virulence genes prior to inoculation

Because of the low susceptibility of cv. Bragg and the derived nodulation mutants to *Agrobacterium tumefaciens* A208, attempts were made to overcome this susceptibility barrier. The soybean susceptibility to the 'supervirulent strain' A281 was also tested (Table 3).

Table 3. Gall size produced after stimulation of *Agrobacterium tumefaciens* A281 and A208 upon inoculation with acetosyringone SIM medium or water. Visual assay was determined thirty five days after inoculation (sixteen galls per treatment : four inoculation sites on four plants).

	Genotypes									
Treatment	Peking		Bragg		nts382		nod49		nod139	
	A208	A281	A208	A281	A208	A281	A208	A281	A208	A281
H_20	++	+	+	+	+	+	+	+	+	+
Aceto.	+++	+	+	+	+	+	+	+	+	+
SIM	++++	+++	+	+	+	+	+	+	+	+

Key: + small galls (<3mm in diameter)
++ medium sized galls (3-5mm in diameter)
+++ large sized galls (5-10 mm in diameter)
++++ very large galls (>10 mm in diameter)
Aceto: Acetosyringone

Attempts were made to increase the susceptibility *in vitro* by the preculture of the *Agrobacterium* in media previously reported to increase virulence (Alt-Morbe *et al.*, 1989).

Agrobacterium preculture in the SIM media produced significantly larger galls in cv. Peking but not cv. Bragg and the derived nodulation mutants (Table 4) (data for cv. Bragg and the mutants was not collected as the galls were too small to weigh). Tobacco plants were used as positive controls in this experiment (Table 5). Figure 16 (Plate 7) shows a typical Peking gall produced from *Agrobacterium* pre-cultured with H_2O, While Figure 17 shows a gall from *Agrobacterium* pre-cultured with acetosyringone. Figure 18 shows a gall produced from *Agrobacterium* precultured with SIM medium. The galls produced from *Agrobacterium tumefaciens* A208 induced with the SIM medium gave an average gall of fresh weight of 1.085 g (dry weight of 0.379 g) compared to a fresh weight of 0.690 g (dry weight of 0.209 g), when the *Agrobacterium* was stimulated with acetosyringone.

Table 4. Fresh and dry weight of galls (mg) produced on cv Peking plants. *Agrobacterium tumefaciens* strains A281 and A208 were pre-treated for five hours prior to inoculation with either acetosyringone SIM medium or water. Four plants were innoculated per treatment at four sites on each plant (sixteen galls) ±standard error. **b)** same treatment with tobacco.

Treatment	***Agrobacterium strain***			
	A208		A281	
	fresh wt (mg)	dry wt (mg)	fresh wt (mg)	dry wt (mg)
H_20	690±82	193±23	14±3	4±1
GUS I	625±50	198±16	13±4	5±2
Acetosyringone	690±68	209±18	65±8	31±4
SIM	1085±126	379±44	550±50	228±22

Table 5. Tobacco (cv. Xanthi), dry gall weights expressed in milligrams. The bacteria were pre-treated with either water, acetosyringone or SIM medium for five hours prior to inoculation.

Treatment	***Agrobacterium strain***	
	A208	A281
	dry wt (mg)	dry wt (mg)
H_20	22±9	N/D
Acetosyringone	61±17	87±9
SIM	460±4	121±32

The effect of duplication of the virB and virG genes from Agrobacterium tumefaciens A281 on gall formation

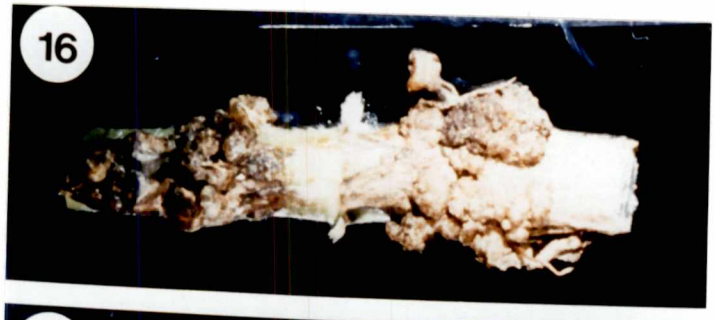
Attempts to increase the virulence of the *Agrobacterium* by the duplication of virulence genes *virB* and *virG* from *Agrobacterium* A281 were investigated. Jin *et al.* (1987) showed that these genes, if duplicated, increased galling on *Nicotiana glauca* and *Kalanchoe daigremontiana.*

Plate 7. Stimulation of *Agrobacterium tumefaciens* gall formation.

Figure 16. Gall formation on a soybean plant cv. Peking thirty five days after inoculation with *Agrobacterium tumefaciens* A208. The bacteria was pretreated for five hours before inoculation with water.

Figure 17. Gall formation (maximum diameter 20mm)on a soybean plant cv Peking 35 days after inoculation with *Agrobacterium tumefaciens* A208. The bacteria was pretreated for five hours before inoculation with 200 µM acetosyringone.

Figure 18. Gall formation (maximum diameter 30mm) on a soybean plant cv. Peking 35 days after inoculation with *Agrobacterium tumefaciens* A208. The bacteria was pretreated for five hours before inoculation with a simple induction medium (SIM: 200µM acetosyringone, 20mM sodium citrate and 2% sucrose pH 5.5).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It was suggested that the inclusion of such genes on the pToK47 plasmid into *Agrobacterium* may increase the virulence and host range of certain *Agrobacterium* (Jin *et al* 1987). The plasmid carrying these genes was mated into strains A281 and A208 and checked for transfer by back-mating. Cultivars Peking, Bragg and the nodulation mutants nts382, nod49 and nod139 were inoculated with these strains. The soybean nodulation mutants tested and showed no difference in size or number of galls formed (Table 6).

Table 6. Introduction of extra copies of *virB* and *virG* in *Agrobacterium tumefaciens* A281 and A208 and their effect on the frequency of gall formation.

	Gall formation			
Genotype	A208	A208+*vir B,G*	A281	A281+ *virB,G*
Peking	32	32	32	32
Bragg	13	12	7	8
nod139	9	10	5	6
nod49	9	7	5	7
nts382	8	9	4	5

A208+*virB, G: Agrobacterium* strain A208 harboring the pToK47 plasmid

A281+*virB,G:Agrobacterium* strain A281 harboring the pToK47 plasmid

Thirty two inoculation sites per treatment were used (eg. eight plants with four inoculation sites per plant).

CONCLUSION

This study demonstrated that there was no correlation between susceptibility to *Agrobacterium* and the supernodulation or non-nodulation phenotypes in soybean. Cultivar Peking was significantly more susceptible than cv. Bragg and the nodulation mutants. It appears that the nodulation mutants may have had lower susceptibility to the *Agrobacterium* strains used than Bragg. Clearly the plant can differentiate between the pathogen and the symbiont.

In vitro susceptibility to *Agrobacterium* was shown to be lower than *in vivo* susceptibility even when the bacteria were stimulated with acetosyringone. This result supports past work on soybean susceptibility to *Agrobacterium* (Owens and Cress, 1985). The reason

for this difference *in vitro* and *in vivo* is not clear. One can hypothesize that galling was increased *in vivo,* because the time allowed for infection *in vivo* is longer, or that the inoculated tissue was growing faster. Also possibly the culture medium *in vitro* suppresses either the bacteria or the plant tissues reponse.

A simple induction medium (SIM) used to stimulate the inoculated bacteria was clearly effective in increasing the mass of the galls formed in a susceptible cultivar such as Peking. However, no such effect was seen with the non-susceptible cultivars such as Bragg and the nodulation mutants. The increased gall size in cv. Peking may have been due to an increase in the initial number of cells transformed.

Inclusion of a plasmid harboring a duplication of the *virB* and *virG* region from A281 on the plasmid pToK47 into both A281 and A208 failed to increase the virulence of the bacteria on cv. Peking or cv. Bragg and its derived nodulation mutants. VirB and VirG proteins are important in signal transduction and pore (VirB) formation in bacterial and plant cell membranes during T-DNA transfer. It appears that this step in the infection process is not limiting in soybean transformation.

The supervirulent strain A281 was weakly pathogenic on the soybean cultivars tested, verifying the results obtained by Bryne *et al.* (1987). This result was contrary to the results obtained by Zhou and Atherley (1990), who suggested that strain A281 was the best available strain to transform soybean.

The use of a GUS intron plasmid and GUS assays in testing the wild-type *Agrobacterium* galls for transformation showed significant advantages over opine assays, which were disadvantageous because of the time required to perform the assay and low levels of endogenous opines in soybeans (Christou *et al.*, 1986). Also there was the posibility of a false positive results due to bacterial expression of nopaline. Often the nopaline assays would be hard to read because of a shadow of nopaline activity in the control lanes. The GUS assay gave a clear positive marker for plant cell transformation and should be the assay of choice.

Since the virulence of the wild-type A208 strain was higher than the disarmed A208 (Hinchee *et al.*, 1988) strain this study only approximated the true transformation frequency with an engineered *Agrobacterium*. However, it was apparent that transformation of cv. Bragg and its nodulation mutants with an engineered

Agrobacterium would be significantly less efficient than transformation of cv. Peking. The commonly used *Agrobacterium* strains may not suitable for the transformation of these nodulation mutants. It is possible that there are other as yet untested wild-type *Agrobacterium tumefaciens or A. rhizogenes* strains which are highly virulent on these cultivars.

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CHAPTER 4

MICRO-PROJECTILE 'GENE GUN' DESIGN, AND OPTIMIZATION OF TRANSFORMATION PARAMETERS

ABSTRACT

Helium and electric micro-projectile gene guns were constructed and compared with a commercially available helium particle gun for their utility in transformation. The frequency of transient gene expression was measured in tobacco (*Nicotiana tabacum*) and cowpea (*Vigna unguiculata* or *sinensis*) leaf cultures as well as in soybean (*Glycine max*) suspension cultures. The 'UT-built' helium gun performed better than the other guns. Baffle size and placement, DNA purity, and phytohormones requirement were optimized for transient gene expression. Stable transformation of soybean suspension cultures was demonstrated by means of GUS assay, PCR analysis and Southern hybridization five months after initial bombardment with the 'UT-built' helium gun.

INTRODUCTION

In recent years, plant transformation strategies have come to rely on micro-projectile gun (particle gun) delivery of DNA into plant cells (also called 'biolistic' transformation) to overcome the transformation barriers of species and cell types. This technology uses micron-sized tungsten (or gold) particles coated with DNA accelerated to high velocities, facilitating penetration of plant cells. This approach has been used to obtain and examine gene expression of both transient and stable transformations of plant cells. In some cases whole transformed plants have been regenerated, (see Mendel, 1990 for a review). More commonly transformations have involved tissue cultured cells transiently expressing the chloramphenicol acetyltransferase (CAT; Klein *et al.*, 1988a) or the ß-glucuronidase gene (GUS; Jefferson 1987).

The first description of the methodology of particle bombardment (Sanford *et al.*, 1987) was concurrent with the report of transfer of tobacco mosaic virus (TMV) RNA and CAT into onion (*Allium cepa*) epidermal cells (Klein *et al.*, 1987). Klein *et al.* (1988a,b) presented many parameters involved in bombardment of micro-projectiles into corn suspension cells. Tobacco leaves and suspension cultures were also transformed with the GUS gene (Klein *et al.*, 1988c). Further application of the biolistic approach came from

transient expression of chimeric genes in tobacco pollen (Twell *et al.*, 1989) and chloroplasts (Daniell *et al.*, 1990).

Another dicotyledonous plant to be successfully transformed using particle bombardment was soybean. Particle bombardment of soybean immature embryos gave rise to kanamycin resistant protoplasts and subsequent callus (Christou *et al.*, 1988). This was followed by stable transformation and regeneration of soybean plants from bombarded immature embryo axes with a GUS construct (McCabe *et al.*, 1988; Christou *et al.*, 1989). Since then Finer and McMullen (1991) and Parrott *et al.* (1992) have produced transgenic soybean plants by bombardment of embryogenic suspension cultures.

Monocotyleydonous plants, and particularly cereal grasses, have been difficult to transform using the bacterial pathogen *Agrobacterium tumefaciens*. However, they have been readily adapted to transformation by particle bombardment. Successful transformations have included transient CAT (Klein *et al.*, 1988a) and GUS (Klein *et al.*, 1988b; Oard *et al.*, 1990) expression in suspension culture cells. Expression of anthocyanin biosynthesis related genes in a variety of tissues (Klein *et al.*, 1989; Ludwig *et al.*, 1990) and most recently the production of regenerated transformed plants (Gordon-Kamm *et al.*, 1990). Significant progress was achieved with rice (*Oryza sativa* L.; Wang *et al.*, 1988; Bruce *et al.*, 1989; Oard *et al.*, 1990), barley (*Hordeum vulgare* L.; Kartha *et al.*, 1989; Mendel *et al.*, 1989), and wheat (*Triticum aestivum* L.em Thell; Oard *et al.*, 1990). Particle bombardment was also used to complement a chloroplast mutant in *Chlamydomonas reinhardti* (Boynton *et al.*, 1988) and a mitochondrial respiration mutant in yeast (*Saccharomyces cereviseae;* Johnston *et al.*, 1988).

Types of micro-projectile gene guns

The types of micro-projectile gene guns used in the described plant cell transformations vary mainly in their method of macro-projectile propulsion. Three basic types of propulsion exist: 1) gunpowder discharge, 2) compressed gas (helium or compressed air) discharge and 3) electric arc discharge. Gunpowder discharge employs a 0.22 caliber blank charge to accelerate a macro-projectile. This macro-projectile acts as a carrier for micro-projectiles coated with DNA. This gun could be leased from the 'DuPont' Company. The compressed gas discharge type or helium gun was first described by Oard *et al.* (1990). Since then the 'DuPont' Company has updated the 'gunpowder' model with the 'helium' gun and has sold the marketing rights to the scientific supply firm BioRad Inc

(California). The commercially available compressed gas type gun is cleaner to use than the gunpowder discharge type, and has the ability to vary the velocity of the macro-projectile by using differing helium pressures. The electric arc discharge gun propels a macro-projectile by producing a high voltage arc through a drop of water which creates a 'plasma' energy burst. This type of gun was first briefly described by Christou *et al.* (1988; Agracetus Inc., Madison WI). Its success was reported to be also due to the ability to vary the velocity of the macro-projectile with the voltage. This was seen as a significant advance over the gunpowder gun. Independent of the group at Agracetus and largely based on first principles, we constructed an electric arc discharge gun. We also constructed a helium discharge gun (Bond *et al.*, 1992) similar to that described by Finer *et al.* (1991) and compared both 'UT-built' guns to the commercially available Helium gun PS 2000 from BioRad, Inc. We found that the 'UT-built' electric gun gave unsatisfactory results, the 'UT-built' helium gun, however, outperformed the commercially available helium gun in the frequency of both transient transformation of various plant tissues and stable transformation of embryogenic soybean suspension cultures. Additional and significant benefits stem from the lower cost and speed of operation seen with the 'UT-built' helium gun.

MATERIALS AND METHODS

Gun designs

The 'UT-built' electric micro-projectile gene gun was of a construction similar to that described by Christou *et al.* (1988). Limited detail in the published description required that we design many of the parts ourselves with help from the Electrical Engineering Department at the University of Tennessee. The electric power supply is displayed in Figure 1(Plate 8), the firing chamber is displayed in Figure 2, and the vacuum system in Figure 3. Figure 4 shows a photo of the gun itself.

The helium micro-projectile gene gun was constructed according to the plans and directions sent to us by Dr John Finer, Department of Agronomy, Ohio State University. The only major modification was an increase in the chamber height from 30 to 35 cm. Figure 5 (Plate 9) shows a diagram of the gun and includes some of the major specifications. The actual working gene gun is depicted in Figure 6. The 'DuPont' PDS 1000 Helium gun (Figure 7) was assembled and operated as specified by the 'DuPont' Biolistic TM operations manual, 1990.

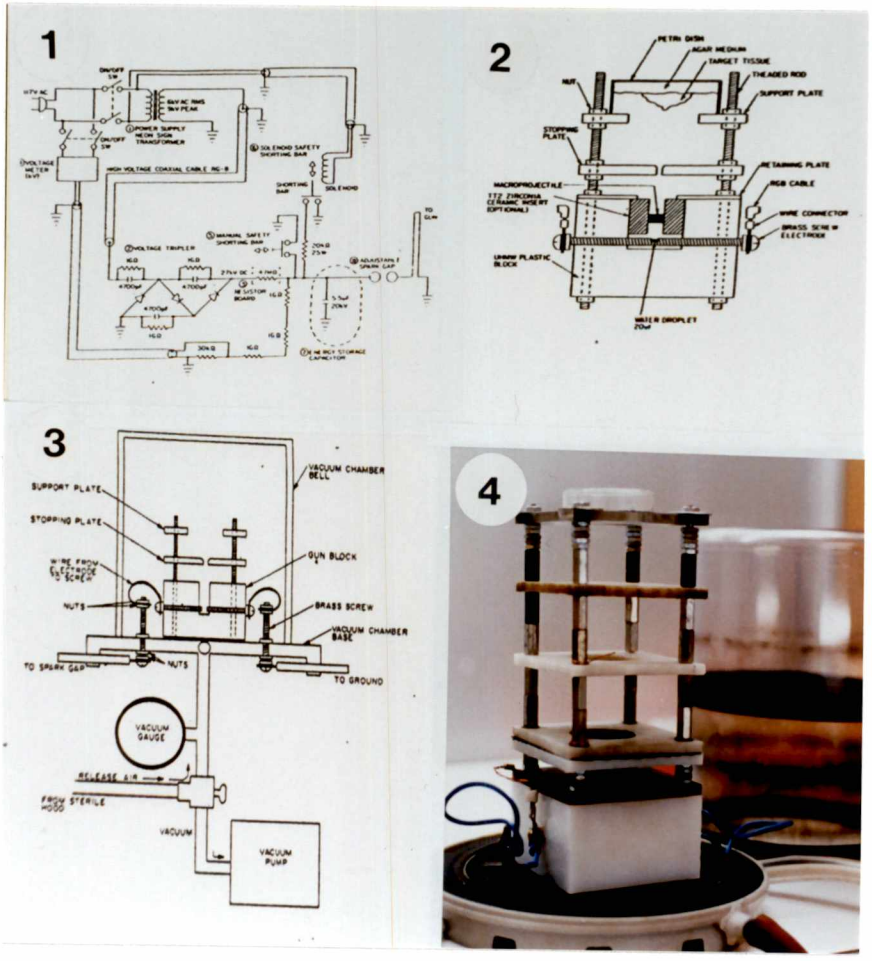
Plate 8. The electric particle gun.

Figure 1. The power supply for the electrical micro-projectile gene gun. The main power supply (117 volts) was directed through an old neon light transformer(1) to produce 9,000 volts (9 KV). The electricity was then directed through a voltage tripler circuit(2) composed of 3 diodes, 3 capacitors and 3 resistors which further increased the voltage to 27 KV. A 5.5 μF capacitor (7) was charged by the 27 KV supply. The rate of charging was measured by a volt-meter(4) connected across the capacitor terminals. The gun was fired by discharging the capacitor at a predetermined voltage through an adjustable spark gap switch(8) composed of two brass door knobs. Extensive safety precautions were taken to ensure the safety of the user. This included using a 2.5 cm thick Lexan™ box enclosing the power supply as well as one automatic(6) and one manual(5) shorting mechanism to avoid electric shock.

Figure 2. The 'firing chamber' of the electrical micro-projectile gene gun. This 'gun' fired a micro-projectile upwards. The propulsion was produced by a 'plasma'. Created by passing 1 to 20 KV through a 20 μl water droplet suspended between two brass electrodes in the firing chamber. This energy burst was used to propel a Nylon™ macro-projectile used as the carrier for the DNA coated tungsten particles. The macro-projectile was accelerated to a velocity determined by the voltage supplied to the gun and then impacted onto a metal stopping plate. This impact propelled the DNA coated tungsten particles through a hole in the stopping plate and through the evacuated chamber into the target tissue. The tissue distance from the stopping plate could be varied from 1 to 15 cm.

Figure 3. The vacuum system to the electric micro-projectile gene gun. The gun stood on a vacuum chamber platform, over which a Lexan™ vacuum bell jar (25x17 cm) was placed to evacuate the chamber to 760mm Hg.

Figure 4. The electric micro-projectile gene gun. High voltage lines come through the chamber's base and connect with brass electrodes in the plastic ultra-high-molecular-weight (UHMW) block which encapsulates the combustion chamber. Plant tissue was placed on one of four platforms at distances of 4, 8, 12 and 16 cm from the stopping plate.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ELECTRODE
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SUPPORT PLATE
STOPPING PLATE
GUN BLOCK
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BRASS SCREW
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TO SPARK GAP
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TO GROUND
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Plate 9. The helium particle gun.

Figure 5. A diagram of the 'UT-built' helium micro-projectile gene gun. Prior to bombardment of plant tissue, a 2 µl suspension of DNA-coated tungsten particles was suspended on a screen in the stainless steel syringe adapter. The gun was supplied with high purity helium gas (National Welders, Knoxville TN) through a gas regulator. The helium pressure was adjusted in the range of 0 to 1250 kPa. A short 50 milli-second burst of helium (normally 500 kPa) was allowed to enter the gun chamber through an electronically triggered solenoid. This propelled the tungsten particles, creating a fine aerosol. The DNA coated tungsten aerosol moved through the evacuated chamber into the target tissue penetrating the plant cell walls. DNA carried into the nucleus was released from the tungsten particles to permit transient gene expression (Yamashita *et al.*, 1991). A small percentage (0.1-1%) of the transiently expressing cells gave stable gene integration in the plant genome .

Figure 6. The 'UT-built' helium micro-projectile gene gun. For aseptic manipulations the gun was kept in a laminar flow chamber.

Figure 7. The "DuPont" PS 2000 helium micro-projectile gene gun. This gun varies from the 'UT-built' helium gun in its method of propulsion. Instead of using a short burst of helium controlled by an electrical solenoid, Kapton^{TM} membranes were designed to rupture at specific helium pressures. Different membranes were available to give a whole range of pressures from 450 to 2200 psi. This provided the propulsion for the DNA coated gold or tungsten particles suspended on a second Kapton^{TM} membrane. This membrane accelerated a short distance before impacting on a stopping screen. The DNA coated particles continued through the screen and into the target tissue placed at various distances in the vacuum chamber.

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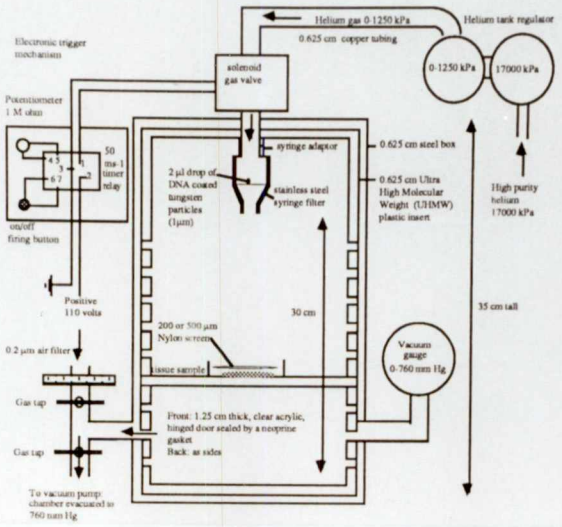
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Potentiometer 1 M ohm
50 ms-1 timer relay
on/off firing button
syringe adaptor
0.625 cm steel box
2 µl drop of DNA coated tungsten particles (1µm)
stainless steel syringe filter
0.625 cm Ultra High Molecular Weight (UHMW) plastic insert
High purity helium 17000 kPa
Positive 110 volts
30 cm
35 cm tall
200 or 500 µm Nylon screen
0.2 µm air filter
Vacuum gauge 0-760 mm Hg
Tissue sample
Gas tap
Front: 1.25 cm thick, clear acrylic, hinged door sealed by a neoprine gasket
Back: as sides
Gas tap
To vacuum pump: chamber evacuated to 760 mm Hg

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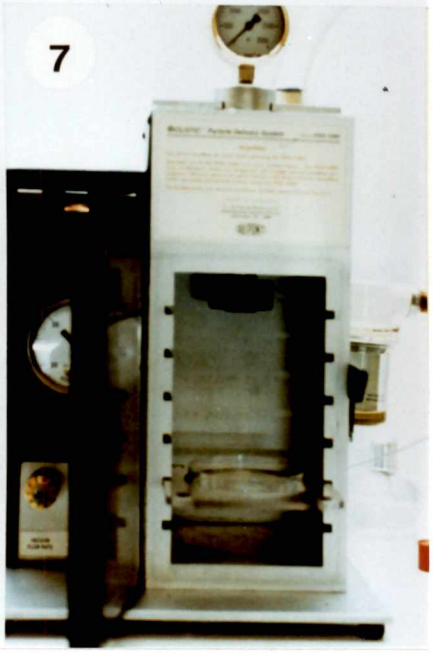

Preparation of DNA and micro-projectiles

All DNA used was prepared by cesium chloride gradient isolation (Sambrook *et al.*, 1989) from a *dam⁻ dcm⁻ Escherichia coli* strain (ATCC culture #47013). This strain which was deficient in the methylation 'machinery' required for DNA methylation, was used to avoid possible problems with the marker gene expression in the plant genome. An association between gene expression levels and methylation of the cytosine and adenine bases in a gene sequence has long been reported in animals and plants (for a review see Cedar, 1988). For transient gene expression studies, a 6.1 kb plasmid (pUC GUS; Figure 8) encoding the GUS reporter gene behind the 35S Cauliflower Mosaic Virus promoter (CaMV) was used. For stable gene expression studies, a 5.2 kb plasmid (pUC HYGRO; Figure 9) encoding the hygromycin B phosphotransferase selectable marker gene behind the 35S CaMV was used in a 50:50 mixture with the p35S GUS plasmid. These plasmids were kindly supplied by Dr. John Finer, Ohio State University. DNA was precipitated using 2.5 M $CaCl_2$ and 0.1 M spermidine according to Klein *et al.* (1988b) onto M10 tungsten powder (approx. 1 μm diameter) supplied by GTE, Sylvania, Towanda, PA, USA.

Culture of tissue

Young leaves from greenhouse-grown tobacco (*Nicotiana tabacum* cv Xanthi) and cowpea (*Vigna sinensis* cv. Coronet) plants were surface-sterilized with 10% Clorox for five minutes and washed four times with sterile water. These leaves were then incubated (adaxialy) for two days before bombardment on a medium (MS 30) composed of MS salts (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose, 2 mg/l naphthalene acetic acid (NAA) and 2 mg/l kinetin and solidified with 8 g/l agar (Difco Bacto agar), pH 5.6. The cultures were maintained under constant illumination (50 μM m^2 s^{-1}) at 25°C. This preculture period was not required but increased transient transformation frequencies.

Embryogenic soybean suspension cultures of cultivars Fayette, Bragg and supernodulating mutant nts1007 (Carroll *et al.*, 1985a; b) were maintained in 150 ml flasks under constant illumination (50 μM m^2 s^{-1}) at 25°C, and constant agitation (150 rpm). Prior to and after bombardment the cultures were grown in 35 ml of liquid suspension medium (modified MS salts) containing 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) as described by Finer and Nagasawa (1988).

Figure 8. The pUC GUS plasmid. This plasmid was used for transient gene expression studies or with the p35S HYGRO plasmid for stable gene expression studies

Figure 9. The pUC HYGRO plasmid. Tissue transformed with this plasmid could be selected for hygromycin resistance. The plasmid was used for stable gene expression studies either on its own or mixed 50:50 with the p35S GUS plasmid.

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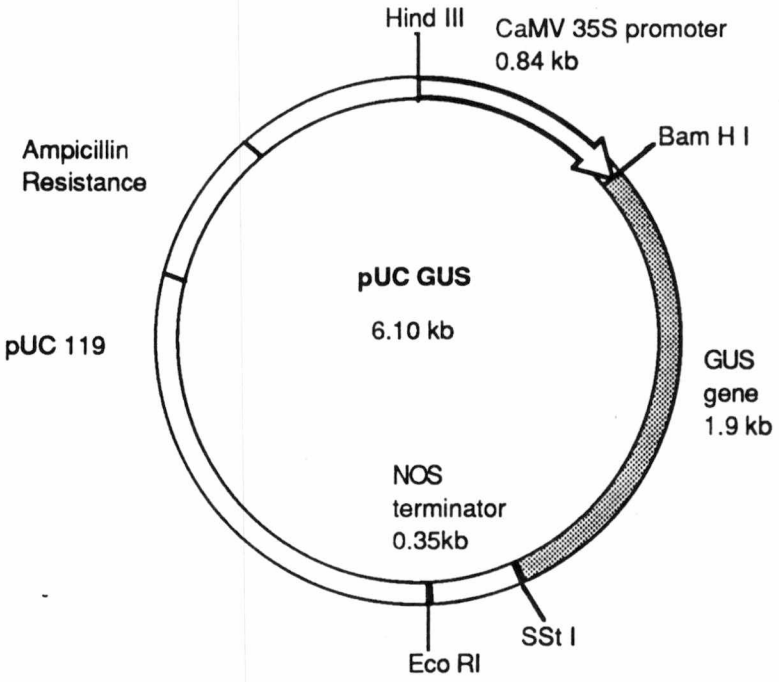
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Bam H I
Ampicillin
Resistance
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pUC 119
GUS
gene
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NOS
terminator
0.35kb
SSt I
Eco RI

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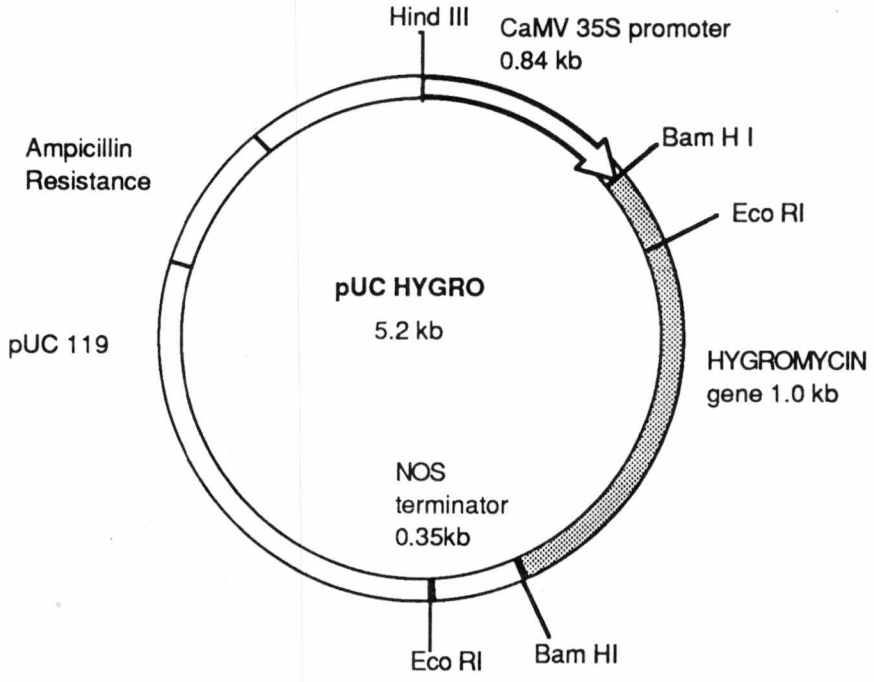
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0.84 kb
Bam H I
Ampicillin
Resistance
Eco RI
pUC HYGRO
5.2 kb
pUC 119
HYGROMYCIN
gene 1.0 kb
NOS
terminator
0.35kb
Eco RI
Bam HI

Inoculum density was kept very low to aid the rapid growth of the suspension cultures (approximately two 3 mm^3 embryogenic clumps per flask). For selection of stable transformants, the suspension cultures were cultured for 2 weeks without selection, then subcultured every week for 10 weeks with 50 mg/l hygromycin added to the culture media. Hygromycin resistant clumps were removed and cultured independently.

Preparation and bombardment of tissue

Approximately 100 mg of soybean embryogenic suspension cultures were placed in an empty 60 mm Petri dish. Any surplus medium was pipetted off to leave air-exposed cell clumps. These were left in the sterile flow hood for approximately 15 minutes until the cell surfaces were visualy dry. Once this state was achieved, the tissue was bombarded. Special care was also taken to ensure that the tobacco and cowpea leaf surfaces were also dry prior to tissue bombardment.

Various sized Nylon^{TM} screens were placed directly on the target tissue prior to bombardment. These screens protected the target cells from the direct helium blast, but permitted the penetration of the DNA coated micro-projectiles.

Bombardment of tissue and subsequent culture

All bombardments were carried out in chambers evacuated to 760mm Hg. Experiments were conducted to investigate several variables which affect transient gene expression such as: 1) distance from tissue to micro-projectile launch, 2) different size screens placed between the tissue and micro-projectile launch, 3) effect of DNA purity, and 4) different micro-projectile velocities. The velocity of the micro-projectiles was varied either by changing the propulsion pressure of the helium gas in the helium guns or by altering the voltage in the electric gun.

After bombardment, the tissues were cultured under conditions identical to those prior to bombardment. This post bombardment period allowed for the phenotypic lag necessary to facilitate the expression of the transferred DNA. Embryogenic suspensions cultured for stable gene integration were cultured under conditions identical to those prior to bombardment for two weeks. The cultures were then subcultured every week with the same suspension medium supplemented with 50 mg/l hygromycin. After ten weeks, green hygromycin resistant clumps were seen. The antibiotic selection was continued for a total

of 20 weeks to produce enough tissue for GUS (Jefferson, 1987), PCR (Mullis and Faloona, 1987) and Southern analysis (Southern, 1975).

Assay of tissue for transient and stable gene expression

The assay for transient gene expression was performed two days after bombardment. This consisted of assaying the tissue for ß-glucuronidase (GUS) activity. Stable gene expression in the tissue was assayed 12 weeks after bombardment. The hygromycin resistant tissue was assayed for GUS activity, the presence of the 35S CaMV promoter by PCR analysis, and gene integration by Southern hybridization.

GUS assay

The GUS assay was performed by incubation of the plant tissue for 12 hr at 37°C in 5-bromo 4-chloro 3-indolyl ß-D glucuronic acid (0.5mg ml^{-1} X-Gluc from Research Organics Inc., Cleveland, Ohio) as described by Jefferson (1987). The tissue was then cleared and preserved in 70% ethanol for two to twelve hours. Transient gene expression (as visualized by blue cells or foci) was examined and counted under a binocular dissecting microscope. Tissue expressing stable GUS activity was compared to non-transgenic tissue.

PCR analysis of transformants

A quick screening for transfer of the p35S GUS plasmid was possible by Polymerase Chain Reaction (PCR) analysis (Mullis and Faloona, 1987). Genomic DNA was isolated from hygromycin resistant suspensions according to Dellaporta *et al.* (1986). DNA transfer was confirmed by PCR amplification (with designed primers 5' CTA CAA ATG CCA TCA TTG CG 3' and 5' ATC ACA TCA ATC CAC TTG CT 3') of a 150bp fragment from the CaMV 35S promoter. This PCR assay indicated the presence of the transgene, but did not show the incorporation status.

Southern blot analysis of transgenic embryogenic suspensions

Genomic DNA from hygromycin resistant embryogenic clumps was isolated according to Dellaporta *et al.* (1986). The DNA was restricted with the restriction enzyme *Eco*RI. Southern blots were probed with a 0.8kb *Bam*HI-*Hin*dIII fragment encoding the 35S

CaMV promoter from the pUC GUS plasmid. Probes were prepared by a random priming reaction using [^{32}P]dCTP-label according to the Boehringer Mannheim Random Primer Labeling Kit. Hybridization and washing were performed at 60^{o}C according to the Zeta Probe^{R}, BioRad hybridization instruction manual. Exposure of the Kodak X-Omatic AR film was for 24 to 72 hrs at -70^{o}C.

RESULTS

The electric micro-projectile gene gun

A number of different electric micro-projectile gene gun designs were tested. One of the later versions is displayed (Figure 4). The 'UT-built' electrical micro-projectile gene gun failed to show transient GUS expression when tested in numerous experiments. Many parameters were tested. Tissue was placed at distances of 1 to 20 cm from the micro-projectile launch. Screens of 200 µm, 500 µm, 700 µm and 1200 µm were placed between the tissue and the micro-projectile launch. The voltage was tested in the range of 4-20 kV.

At numerous times false GUS activity was observed in tobacco leaves (Figure 10, Plate 10) and embryogenic soybean suspension cultures (Figure 11). This was often associated with apparent cell damage. This effect was also observed in control tissue shot only with tungsten particles. This intrinsic background activity was distinguished by a more diffuse nature and a light blue/turquoise color. Positive GUS expression was associated with a deep royal blue color localized within a single or small group of plant cells. Only once was a dark blue spot found in *Glycine max* petiole tissue (Figure 12). Even this spot was not a discrete focus as seen in tissue bombarded with the helium guns and could possibly be due to contaminating bacteria expressing GUS activity.

The helium micro-projectile gene guns

Plant tissue bombarded with both the commercially available (PDS 1000) and the 'UT-built' helium micro-projectile gene guns showed high levels of transient GUS activity. Tissue damage was observed after bombardment with both helium guns, but the damage was minor when compared to that inflicted by the electric micro-projectile gene gun. An example of tissue damage in cowpea leaves bombarded at close distance is shown in Figure 13.

Plate 10. Plant tissue bombarded with the particle gun.

Figure 10. False GUS activity in tobacco leaves two days after bombardment with the electric particle gun.

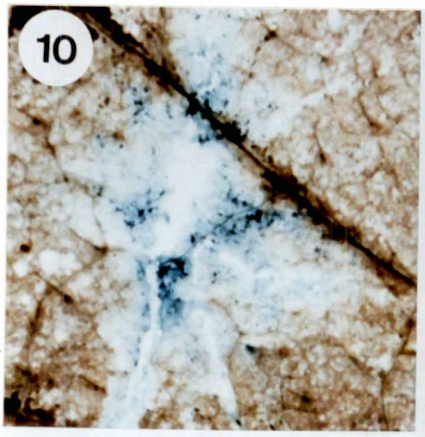
Figure 11. False GUS activity in soybean embryogenic suspension cultures two days after bombardment with the electric particle gun.

Figure 12. GUS activity in soybean tissue two days after bombardment with the electric particle gun. Cotyledonary petiole tissue from *Glycine max* cv. Peking displayed a dark blue area of GUS activity two days after being shot with the 'UT-built' electrical particle gun. It is also possible that this GUS activity was due to bacterial contamination of the tissue.

Figure 13. Tissue damage seen in a cowpea leaf tissue after bombardment with the 'UT-built' helium gun. A 500 μm Nylon screen was placed over the leaf tissue to protect the leaf cells. During bombardment some leaf cells were exposed to the helium blast producing a "waffle effect" on the leaf. Damage like this kills the cells which could be transformed. This tissue damage was not observed when the tissue was placed 10 or more cm from the blast.

Figure 14. Transient GUS activity in a cowpea leaf two days after bombardment with the 'UT-built' helium gun. As many as 2000 foci per leaf expressed GUS activity after one bombardment.

Figure 15. Transient GUS activity in an embryogenic suspension culture of the soybean nodulation mutant nts1007. The GUS activity is characterized by discrete dark blue foci.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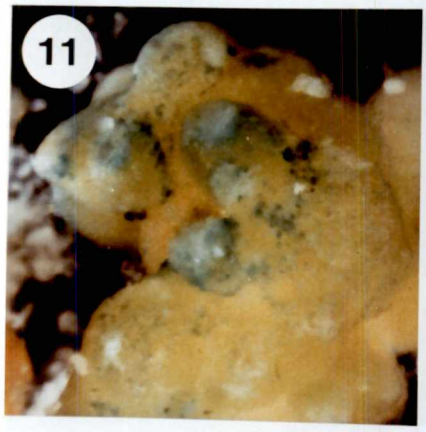
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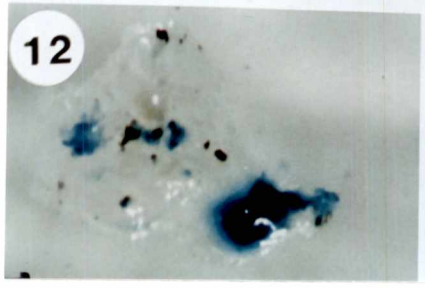
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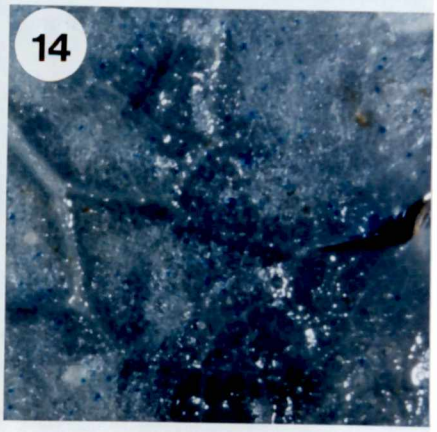
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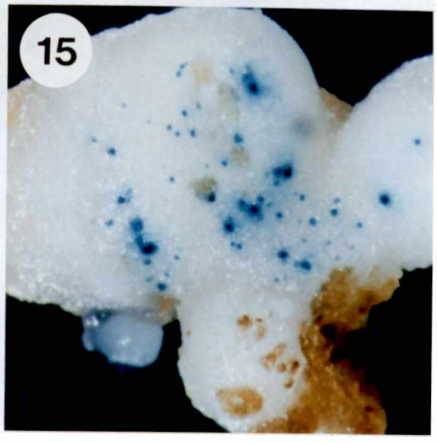
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Cowpea leaves (Figure 14) and soybean embryogenic suspension cultures (Figure 15) displayed high levels of transient GUS activity when bombarded with the 'UT-built' helium gun.

Comparison of helium guns

The 'UT-built' and 'DuPont' helium guns differed in several design parameters. These parameters include: propulsion pressures and mechanisms, bombardment distances and baffle use. Dispite these differences, comparisons were made between these guns by evaluating the frequency of transient gene expression over the range of variable bombardment conditions. Most of the comparisons were preformed only once as the 'DuPont' helium gun was only available for a two week period. Thus many of these results should be considered preliminary. Care was taken to assure that the tissues and culture conditions were identical. In the transient assays, each GUS blue spot or foci was considered one transiently expressing cell.

a) frequency of transient gene expression

Experiments were performed to compare the number of tobacco leaf cells expressing GUS activity two days after bombardment with the 'DuPont' and 'UT-built' helium guns (see Figures 16 and 17). The maximum number of cells transiently expressing GUS activity with the commercial helium gun was 190 per leaf. Whereas the maximum number obtained with the 'UT-built' helium gun was 780 per leaf. A similar experiment was carried out to compare the number of embryogenic soybean suspension cells expressing GUS activity two days after bombardment with both guns (see Figures 18 and 19). The maximum number of cells transiently expressing GUS activity with the commercial helium gun was 520 per gram (fresh weight) of tissue, whereas the maximum number obtained with the 'UT-built' helium gun was 6000 per gram (fresh weight) of tissue. Thus the 'UT-built' helium gun displayed approximately four times the number of tobacco leaf cells and ten times the number of embryogenic soybean suspension cells expressing GUS activity over the 'DuPont' gun.

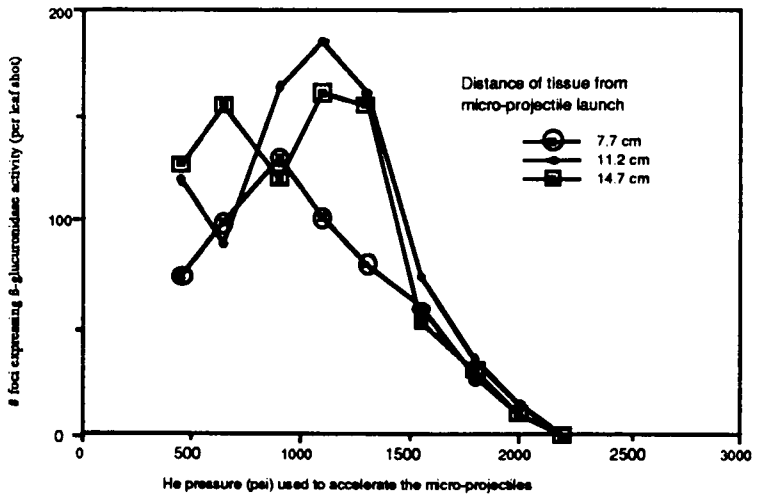

Figure 16. Transient GUS activity in tobacco leaf cells shot with the 'DuPont' helium gun. Each line on the chart represents a distance (shelf level) from the micro-projectile launch. Data from a single experiment (one replicate per data point).

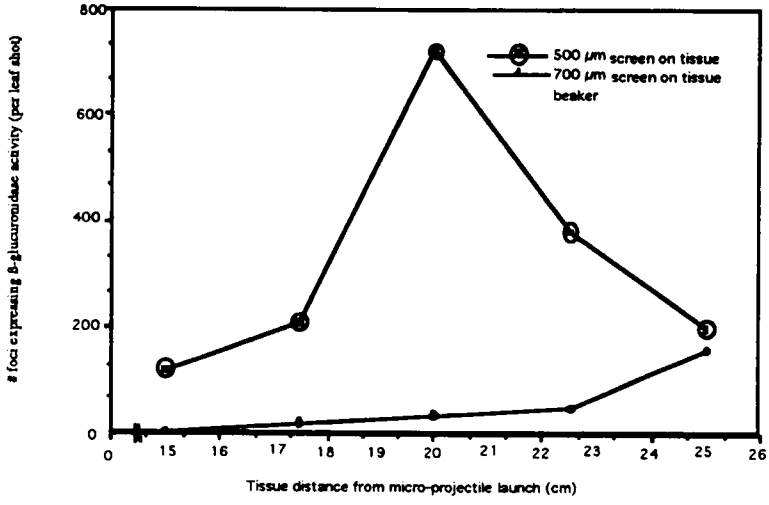

Figure 17. Transient GUS activity in tobacco leaf cells shot with the 'UT-built' helium gun: One helium pressure (70 psi) was used and the distance (shelf level) from the micro-projectile launch was varied. The lines represent the effect of placing either a 500 μm or 700 μm Nylon screen directly on the leaf tissue during bombardment. Data from a single experiment (one replicate per data point).

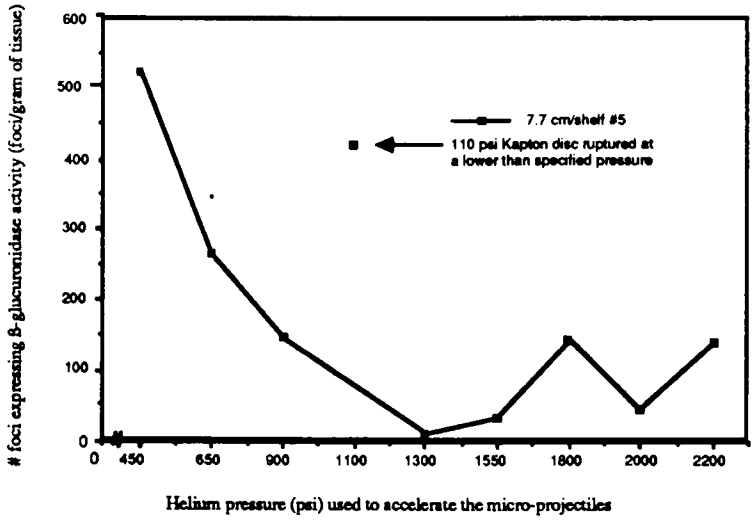

Figure 18. Transient GUS expression in soybean suspension culture cells bombarded with the 'DuPont' helium gun. One shelf level (7.7 cm from the micro-projectile launch) and a range of helium propulsion pressures were used. Data from a single experiment (one replicate per data point).

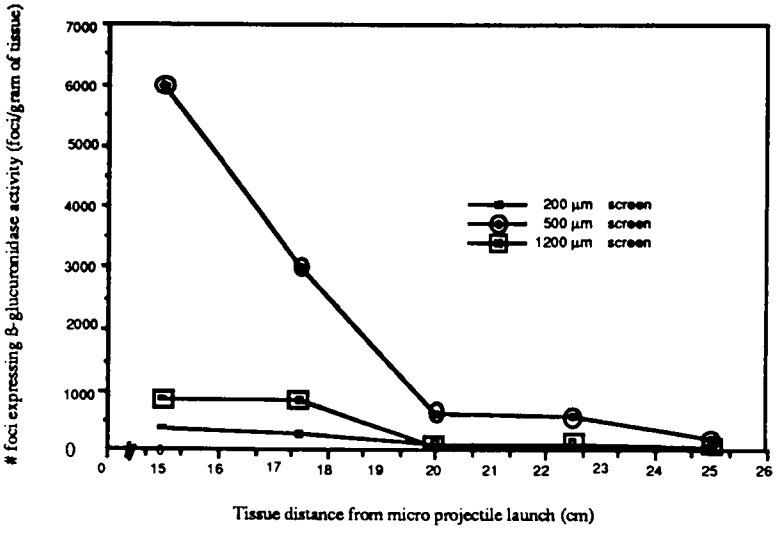

Figure 19. Soybean suspension culture cells expressing GUS activity two days after bombardment with the 'UT-built' helium gun. The effect of tissue distance from the micro-projectile launch and the effect of three screen sizes (200 µm, 500 µm and 1200 µm) placed directly on the tissue are represented. Data from a single experiment (one replicate per data point).

b) effect of baffles on transient gene expression

Screen size was important in the frequency of soybean suspension cells expressing GUS activity (Figures 17 and 19). It was observed that 1200 and 700 μm screens offered little or no protection from the blast. The 500 μm screen was optimal for transient expression. The 200 μm screen appeared to stop too many micro-projectiles and lowered the transient GUS expression.

c) The effect of tissue distance and propulsion pressure on the frequency of cells expressing GUS activity with the helium micro-projectile gene guns

Different tissue types were shown to require different optimal distances from the origin of the micro-projectile launch as well as different propulsion pressures (see Table 1). Tissue placed too close to the origin of the blast would be subjected tissue damage. If the tissue was placed too far away, the tungsten particles would not have the momentum to penetrate the plant cells. Factors such as the thickness and composition of the cell wall, the size of the plant cells and the degree of pubescence also affect the frequency of cells showing transient gene expression. Non-pubescent leaves such as cowpea were the best targets for bombardment, whereas pubescent leaves such as African violet are the worst targets tested (data not shown). The 'DuPont' helium gun displayed the most variability in transient expression levels with different helium pressures (cf. Figures 16 to 19). The reason for this variability was assumed to be caused by the Kapton^{TM} rupture discs, which often ruptured at incorrect pressures.

Comparison of construction and operation costs of the electric and helium micro-projectile gene guns.

Table 2 compares the construction/lease and operation costs of each helium particle gun. This table shows that operating the 'UT-built' helium gun as opposed to the 'DuPont' helium gun can result in significant savings. Operation and preparation time were also significantly shorter with the 'UT-built' helium gun. The time required to precipitate the DNA on the tungsten or gold particles and then load them onto the Kapton^{TM} carrier sheets was estimated to be 3 minutes per disc prepared and three minutes per disc shot. In contrast, the time to prepare the tungsten and DNA and load it into the syringe adapter was approximately one minute. Less than one minute was required to shoot the 'UT-built' helium gun.

Table 1. Comparison of the optimal parameters for maximal frequencies of transient GUS expression with the commercial and the 'UT-built' helium guns. The frequency of transient expression for the leaf cultures of tobacco and cowpea is represented as foci per leaf per shot. For soybean suspension cultures the units are foci per gram fresh weight per shot.

Tissue type	'DuPont' helium gun			'UT-built' helium gun		
	(psi)	shelf level	foci number	(psi)	shelf level	foci number
Tobacco leaves	1000	11.2	190	80	20	710
Cowpea leaves	ND	ND	ND	80	25	2000
Suspension cultures	450	11.2	520	80	15	6000

* pressure in PSI (pounds per square inch)

ND = not determined; shelf level is in centimeters from nozzle to shelf.

Table 2. Approximate construction, leasing, and operating costs of both the 'DuPont' helium gun and the 'UT-built' helium and electric guns.

Gun Type	Cost of construction/lease	Operation costs per shot
'DuPont' helium gun	$7,500 per year*	$2.00 per shot
'UT-built' electrical gun	$3,000 total**	$0.04 per shot
'UT-built' helium gun	$300 total**	$0.0001 per shot

* $3,000 per year thereafter for the next 4 years, or $9,500 purchase.

** excluding labor costs

Effect of DNA quality on the number of cells expressing GUS activity

An experiment was performed to investigate the effect of DNA purity on transient GUS expression. The methods employed for DNA isolation were: standard alkaline lysis (mini-prep) (Bassam *et al.*, 1987), column purification (5'-3'Inc pZ50 columns) or cesium chloride gradient purification (Sambrook *et al.*, 1989). Agarose electrophoresis and ethidium bromide staining of the DNA (Figure 20, Plate 11) displayed impurities in the column and mini-prep purifications. Spectrophotometric analysis of the DNA showed differences in the DNA purity between the different isolation protocols. Cesium chloride purified DNA displayed a 260/280 nm ratio of 1.6 whereas the mini-prep and column DNA displayed a 260/280 ratio of 1.0 (Figure 21). It appears that there may have been some polysaccharide and RNA contamination of these samples.

Cesium chloride purified DNA produced at least four times the frequency of transiently expressing GUS foci than mini-prep or column purified DNA (see Figure 22). Personal communications with other laboratories (Wooster, OH and Lexington, KY) supported this result.

Effect of screen position on the frequency of cells expressing GUS activity

The number of plant cells surviving bombardment depended upon the amount of cell damage inflicted during the bombardment process. Consequently the number foci or cells transiently expressing GUS activity was limited by the amount of damage inflicted on the target tissue. Thus baffles or screens were employed to reduce tissue damage due to the shock wave, while still allowing micro-projectile penetration. A very fine screen offered excellent protection to the cells but hindered or slowed the micro-projectile progress, limiting the number of particles entering the cells.

The effect of baffle position on transient GUS expression in cowpea leaves bombarded with the 'UT-built' helium gun was investigated. A preliminary 'shaving foam' experiment (Sanford *et al.*, 1991) was performed with Nylon screens of 200, 500, 700 and 1200 μm placed on each shelf at distances of 2 to 25 cm from the launch of the micro-projectiles and 0 to 25 cm from the target. In this experiment the baffling effect was observed by the extent of the dispersal of the shaving foam in a Petri dish (data not shown).

Plate 11. DNA purity and transient gene expression

Figure 20. Agarose gel electrophoresis and ethidium bromide staining of the undigested pUC GUS plasmid. The DNA in lane 1 was purified by standard alkaline lysis, in lane 2 alkaline lysis followed by column purification, and in lane 3 alkaline lysis followed by cesium chloride centrifugation. It appeared that there may have been polysaccharide contamination and residual amounts of RNA in lanes 1 and 2. Some impurities also appeared in the loading wells of these lanes. The DNA in lane 3 appeared to be clean. The DNA was measured on a flourometer (Hoefer Sci. Inst. TKO 100) and 100 ng was loaded in each well.

Figure 21. Spectrophotometric analysis of DNA purified by either cesium chloride centrifugation, standard alkaline lysis or alkaline lysis followed by column purification. These spectra showed that the DNA from standard alkaline lysis and alkaline lysis followed by column purification contained some impurities. The cesium chloride purified DNA appeared clean.

Figure 22. The effect of DNA purity on the number of foci per gram fresh weight transiently expressing GUS activity two days after bombardment with a 'UT-built' helium gun. The black columns represent standard alkaline lysis (mini-prep). The striped columns represent column purified DNA. The gray column represents cesium chloride purified DNA. Two distances from the micro-projectile launch are represented. Each data point is replicated four times and the standard error is represented as error bars.

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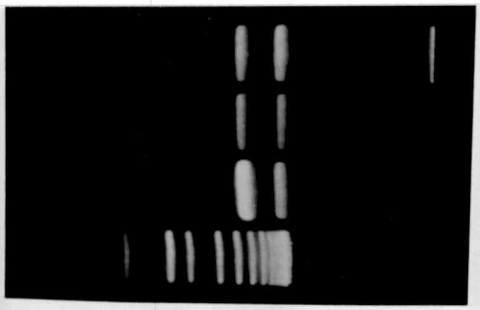
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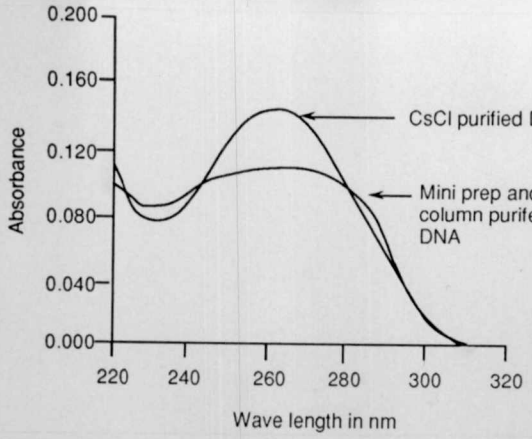
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Wave length in nm

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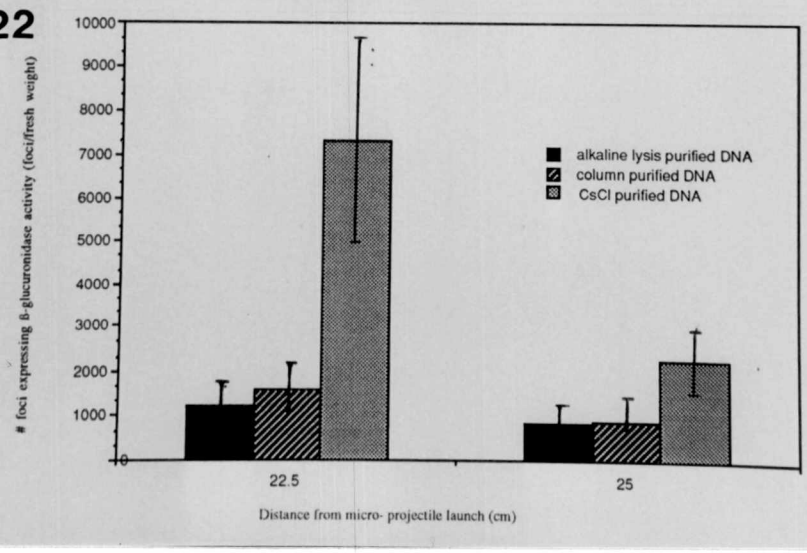
foci expressing ß-glucuronidase activity (foci/fresh weight)
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9000
8000
7000
6000
5000
4000
3000
2000
1000
0
alkaline lysis purified DNA
column purified DNA
CsCl purified DNA
22.5
25
Distance from micro- projectile launch (cm)

The closer the screen to the tissue the smaller the tissue damage and the greater the transient GUS expression (Figure 23). In this experiment a 700 µm screen was either placed on the cowpea leaf itself or mounted on an inverted 500 ml plastic flask, 10 cm above the tissue. A screen placed on the target tissue gave up to 10 X more cells expressing GUS activity 2 days after bombardment (Figure 23).

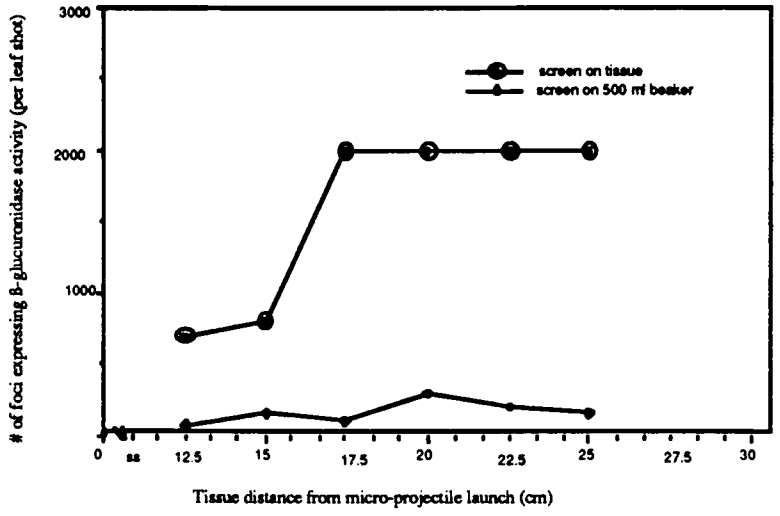


Figure 23. Effect of screen position on transient GUS expression in cowpea leaves. The tissue was bombarded with the 'UT-built' helium gun and assayed two days later. The preferred position for the 700 µm screen was resting on the tissue and not 10 cm above the tissue mounted on an inverted 500 ml beaker. The maximum number of foci transiently expressing GUS activity is 2000 per leaf. The best tissue distance from the micro-projectile launch was in the 17.5 to 25 cm range. Data from a single experiment (one replicate per data point).

Addition of phytohormones to the culture media

The addition of phytohormones to the culture media before and after bombardment of the tobacco and cowpea leaf cultures increased the frequency of cells showing transient gene expression (data not shown). It can be hypothesized that this increase in transient gene expression was due either to an increase in the survival frequency of cells or an induction of cell divisions. Foci of GUS expression in media with phytohormones appeared larger than those bombarded on a medium without hormones (data not shown). This may either

be due to stimulation of the plant's transcription and translational apparatus and leaching of the ß-glucuronidase enzyme to other cells or due to cell divisions post-bombardment.

Stable transformation events

Soybean embryogenic suspension cultures were bombarded with a mixture of pUC GUS and pUC HYGRO plasmids. Bombardments were performed at the optimal conditions determined for transient gene expression in each helium gun. Stable transformants were found after 10 weeks of selection in culture media supplemented with hygromycin (Figure 24, Plate 12). Whole GUS positive embryogenic clumps could be obtained after another 6 weeks of selection (Figure 25). These cultures were analyzed for the presence of foreign DNA using PCR analysis (Figure 26) and foreign DNA integration by Southern hybridization (Figure 27). Although suspension cultures were shot and selected with both helium guns, only the 'UT-built' helium gun yielded stable transformants. However, the commercial 'DuPont' helium gun was only available for a limited period (two weeks). Suspension cultures used in stable transformation experiments were bombarded and baffled with 700 and 500 µm screens. Only cultures bombarded with the 500 µm screens gave stable transformants. Flasks which produced hygromycin resistant clumps typically produced three to seven independent clumps.

CONCLUSION

The results presented here show that construction of a 'UT-built' helium particle gun is a very practical alternative to leasing or purchase of a commercial helium gun. Not only was the construction cost approximately the same as leasing the commercial gun for two weeks, the operational costs were a fraction.
The helium gun was constructed in two days. Transient levels of GUS expression were significantly higher than those demonstrated with the commercial helium gun. Unfortunately, replicated experiments with the commercial helium gun were not possible due to the limited time that the instrument was available.

Important parameters were delineated for optimum transient gene expression in soybean suspension cultures as well as in tobacco and cowpea leaves. Tissue damage during bombardment appears to be the most crucial variable in successful transformation experiments.

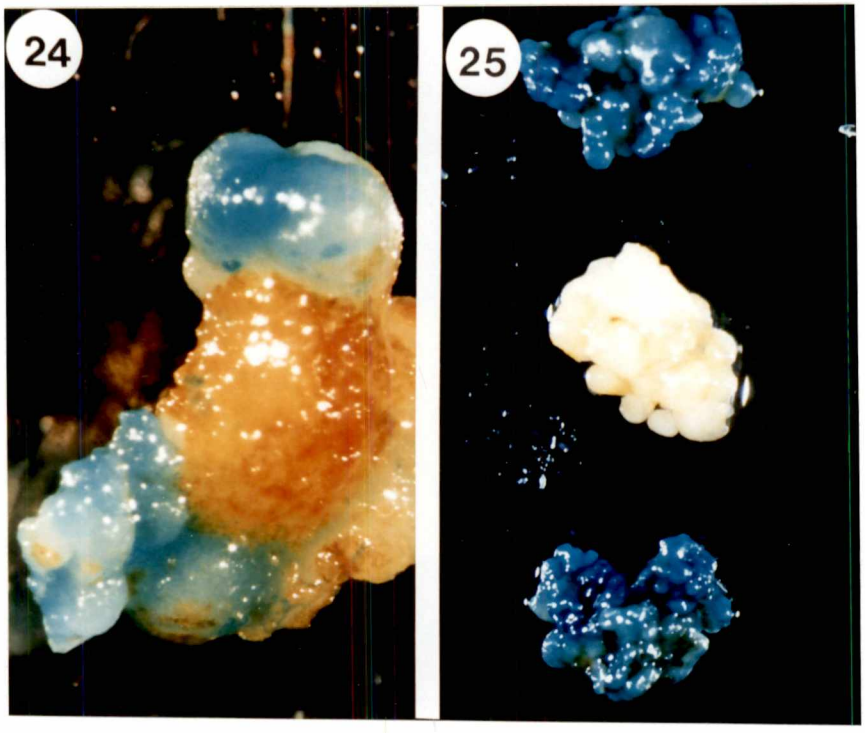
Plate 12. Stable gene expression.

Figure 24. GUS positive embryogenic clumps grew from apparently 'dead' suspension cultures after ten weeks of selection in culture medium supplemented with hygromycin. The suspension cultures appeared brown and 'dead' for seven to ten weeks. Small yellow/green hygromycin resistant clumps would then grow from this 'dead' tissue.

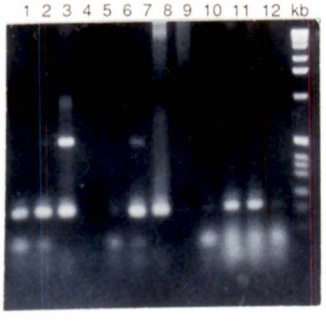
Figure 25. Homogeneous GUS positive embryogenic clumps after 16 weeks of selection. These transgenic clumps were compared to a non-transgenic suspension clump in a GUS assay. The transgenic clumps (top and bottom) exhibited a dark blue GUS reaction whereas the non-transgenic clump (center) exhibited no GUS activity.

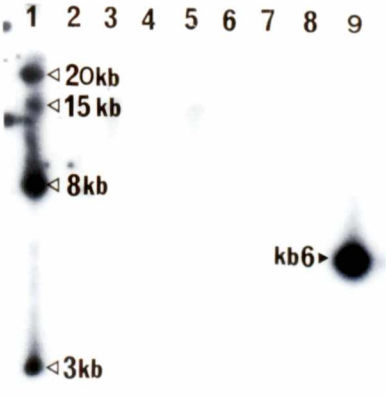
Figure 26. Hygromycin resistant GUS positive suspension cultures analyzed for the presence of foreign DNA using PCR analysis. Primers were designed to amplify a 150 bp fragment of the CaMV 35S promoter. This promoter 'drives' both the GUS and hygromycin resistance genes. The template DNA in lanes 1, 2 and 3 were 1 pg, 10 pg and 100 ng of pUC GUS plasmid DNA amplified with 1X primer concentration. Lane 4 was 100 pg of pUC GUS plasmid DNA amplified with 1X primer concentration. Lanes 5, 6 and 7 were 1 pg, 10 pg and 100 ng of a separate pUC GUS plasmid isolation amplified with 0.1X primer concentration. Lane 8 contained 100ng of pUC GUS plasmid DNA but no primers. Lane 9 was 100 ng of control non-transgenic DNA. Lane 10 and 11 was 100ng of DNA isolated from hygromycin resistant embryogenic suspension clumps. Lane 12 was was 100 ng of control non-transgenic DNA. Lanes 9 to 12 used a 1X primer concentration.

Figure 27. Hygromycin resistant suspension cultures analyzed for the integration of foreign DNA by Southern hybridization. Lanes 1, 2 and 3 were DNA samples (5 µg/lane) from hygromycin resistant cell clumps. Lane 4 was blank. Lanes 5, 6 and 7 contained DNA from non-transgenic suspensions. Lane 8 and 9 contained 10 and 100 pg of pUC GUS plasmid DNA respectively. All the DNA samples were digested with *Eco*RI before electrophoresis, blotting and hybridization on to a Nylon membrane. The DNA was probed with a 0.8kb 35SCaMV promoter fragment labeled with [^{32}P]dCTP. The autoradiograph was developed for 48 hrs.


24
25

26


1 2 3 4 5 6 7 8 9 10 11 12 kb
150 bp


1 2 3 4 5 6 7 8 9
20kb
15 kb
8kb
kb6
3kb

27

This damage can be limited and still allow cell transformation by the selection of the correct helium pressure for micro-projectile propulsion (data not shown for the 'UT-built' gun), and the selection of the correct distance and baffle size.

Effective use of different sized screens or baffles was demonstrated in varying the transient expression frequency of the GUS gene. This manipulation has proven to be just as effective as varying the helium propulsion pressure. It also offers fine tuning of the system by effectively protecting the target tissue from the physical shock of bombardment. Baffles were shown to be most effective when placed directly on the plant tissue. The 500 μm baffle appeared to be the best for the production of stable and transient transformants.

The electric micro-projectile gene gun caused extensive tissue damage even at low voltages. Extensive use of baffles did not help in this case. The physical shock from bombardment may have been the reason for its lack of success. In light of the success of the helium gun, no further analysis of the electric gun is planned.

If the helium propulsion pressures during bombardment were dropped too far, and the distances from the micro-projectile launch increased to its maximum, transformation frequency decreased. Thus there was a definite window for cell transformation without cell death and with limited cell damage. However, to transform the plant cells by particle bombardment a degree of damage to the plant cells was to be expected.

DNA precipitation onto the tungsten particles using cesium chloride purified DNA produced less aggregation of the tungsten particles than DNA purified by other procedures. It is possible that aggregation was caused by contamination of the DNA with high molecular weight polysaccharides. Such contamination was likely to affect the number of cells that were successfully transformed. If any more than ten particles entered an individual cell, the cell is likely to die (Klein *et al.*, 1988b). It was also possible that any contaminants in the DNA, such as polysaccharides or RNA, increased the cell shock or interfered with the transcription of the marker gene.

Stable transformation of soybean embryogenic suspension cultures was demonstrated by bombardment with the 'UT-built' helium gun. This was possible by application of the optimal parameters elucidated for transient gene expression in soybean embryogenic suspension cultures.

Stable transformants were produced from cultures bombarded while baffled by a 500 μm screen. No stable transformants were produced when cultures were baffled by a 700 μm screen. Approximately 100 mg of suspension cultures were bombarded at one time. The average number of transiently expressing cells or foci was 600 per 100 mg fresh weight. Between three to seven independent hygromycin resistant transgenic clumps were obtained per flask (100 mg of bombarded tissue) (data not shown). These findings indicate that approximately 1% of the transiently expressing cells survived to become stably integrated. GUS assays of transgenic suspensions 20 weeks after bombardment showed that GUS expression was still high. Southern analysis indicated that the DNA is integrated at various regions in the genome.

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CHAPTER 5

PARTIAL SYMBIOTIC CHARACTERIZATION OF *AGROBACTERIUM RHIZOGENES* INDUCED ROOTS OF *GLYCINE MAX*, MODIFICATION OF NODULE PHENOTYPE AND EXAMPLES OF CLASSICAL AND NON-CLASSICAL TRANSFORMATION EVENTS

ABSTRACT

An *Agrobacterium rhizogenes* cucumopine strain (K599) was used to induce transgenic hairy roots at high frequency on hypocotyls of soybean cultivars Peking, Bragg and its derived nodulation mutants nts382 (supernodulating), nod49 and nod139 (non-nodulating). Transgenic roots were nodulated in soil with *Bradyrhizobium japonicum*. Approximately 90% of the *G. max* plants showed nodule phenotypes (nodule number, shape and nitrogen fixing ability) consistent to that of the parental plants with non-transgenic roots. A small group of transgenic plants (10%) exhibited an altered nodule phenotype and higher nodule numbers than non-transgenic plants. The nodules were typically non-fixing (Fix-), elongated or budding and possessed active meristems as in indeterminate nodules. The plants with modified nodule structures are thought to be due to the incorporation and unusually high expression of the *rol* genes (*rol A, B, C, D*) from the Ri plasmid. The variation in nodule morphology between these groups is thought to be due to a positional effect of *rol* gene integration. Transformation was confirmed by opine assay, NPT II assay, GUS assay (GUS intron gene) and Southern hybridization. Transgenic roots analyzed by Southern hybridization for integration of the Ri plasmid T-DNA showed classical integration patterns. Southern analysis of the same plants also indicated that the binary Ti plasmid p35S GUS I (intron containing GUS gene) did not show classical integration even though the roots were GUS positive. This indicates that the p35S GUS I plasmid may exist as an extra-chromosomal element. This transformation system is rapid (3 months from inoculation to nodulation of plants in the greenhouse) and highly efficient. From 15 to 85% (this frequency was genotype dependent) of the original inoculations gave hairy roots at the wound site. When tested for GUS activity 70 to 100% of these roots tested positive for GUS activity. This transformation system has potential for the study of the genetics of nodulation and gene expression in soybean.

INTRODUCTION

Agrobacterium rhizogenes is the causal agent of hairy root disease. This bacterium is virulent on a wide range of dicotyledonous plants (De Cleene and De Ley, 1981). It transforms plants by insertion of a region of DNA called T-DNA (transfer DNA) from the root-inducing (Ri) plasmid into the plant genome (Chilton *et al.*, 1982; for a review of molecular biology of the Ri plasmid; Sinkar *et al.*, 1987).

The *A. rhizogenes* strains are classified by the opines which are synthesized by the Ri T-DNA. The opines (which are conjugated amino acids and carboxylic acids) synthesized by these genes serve as a metabolite for the *Agrobacterium* (Petit *et al.*, 1983). To date four different opine types have been found in *A. rhizogenes* induced roots. These are agropine, mannopine, cucumopine and milkopine (Davioud *et al.*, 1988; Filetici *et al.*, 1987; Isogai *et al.*, 1988; Petit *et al.*, 1985).

In strains of *A. rhizogenes* that produce agropine the T-DNA is split into two regions T_L and T_R, each approximately 15-20 kb in size. Both T-DNA regions are integrated separately into the plant genome (Petit *et al.*, 1983). Agropine strains have both the agropine synthesis and auxin indole acetic acid (IAA) synthesis genes referred to as *aux* 1 and 2, or *tms* 1 and 2 on the T_R segment. Cucumopine and mannopine *A. rhizogenes* strains have only one T-DNA region between 34 to 42 kb in size.

The function of rol genes in Agrobacterium rhizogenes transformation

On the T_L segment of the agropine strain there are 18 open reading frames of 255 nucleotides or more (Slightom *et al.*, 1986). Transposon mutagenesis of this region has revealed the presence of at least four genetic loci (*rolA, B, C* and *D*) which also affect root induction. The *rol* (root morphogenesis loci) and *aux* genes are responsible for root induction either individually or together depending on the plant species or type of tissue (White *et al.*, 1985).

Interestingly, cucumopine and mannopine *A. rhizogenes* strains do not have *aux* genes, only *rol* genes and yet they still produce a hairy root phenotype. Also, although *aux* genes have been identified in agropine *A. rhizogenes* strains, their presence is not required for hairy root induction in tobacco (Vilaine *et al.*, 1987). In fact the whole T_R fragment is not required for hairy root production. Transgenic plant lines elicited by the agropine-type *A.*

rhizogenes strains do not always synthesize agropine (Petit *et al.*, 1983). In these cases it seems that sufficient auxin is supplied by the plant and that the *aux* genes may have a role in supplementing auxin only when it exists in limiting amounts (Grierson and Covey, 1988).

The *rolA, B, C* and *D* loci have been shown to affect virulence and hairy root formation. At one time it was thought that the *rol* genes also synthesized auxins causing the characteristic hairy roots much in the same way that *Agrobacterium tumefaciens* initiate callus production by transforming cytokinin synthesizing genes (Zambryski *et al.*, 1989). However, it now appears that the mechanism is much more complex. Shen *et al.* (1988) reported that transformed roots of this type contained less auxin but were 10 to 100 times more sensitive to the presence of auxins than normal roots. Because these roots did not show altered, auxin-independent proton excretion, altered ethylene sensitivity or increased auxin uptake, they suggested that the transformed genes affected the auxin receptor-transduction system. Shen *et al.* (1988) proposed that increased auxin sensitivity induced root induction. This result is supported by the work of Maurel *et al.* (1991) with tobacco protoplasts. They reported that sensitivity to auxin by transformation of single *rol* genes could be increased 30 fold by the addition of all the *rol* genes on the T_L-DNA segment of an agropine strain.

Roots are known to be initiated by endogenous concentrations of auxins. However, Croes *et al.* (1989) detected no differential auxin sensitivity between normal and 'rhizogenes' root cultures. Quattrochio *et al.* (1986) showed that anti-auxins had an inhibitory effect on the growth of 'rhizogenes' potato roots. Cardarelli (1987) and Spena *et al.* (1987) both showed that when the *rolA, rolB* and *rolC* genes were expressed individually, transgenic roots were still produced but at an attenuated level. The effects of each *rol* gene on root initiation and growth are different (Schmülling *et al.*, 1988; 1989). The *rolB* and *rolC* genes seem to be involved in root branching, but the level and region of their expression are different. Expression of *rolB* is in the root cap whereas *rolC* is expressed in the phloem area (Schmülling *et al.*, 1988). Mutations in the *rolA* gene (*rolA*$^-$) result in the formation of long straight roots. A *rolB* mutation eliminates both callus and root formation at the wound site. *The rolC* and *rolD* mutations are more subtle. Root growth is retarded with a *rolC* mutation, whereas a *rolD* mutation give rise to *Agrobacterium tumefaciens* like tumors (White *et al.*, 1985). The primary determinate in the severely wrinkled leaf phenotype seen in *A. rhizogenes* transformed plants is the *rolA* gene (Sinkar *et al.*, 1988).

The rol gene proteins

Estruch *et al.* (1991a) studied the rol C peptide and found that it was a non-transported 20 kDa cytosolic protein. Plants expressing this protein showed dwarfing and reduced apical dominance. Chimeric plants for *rolC* expression showed leaves with pale green sectors with sharp borders, indicating lack of diffusion or transportation of this protein. This is compatible with the hypothesis that plant growth and morphogenesis is altered by the rol proteins which modify endogenous plant hormones (Estruch *et al.*, 1991a). Estruch *et al.* (1991b) later found that the rolC protien releases cytokinins from glucoside conjugates Also the rolB protien was found to hydrolyses indole glucosides thus releasing auxins from its conjugate (Estruch *et al.*, 1991a). The combined action of these two genes is thought to modulate the intracellular concentration of auxins and cytokinins to produce 'rhizogenes' roots.

Production of transgenic plants from Agrobacterium rhizogenes transformed roots

A. rhizogenes induced hairy roots of about 50 different plant species have been cultured and transgenic plants regenerated either by spontaneous shoot formation or through a callus stage initiated on a medium supplemented with auxins and cytokinin (for a review, Potter, 1991). Phelep *et al.* (1991) used the *A. rhizogenes* strain K599 as well as the agropine strain A4 to transform the woody non-legume *Allocasuarina.* Their study was characterized by careful interpretation of Southern hybridization data which showed covalent integration of *Agrobacterium* sequences into plant DNA. Limited success has been reported in the *Glycine* genus. Only in the wild soybean *Glycine canescens* have whole plants been regenerated from transgenic roots (Rech *et al.*, 1988).

Desired sequences can be transferred into plants using A. *rhizogenes* mediated transfer. This can be achieved either by inserting the sequence into an *A. tumefaciens* derived binary plasmid (Simpson *et al.*, 1986) or integrating the sequence into the pRi T_L-DNA segment of the Ri plasmid (Stougaard *et al.*, 1987a).

Application of A. rhizogenes mediated gene transfer techniques to the genetics of nodulation in legumes

Most transformation systems developed to study the genetics of nodulation in legumes use *A. rhizogenes* derived roots or plants. Stougaard *et al.* (1987b) were the first researchers to use *A. rhizogenes* derived transgenic plants to study the genetics of nodulation. They demonstrated nodule specific expression of a complete soybean leghemoglobin gene in the root nodules of transgenic *Lotus corniculatus*. Later work focused on using the 5' promoter sequence from the leghemoglobin gene fused to the CAT reporter gene in order to show tissue specific expression in transgenic nodules of *Lotus corniculatus* (de Bruijn *et al.*, 1989; de Bruijn *et al* ., 1990; Jensen *et al.*, 1988; Szabados *et al.*, 1990). Similar work was carried out using the *Parasponia* leghemoglobin promoter and GUS reporter gene fusions in transgenic *Lotus* and tobacco (Bogusz *et al.*, 1990). Miao *et al.* (1992; submitted) used the same promoter reporter gene fusion technique in *L. corniculatus* to study the tissue specific expression of nodulin 26 (nodulin 26 promoter fused to the GUS gene). Nodulin 26 is a major protein of the peribacteroid membrane. GUS expression was seen at emerging nodules and root primordia. Roots expressing anti-sense N-26 showed retarded hairy root growth (*A. rhizogenes*) in *Vigna aconitifolia*. Anti-sense studies were also conducted with nodulin 35. Soybean nodulin-35(N-35) encodes a subunit of uricase localized in the peroxisomes of uninfected nodule cells (Nguyen *et al.*, 1985). Lee *et al.* (1992; submitted) introduced an antisense cDNA clone into *Vigna* behind a CaMV-35S promoter. Nodules formed on the resultant transgenic roots were smaller in size and plants exhibited a yellow leaf phenotype. Uricase activity was reduced by 50-60% as shown by enzymatic assays. Electron microscopy also showed that the size of the peroxisomes was reduced.

Application of gene transfer techniques to the genetics of nodulation in soybean

No transformation systems has been used for the study of symbiotic nitrogen fixation in soybean to date. This is in part because no suitable transformation system exists for such a study in soybean. Transformation of *G. max* was reported via particle delivery (McCabe *et al.*, 1988, Finer *et al.*, 1991) and *A. tumefaciens* (Hinchee *et al.*, 1988). However, these approaches suffer from a low frequency of transformants and a long culture procedure. To date none of these transformation systems has been used in the study of symbiotic nitrogen fixation in soybean.

Susceptibility of soybean to Agrobacterium rhizogenes

G. max still evades regeneration from *A. rhizogenes* - induced roots. Even production of these roots has proven difficult. Owens and Cress (1985) tested the responses of 26 soybean genotypes to *A. rhizogenes* strain A136 harboring the pRiA4b (Ri) plasmid. Seven out of 26 of these genotypes produced roots at the infection site. Attempts to culture these roots failed and assays for 'hairy root' markers (opine content) were not performed. Simpson *et al.* (1986) was able to use *A. rhizogenes* roots to introduce transgenic sequences from an *A. tumefaciens* derived binary plasmid conferring kanamycin resistance. In these dual plasmid systems both oncogenes and the engineered transgenic sequence can be transferred. This plasmid functioned in *A. rhizogenes* (agropine strain A4) and produced roots which expressed the transgenic sequence in tomato, tobacco and alfalfa. Although Simpson *et al.* (1986) demonstrated a relatively high frequency of transgenic roots on alfalfa (33% nopaline positive, 4% kanamycin resistant), tomato (33% nopaline positive, 19% kanamycin resistant) and tobacco (19% kanamycin resistant, nopaline not determined), in soybean only 2% of the roots were nopaline positive and none were kanamycin resistant. In soybean a high percentage of non-transgenic roots were produced from the inoculation/wound site (Simpson *et al.*, 1986). The ability of soybean to show intrinsic nopaline activity (Christou *et al., 1986*), coupled with fact that none of the roots showed kanamycin resistance seriously limited the use of *A. rhizogenes* to produce transgenic roots in soybean.

Not until Savka *et al.* (1990) was an *A. rhizogenes* strain (cucumopine strain K599/pRi2659) found that produced *A. rhizogenes* roots at a high level in *G. max* (37% of the cotyledons infected on the 10 genotypes tested). Root cultures derived from these infection events were positive for cucumopine production and grew rapidly in culture. Savka *et al.* (1990) used these cultures to propagate soybean cyst nematodes *in vitro*.

Classical and non-classical transformation as indicated by Southern blot analysis

Classical integration of the T-DNA into the plant genome is indicated by different sized border fragments hybridizing in a Southern blot (plant/plasmid border fragments). These fragments must be different from the restriction fragment sizes observed by digestion of the transferred plasmid alone. If a hybridization pattern identical to that obtained by the restriction digest of the plasmid is obtained then this indicates non-classical plasmid DNA transfer and maintenance or bacterial contamination of the tissue.

Research in this paper

This study demonstrates that transgenic roots of several soybean lines can be produced with relative ease. The plants studied include a range of soybean nodulation mutants which either cannot nodulate (Carroll et al., 1986) or cannot control the degree of nodulation (supernodulate; Carroll *et al.*, 1985a, b). This study shows that the transgenic roots nodulate (contrary to Beach and Gresshoff 1986) and that the degree of nodulation, conditioned by the host's genotype, is maintained in transgenic roots. A small sub-set of these plants (15%) exhibited an alteration of nodule phenotype. This alteration suggests that plants with normally determinate nodule structures (soybean) can be genetically engineered to produce 'indeterminate- like' nodules (eg. alfalfa) by the addition of a limited number of transgenes. Also observed are examples of classical and non-classical transfromation events in the same transgenic tissue.

MATERIALS AND METHODS

Bacterial culture

Agrobacterium rhizogenes cucumopine strain K599 (pRi2659) was kindly supplied by Dr. Steven Farrand, Department of Plant Pathology University of Illinois, Urbana 61801. The bacteria was grown in 25 ml of liquid Bergersen's Modified Medium (BMM, Bergersen, 1969) under constant agitation (150 rpm) at 28°C for four days. Acetosyringone (200 µM) was added to the medium just prior to pelleting the bacteria at 4 K rpm. The bacterial pellet was resuspended in 1 ml of the supernatant by vortexing. Approximately 10 µl of this bacterial solution was used for each plant inoculation

Plant inoculations were either performed with the *A. rhizogenes* bacterium harboring the Ri plasmid alone, or with *A. rhizogenes* harboring an additional engineered binary plasmid. The disarmed binary vector p35S GUS intron, (see Figure 1) encoding the GUS intron and NPT II (kanamycin resistance)genes, was obtained from Dr. Guy Vancanneyt, Institut für Genbiologische Forschung, Ihnnestrasse 63, D-1000 Berlin 33, Germany. This vector was transformed into the *A. rhizogenes* strain K599 by the triparental mating technique, using the mobilizing plasmid pRK2013 (Van Haute *et al.*,1983). The transformed *Agrobacterium* was selected on minimal BMM medium supplemented with 100 µg/ml kanamycin.

Agrobacterium rhizogenes strain K599 was also transformed with the KYLX p3-1 GUS binary plasmid. This construct was only used to demonstrate the expression of GUS activity in *Agrobacterium* when a construct without an intron was used. The KYLX p3-1 GUS binary plasmid is almost identical to the p35S GUS intron plasmid except that the GUS gene does not have an intron in the coding region. This plasmid was kindly donated by Dr. Arthur Hunt, University of Kentucky, Lexington. Tetracycline (25 µg/l) was used to select *Agrobacterium* harboring this plasmid (12.2µg/l for *E.coli*).

Bradyrhizobium japonicum strain USDA110 was used for all plant inoculations. The bacteria were grown for 4-5 days in liquid BMM medium under constant agitation at 150 rpm at 28°C, prior to plant inoculation. The bacteria were used for inoculation when in late log phase.

Assay for GUS activity in Agrobacterium harboring the KYLX p3-1 GUS plasmid or the p35S GUS intron plasmid

Agrobacterium rhizogenes strain K599 transformed either with the p35S GUS intron or the KYLX p3-1 GUS plasmid were assayed for their ability to express GUS activity. *Agrobacterium* were grown for two days in liquid BMM were assayed for GUS activity by incubation of 100 µl of bacteria in 100 µl of 5-bromo-4-chloro-3-indolyl-ß-glucuronide (X-Gluc) at 37°C for four hours. The results of the assay were scored by the presence or absence of a blue color.

Culture and inoculation of soybean seedlings

Seeds of cultivars. Peking, Bragg and the derived nodulation mutants, supernodulation mutant nts382 (Carroll *et al.*, 1985a, b) and non-nodulation mutants nod49 and nod139 (Carroll *et al.*, 1986) were surface-sterilized. This was performed by first soaking the seeds in a aqueous soap solution containing 100 µg/ml Captan™ (a fungicide) for 10 minutes. (constant agitation). This solution was then decanted and a 40% solution of Chlorox added with 1-2 drops of Tween 20. After constant agitation for 10 minutes this solution was also decanted and the seeds washed 5 times with sterile distilled water. Finally, the seeds were dusted with Captan and Banrot fungicides and left to stand for 1 hr in a laminar flow hood. All manipulations hereafter were performed aseptically in a flow hood. Seeds were then plated on a medium composed of 1/10th Gamborg's B_5 salts

(Gamborg *et al.*, 1968), 30 g/l sucrose, 8 g/l Difco Bacto agar, pH 5.7, and autoclaved for 20 mins at 120°C. Approximately 10 seeds were plated on each 20x100 mm Petri dish and incubated at 25°C under a constant fluorescent light regime of 300 $\mu M\ s^{-1}\ m^{-2}$ for four days prior to inoculation with *Agrobacterium*. Soybean seedlings of cv. Peking, Bragg and the derived nodulation mutants were then lightly wounded with a sterile scalpel on the hypocotyl just beneath the cotyledons and approximately 10 µl of the *Agrobacterium* suspension was used to inoculate these wounds. The inoculated seedlings were cultured in Magenta jars (a 10x7x7 cm autoclavable clear plastic vessel, Magenta Corp., Chicago.) on 1/10th B_5 medium under conditions identical to those used for seed germination.

Selection and subsequent culture of transgenic roots

As soon as the putative transgenic roots appeared from the wound site (2-4 weeks) the seedlings were removed from the Magenta jars and the hypocotyl cut below the original wound site. The manipulated seedling was then transferred to a new Magenta jar containing approximately 20 mls of Mon Mor (MM) medium (Savka *et al.*, 1990). The medium was supplemented with 100 µg/ml kanamycin to select for transgenic roots and 100 µg/ml cefotaxine, 500 µg/ml carbenicillin, 75 µg/ml amoxicillin and 37.5 µg/ml clavulanic acid to kill all residual *Agrobacterium* cells. After 1-2 weeks of culture in the liquid MM medium under culture conditions identical to those used for seed germination, enough root material was produced to: a) check for residual bacterial contamination, b) assay for GUS and NPT II activity, c) propagate the roots independently of the explant shoot, and d) transfer the explant with the transgenic roots to soil.

Evaluating root tissue for residual Agrobacterium contamination

Small (5 mm) roots sections were aseptically ground in an Eppendorf tube using a mini-pestle and 300 µl of a 0.8% NaCl solution. A 100 µl aliquot of this slurry was plated on BMM medium supplemented with 100 µg/ml kanamycin (kanamycin was only included if the roots contained the binary GUS I plasmid). Alternatively, the 5 mm root sections were directly placed on this selective medium and left to culture for 4-7 days at 28°C. Bacterial contamination was assessed by the presence or absence of colony growth.

Culture of the transgenic roots independent of the explant

Transgenic roots growing from the explant shoot which had been cleared of residual bacterial contamination could be sub-cultured as independent root cultures. The root tissue was rapidly multiplied by inoculation of 2 cm root fragments into 16 ml of liquid MM media in a 100x20 mm sealed Petri dish (Parafilm^{TM}) supplemented with kanamycin 100 µg/l (kanamycin was only included if the roots contained the binary GUS I plasmid). The cultures were maintained in 100x25 mm Petri dishes in an illuminated incubator at 25°C, and a constant fluorescent light regime of 50 µM s^{-1} m^{-2}.

GUS assay of root material

The root tissue was assayed for ß-glucuronidase (GUS) activity by incubation for 12 hrs in 5-bromo 4-chloro 3-indolyl ß-D glucuronic acid (X-Gluc) at 37°C as described by Jefferson (1987a, b). The appearance of a dark blue color was taken as evidence of GUS activity. The root tissue was then preserved in either 70% ethanol or autoclaved in 75% lactic acid for 10 minutes at 15 psi (O'Brian and McCully, 1981) which served to slightly clear the root tissue.

Kanamycin selection of transgenic roots

The effect of kanamycin levels of 0, 25, 50, 75, 100, 125, 150, 175 and 200 µg/ml on 'rhizogenes' root growth was measured over a 30 day period. Growth of *A. rhizogenes* induced roots harboring the p35S GUS I plasmid were compared to control non-transgenic roots and *A. rhizogenes* derived roots without the binary GUS I plasmid. The roots were either grown on solid 1/10th B_5 medium or in liquid BMM medium.

Attempted regeneration of plants from transgenic A. rhizogenes derived roots

Rech *et al.*, (1988) induced regeneration of plants from *A. rhizogenes* roots of *Glycine canescens* by induction of shoot formation using a B_5 based medium supplemented with BAP at 44µM and IBA at 2.5 µM. This regeneration medium and a range of BAP concentrations were investigated for the *A. rhizogenes* induced roots of soybean. Roots were plated on a solid B_5 medium (identical to 1/10th B_5 medium except that the B_5 salts is added at full strength) supplemented with 2.5 µM IBA and a range of BAP concentrations (0, 1, 3, 5, 25, 50, 100, 200, 400 µM). Culture conditions were as

previously described for *A. rhizogenes* roots. The cultures were maintained for 3 to 4 months and inspected regularly for signs of shoot induction.

Neomycin Phosphotransferase (NPT II) assay

Roots testing negative for the presence of bacteria and positive for GUS activity and kanamycin resistant growth were assayed using the radioactive neomycin phosphotransferase dot blot assay as described by McDonnell *et al.* (1986). Tissue was either taken from *in vivo* grown roots or *in vitro* roots and nodules. Control tissue was taken from non-transformed root and nodule tissue, and *Bradyrhizobium japonicum* (USDA110) cells.

Opine assay

The roots were assayed for cucumopine content by high voltage paper chromatography as described by Savka *et al.*, (1990). For the detection of mannityl opines, approximately 0.3g of root tissue was macerated in 100µl of 70% ethanol containing 10µl of the electrophoresis running buffer (formic acid /acetic acid /water, 3:9:91 v/v/v, pH=1.9). For detection of cucumopine, root tissue was macerated in distilled water. In each case supernatants were recovered following centrifugation. Twenty microliters of the supernatant extract was spotted on Whatman 3MM paper. The spots were allowed to dry and the papers wetted with the running buffer and subjected to high voltage paper electrophoresis (HVPE) at 4,0000 V for 12-15 min. Spots were identified as opines by comparing their electrophoretic mobilities and staining properties with those of authentic standards. Mannityl opines were visualized by staining with an alkaline silver nitrate reagent (Savka *et al.*, 1990). Extracts prepared from non-transgenic root tissue and authentic hairy roots of *Nicotiana tabacum* L. var. Xanthi NG were included on the electrophoretograms as positive and negative controls.

Southern blot analysis of transgenic roots

Southern hybridization analysis was performed essentially according to Southern *et al.*, (1975). Genomic DNA from transgenic and non-transgenic control roots grown *in vitro* and roots and nodules grown *in vivo* were isolated according to Dellaporta *et al.*,(1983). Purified DNA was digested with either *Pvu*II or *Eco*RI restriction endonucleases and separated by agarose gel electrophoresis (10 µg /lane, 0.9% agarose) and transferred onto

Zeta-Probe^R Nylon membrane by vacuum blotting (LKB). Blots were probed for the transfer of both T-DNA from the Ri and the binary p35S GUS intron plasmid. Probing for Ri T-DNA transfer was performed with a 4 kb *Hind* III right border T-DNA probe for the pRi2659 plasmid. Probing for the p35S GUS intron T-DNA transfer was performed with either the 0.8 kb *Hind*III/*Bam*HI 35S promoter fragment, the 2.9 kb *Hind*III 35S/GUS fragment or the whole p35S GUS I plasmid. Probes were prepared by a random priming reaction using [^{32}P]dCTP-label according to the Boehringer Mannheim Random Primer Labeling Kit. Hybridization and washing were performed at 60°C according to the Zeta Probe^R, BioRad hybridization instruction manual. Kodak X-Omatic AR film was used for autoradiograms and exposured for 24 to 72 hrs at -70°C.

Nodulation of the A. rhizogenes rooted explants

Explants with roots testing positive for NPT II activity and showing gene integration via Southern blot analysis were transferred to the greenhouse. Control plants of all the genotypes were propagated at this time by sowing seeds and culturing them under identical conditions to the plants with transgenic roots. All of the plants were potted in 3" pots in a 50/50 mixture of fine Vermiculite^{TM} and Faffard^{TM} soil-less compost. The greenhouse temperature range was between 15 and 28°C, and light intensity between 300 and 500 $\mu M\ s^{-1}\ m^{-2}$. The plants were slowly acclimatized to greenhouse humidity over a seven day period with the aid of a plastic propagator. Inoculation of the plants with *Bradyrhizobium japonicum* USDA110 at an inoculum density of approximately $2x10^6$ cells was performed at transfer to soil and again seven days later. Care was taken to keep the soil as dry as possible without letting the plants wilt as the plants with transgenic roots seemed more susceptible to rotting than non-transgenic roots. Plants were removed from the soil four weeks after transfer to soil. The roots were washed under water, the nodule number was counted and nodule phenotype examined. The roots and nodules of these transgenic plants were compared to control plants of all the plant genotypes. The transgenic nature of the nodulated roots was confirmed by GUS assay and Southern blot.

Acetylene reduction activity of transformed nodules

Transgenic and non-transgenic control nodules were tested for their acetylene reduction activity (ARA) according to Bergersen (1979) on a Gas Chromatograph (G.C.). The transgenic nature of these nodules was confirmed after the acetylene reductase assay by performing a GUS assay on the nodules.

Confirmation of transgenic nature of the nodulated root systems

Southern hybridizations were repeated on the DNA from root and nodule tissue collected from the greenhouse grown plants. These were probed with the same probes as previously mentioned to check for transfer of both the p35S GUS intron T-DNA and the Ri plasmid T-DNA. A quick screening of all plants was possible by PCR amplification (with designed primers from National Biosciences, 3650 Annapolis Lane, Plymouth MN) of either a 500 base pair fragment of the cucumopine gene located in the T-DNA from the Ri plasmid (primers: 5' CTA ACT GGT GGG CTC CAT CT 3' and 5' CAT GCT GCG CGA CCA GTC GA 3') or a 150 bp fragment from the CaMV 35 S promoter located on the p35S GUS intron plasmid (primers: 5' CTA CAA ATG CCA TCA TTG CG 3' and 5' ATC ACA TCA ATC CAC TTG CT 3'). This PCR assay showed the presence of the transgene but did not show the incorporation status.

Histological analysis of transgenic roots and nodules

Transgenic roots and transgenic nodules of modified morphology were fixed in 3% Karnovsky fixative (Karnovsky 1965) buffered with 0.1M sodium cacodylate (pH 7.2) overnight at 4°C. Vacuum was applied to the cold samples to facilitate rapid penetration of fixative. The samples were then washed with 0.1M cacodylate buffer, post fixed with 2% osmium tetroxide for two hrs at 4°C, dehydrated in an acetone series and embedded for longitudinal sectioning low viscosity epoxy plastic (Spurr 1969). Thick plastic sections (0.5-2μm) were collected on glass slides and stained with toluidine blue for light microscopy. The abnormal transgenic nodules where examined for any abnormal structures. The transgenic roots were examined for possible *Agrobacterium* contamination.

RESULTS

The first results confirmed that *Agrobacterium* harboring the p35S GUS intron (GUS I) plasmid (see Figure 1 for the plasmid map, Plate 13) did not express GUS activity (Figure 2). This confirmed that by using the p35S GUS intron plasmid, the potential for false positive GUS reactions in the plant tissue by contaminating bacteria had been removed.

Plate 13. Production of transgenic soybean roots.

Figure 1. The p35S GUS Intron plasmid. This binary Ti plasmid carries the GUS and NPT II genes within its T-DNA borders. The intron in the GUS gene can only be spliced by a eukaryotic cell. Therefore *Agrobactrium* cannot express GUS activity. Digestion of this plasmid with *Pvu*II produces 4 fragments, a 3 kb fragment internal to the T-DNA borders, a 4.3 and 5 kb border fragments and a 1.7 kb fragment external to the T-DNA borders.

Figure 2. A GUS assay of *Agrobacterium rhizogenes* harboring the KYLX p3-1 GUS plasmid or the p35S GUS intron plasmid. The top three wells demonstrated that *Agrobacterium* harboring the p35S GUS intron plasmid cannot express GUS activity. The bottom three wells demonstrated that *Agrobacterium* harboring the KYLX p3-1 GUS plasmid which did not have an intron in the GUS gene can express high levels of GUS activity. This experiment showed the importance of using a GUS intron gene construct to avoid false positives in experiments involving *Agrobacterium* .

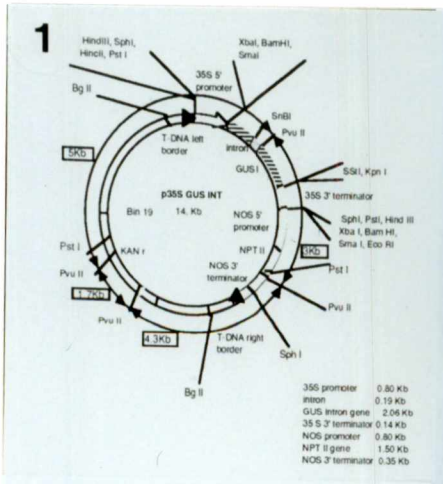
Figure 3. Development of *Agrobacterium rhizogenes* induced roots from the hypocotyl of soybean nodulation mutant nod 49 two weeks after inoculation. Callus initially formed at the wound site on the soybean hypocotyl one week after wounding. Roots would initiate from the wound site 2 to 4 weeks after wounding.

Figure 4. *A. rhizogenes* induced roots under antibiotic selection. The 'rhizogenes' induced root on the left harbors the p35S GUS intron plasmid and showed extensive growth over a 7 day period on solid Mon Mor supplemented with kanamycin 100 μg/ml. The 'rhizogenes' induced root on the right harbored no engineered plasmid. This root did not grow and finally died over the seven day period.

Figure 5. Test for *Agrobacterium* contamination of root cultures. After 1 to 2 weeks of antibiotic treatment in liquid MonMor medium the *A. rhizogenes* induced roots were screened for bacterial contamination. Roots were plated on a minimal BMM medium supplemented with Kanamycin 100μg ml^{-1} for 4 days at 28^0C. Roots segments A to H were from transgenic GUS positive roots which completed the 2 week antibiotic treatment and showed the effective removal of contaminating bacteria. Root segments I and J were from transgenic GUS positive roots prior to antibiotic treatment and served as the positive control. Root segment N in the center for the plate was from an aseptically grown non-transgenic root and served as the negative control.

Figure 6. Culture of transgenic NPT II and GUS positive (transformed with the p35S GUS intron plasmid) soybean cv. Bragg, roots in liquid Mon Mor medium.

Figure 7. Callus generated from transgenic roots of nod49 cultured on a solid B_5 medium supplemented with 25 µg/ml BAP and 2.5 µg/ml IBA after 2 weeks of culture.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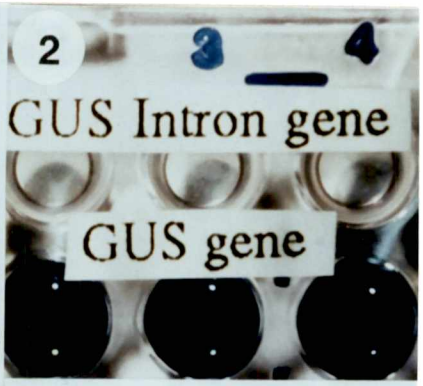
HindIII, SphI, HincII, Pst I
XbaI, BamHI, SmaI
35S 5' promoter
Bg II
SnBI
Pvu II
T-DNA left border
intron
GUS I
SSII, Kpn I
35S 3' terminator
p35S GUS INT
Bin 19
14. Kb
NOS 5' promoter
SphI, PstI, Hind III Xba I, Bam HI, Sma I, Eco RI
NPT II
KAN r
NOS 3' terminator
Pst I
Pvu II
5Kb
1.7Kb
4.3Kb
T-DNA right border
Sph I
Bg II
35S promoter 0.80 Kb
intron 0.19 Kb
GUS intron gene 2.06 Kb
35 S 3' terminator 0.14 Kb
NOS promoter 0.80 Kb
NPT II gene 1.50 Kb
NOS 3' terminator 0.35 Kb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GUS Intron gene
GUS gene


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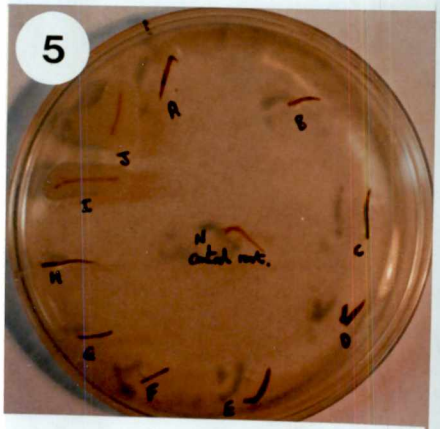
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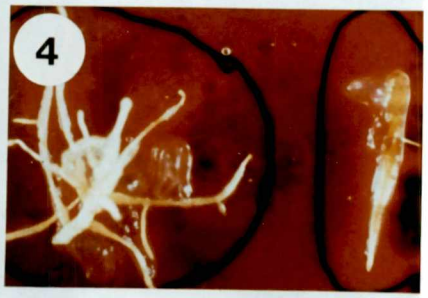
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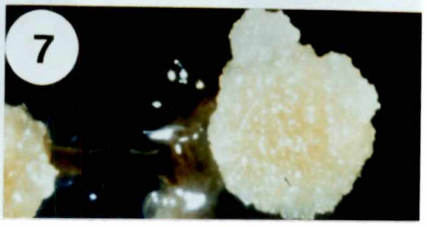
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Roots were initiated from the wound site 2 to 4 weeks after wounding (see Figure 3). The roots were assayed for GUS activity at this stage and again 1 to 2 weeks after transfer to liquid Mon Mor medium. This period in liquid medium facilitated the removal of residual *Agrobacterium* cells with antibiotics and selection of kanamycin resistant roots. Kanamycin in the range of 75 to 125 µg/ml proved effective at killing non-transgenic and *A. rhizogenes* induced roots, but not 'rhizogenes' roots harboring the NPT II gene on the p35S GUS intron plasmid. A kanamycin level of 100 µg ml was chosen as optimal selection level (Figure 4). Higher levels (up to 200 µg/ml) of kanamycin did not kill the transformed roots but slowed their growth considerably.

Tansgenic roots were screened for *Agrobacterium* contamination two weeks after transfer to liquid MM medium supplemented with antibiotics. Roots that had been through this culture period appeared to be free of contaminating *Agrobacterium* (Figure 5). Transgenic roots which had not been through this culture period showed bacterial contamination. These transgenic roots could be maintained in culture (liquid MM medium) for periods in excess of six months (Figure 6). Regular sub-culturing (every one to two weeks depending on culture growth) was required to maintain rapid growth.

Attempted regeneration of plants from Agrobacterium rhizogenes roots

Regeneration of whole plants from the transgenic *A. rhizogenes* roots was attempted. Transgenic roots on B5 medium supplemented with IBA at 2.5µM and BAP levels of 0, 1, 3, 5, 25, 50, 100, 200,400 µM. Levels in the range of 3 to 100µM induced green callus production from the roots and inhibited root growth (figure 7). However, no shoot induction was noted over a culture period of 4 months. BAP levels of 0 and 1 µM did not influence normal root growth or induce callus production. The high BAP levels of 200 and 400 µM were toxic to root and callus growth.

GUS assay of transgenic roots

There was a large variation in the virulence of *A rhizogenes* to the soybean genotypes. Table 1 shows the percentage of seedlings of each soybean genotype which show GUS activity in their roots. Cultivars 'Peking,' Bragg and mutant nts382 showed the highest frequency of transgenic root production. Non-nodulating mutants nod49 and nod139 showed the lowest frequency of 'rhizogenes' root production. The percentage of 'rhizogenes' roots which tested GUS positive was high (70 to 100%). This suggested that

the frequency of co-transfer of the Ri plasmid T-DNA and the binary plasmid was high. This result also suggested that almost all of roots initiating from the wound site were transgenic for the Ri T-DNA and/or the p35S GUS I plasmid DNA.

Table 1. Frequency of *A. rhizogenes* induced roots and percentage of those roots testing positive for GUS activity in different soybean genotypes 4 weeks after inoculation.

Genotype	Number of plants inoculated	Number of plants producing hairy roots	Percentage of roots testing GUS positive
Bragg	20	10	80
nts382	20	14	70
nod49	20	4	75
nod139	20	7	100
Peking	20	19	89

Plants with *A. rhizogenes* induced roots (Figure 8, Plate 14) were transferred to the greenhouse and inoculated with *Bradyrhizobium japonicum* USDA110. These plants were grown for approximately four weeks (Figure 9). The majority of these plants (90%) exhibited dark green leaves. Approximately 15% of the plants had light green leaves and appeared nitrogen starved. Roots and nodules were taken from these plants and assayed for GUS activity (Figures 10, 11 and 12).

GUS activity varied between the transgenic plants. Some plants exhibited GUS activity in all root and nodule tissue other plants only expressed GUS activity in meristematic regions such as root tips, vascular tissue and nodule inner cortex (Figure 13 to 17, Plate 15).

The non-nodulating mutant plants nod139 and nod49 (Figure 10) did not nodulate. The supernodulating nts382 plants remained supernodulating (Figure 11, 15,16 and 17). Plants with a wild-type nodulation phenotype such as cv. Bragg and Peking remained wild-type (Figure 12 and 14).

Plate 14. Nodulation of trangenic soybean roots

Figure 8. A soybean plant cv. Bragg 5 weeks after inoculation with *A. rhizogenes* harboring the p35S GUS intron plasmid. This plant had been cultured for 2 weeks in liquid Mon Mor medium with antibiotic selection for transgenic kanamycin resistant roots and removal of contaminating *Agrobacterium.* All of the roots on this plant tested GUS positive.

Figure 9. Soybean plants with 'rhizogenes' induced roots growing in the greenhouse. The dark green plants on the left represent the majority (85%) of the plants produced with transgenic roots and nodules. These plants nodulated normally and there nodules fixed nitrogen. The plants on the right represented 15% of the plants produced with transgenic roots. These plants exhibited abnormal nodule phenotypes and were non-nitrogen fixing.

Figure 10. A GUS positive root from soybean nodulation mutant nod139.

Figure 11. The developing nodules (see arrows) from a transgenic GUS positive root of soybean mutant nts382.

Figure 12. A mature nodule from a transgenic GUS positive root of soybean cv. Peking. The two regions of the root that appeared unstained for GUS activity may be due to the tissue being chimeric for the transgene integration or expression or due to an experimental artifact such as uneven penetration of the X-GLUC substrate.

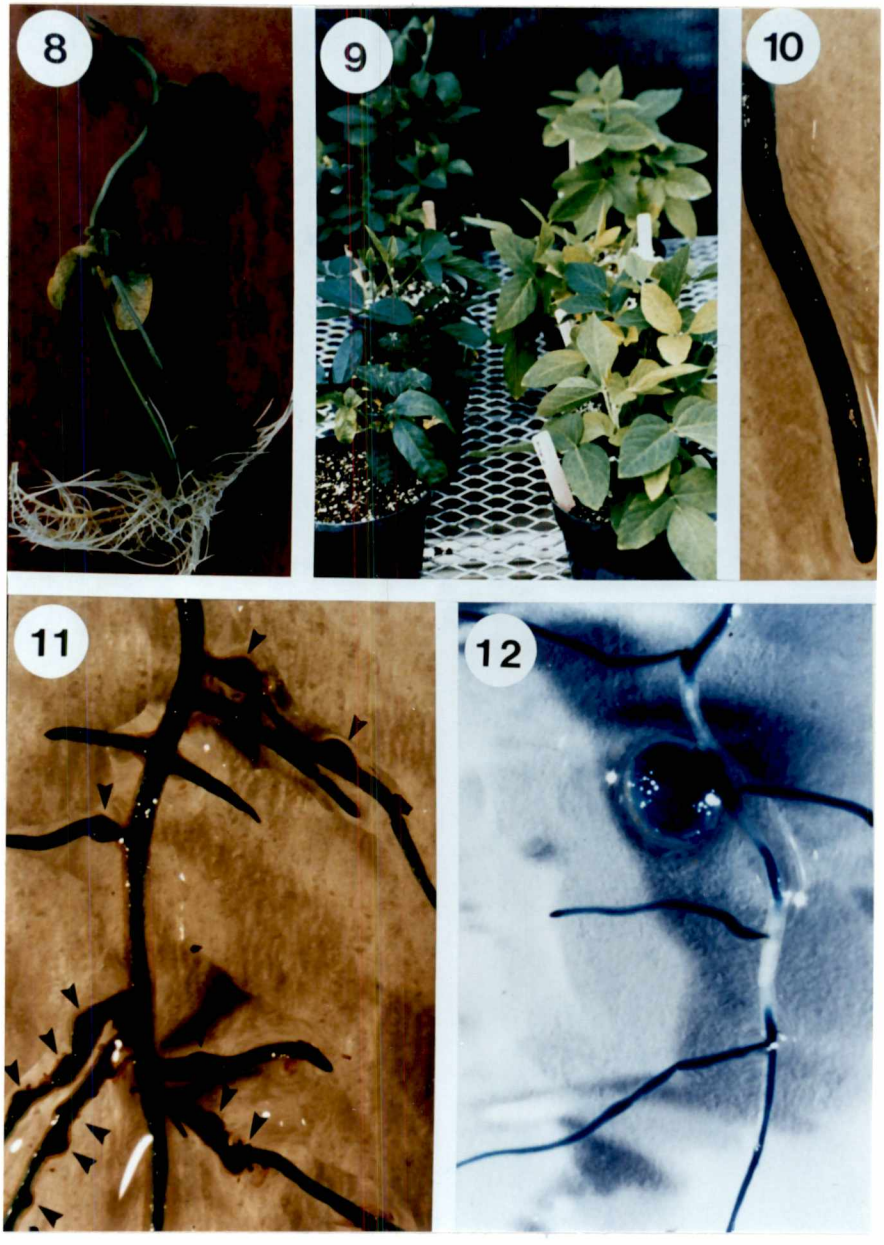
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Plate 15. Gene expression in nodulated transgenic roots.

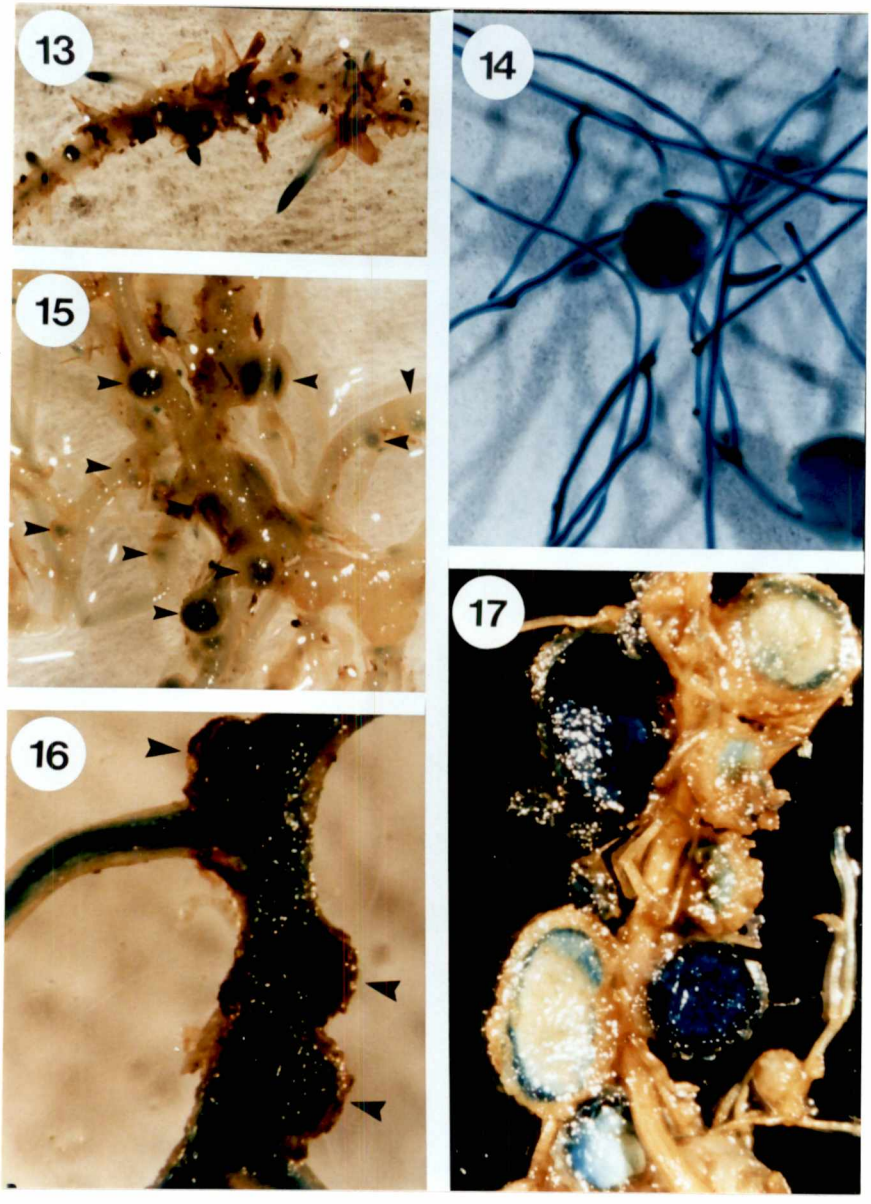
Figure 13. A transgenic root from soybean nodulation mutant nts382. This roots demonstrates that lateral root primordia are areas of high GUS expression.

Figure 14. A transgenic GUS positive root from soybean cv. Bragg. This root showed GUS expression such as root tips and nodule inner cortex, but not in other areas of the root.

Figure 15. A transgenic root from soybean nodulation mutant nts382. This root showed nodules as areas of GUS expression but not older root tissue.

Figure 16. A transgenic root from soybean nodulation mutant nts382. This root showed high GUS expression throughout the root and nodule tissue.

Figure 17. A transgenic root from soybean nodulation mutant nts382. This root showed nodules (specifically the nodule inner cortex) as areas of GUS expression but not older root tissue. Root meristems also showed GUS activity.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Nodule phenotype and GUS activity in transgenic nodules

Nodule inner cortex tissue (NIC) was always the region of highest GUS activity (Figures 18 to 21, Plate 16). The inner cortex was rich in vascular tissue which also appeared another region of high GUS activity in the roots. The vascular tissue in the nodule cortex could clearly be seen when the GUS stained nodule was cleared by autoclaving the tissue in 70% lactic acid (Figure 20). The bacterial infection zone was apparently devoid (Figure 18 and 21) or low in GUS activity (Figure 19) depending upon whether the nodule was assayed for GUS activity intact or sectioned. This suggested that the GUS reaction was a limited either by lack of diffusion of the X-Gluc substrate into the infected zone, or the nodule's oxygen diffusion barrier limited the oxygen requiring GUS reaction (Jefferson 1987a; b) in the infected region. The 35S CaMV promoter is considered a constitutive promoter. Some plants showed root systems with constitutive GUS expression (Figures 10, 11 and 14). However, our results also prove that there can be preferential expression of GUS in specific tissues (nodule inner cortex and root tips; Figures 13, 15, 17).

Comparison of nodule number per plant in transgenic and non-transgenic soybean roots

Nodule number of the plants with transgenic roots was compared to plants with non-transgenic roots of the same genotype cultured under identical conditions (Table 2).

Nodules with altered phenotype

Approximately 85% of the plants (cv. Peking) with *'rhizogenes'* induced roots showed a normal nodule phenotype (spherical nodules) and fixed nitrogen. However, a small subset of these plants (15%) showed an extremely altered nodule phenotype. At times these nodules would branch or bud from a mother nodule (Figure 22, Plate 22). The nodules were unlike classical spherical soybean nodules, but appeared elongated (Figure 23 and 24). These nodules appeared to have an infection zone which followed an actively growing nodule meristem (Figure 25). These abnormal transgenic nodules did not fix nitrogen (Nif^-). Also unlike normal soybean nodules these nodules senesced rapidly (2 months). Indeterminate nodules senesce early, however, determinate nodules usually last the life time of the plant.

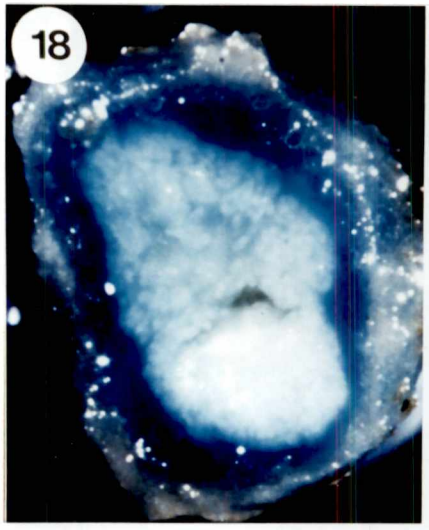
Plate 16. Gene expression in sectioned nodules.

Figure 18. A cross-section of a transgenic GUS positive nodule from soybean cv. Peking. The nodule inner cortex was highly stained when the nodule is incubated in the X-gluc assay solution intact. This nodule had a normal spherical nodule phenotype.

Figure 19. A cross-section of a transgenic GUS positive nodule from soybean nodulation mutant nts382. The nodule cortex and infection zone was stained GUS blue when the sectioned nodule was incubated in the X-gluc assay solution. This nodule had a normal spherical nodule phenotype.

Figure 20. The result of clearing of a GUS stained nodule from cv. Peking by autoclaving in 70% lactic acid. This cross-sectioned nodule clearly exhibited branching vascular tissue in the nodule cortex. This nodule had a normal spherical nodule phenotype.

Figure 21. A cross-section of a transgenic GUS positive nodule from soybean nodulation mutant nts382. The nodule cortex was highly stained when the nodule was incubated in the X-gluc assay solution intact. This nodule had a normal spherical nodule phenotype.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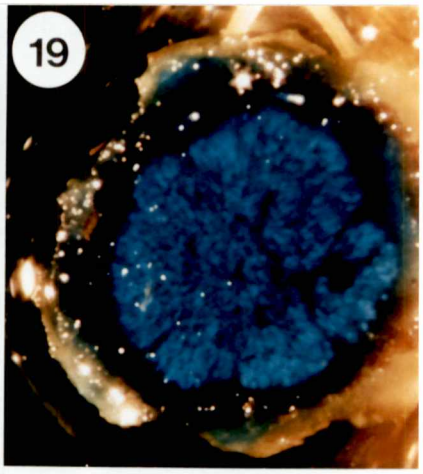
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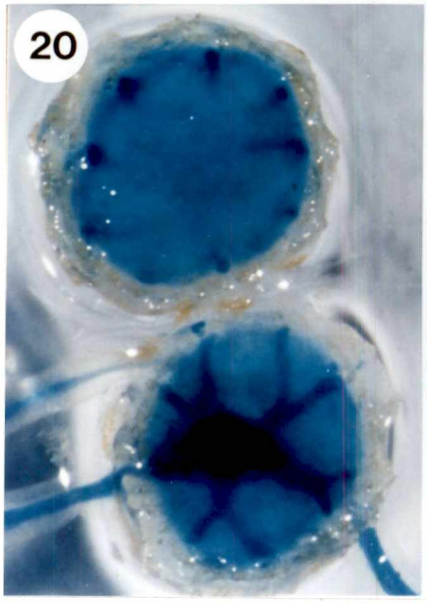
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21

Table 2. Comparison of nodule numbers on transgenic *A. rhizogenes* induced and normal seedling roots. This table shows that there is no difference in the average nodule number of any of the transgenic Ri rooted explants with normal nodule phenotype (no elongated nodules), when compared to the average number of nodules on non-transgenic roots of the same cultivar or mutant type.

Genotype	Nodule number	
	non-transgenic root system	transgenic root system
nts382	255±75	246±39
nod49	0	0
nod139	0	0
Bragg	26±6	24±7
Peking	12±3	12±2

The mean nodule number (± SE) in transgenic (*A. rhizogenes* induced roots) and non-transgenic root systems (wild-type roots) of nodulation mutants nts382, nod139, nod49 and wild-type plants Bragg and Peking. Five plants were used per sample (four plants for nod49). Nodule number was measured 4 weeks after inoculation with *Bradyrhizobium japonicum* USDA110.

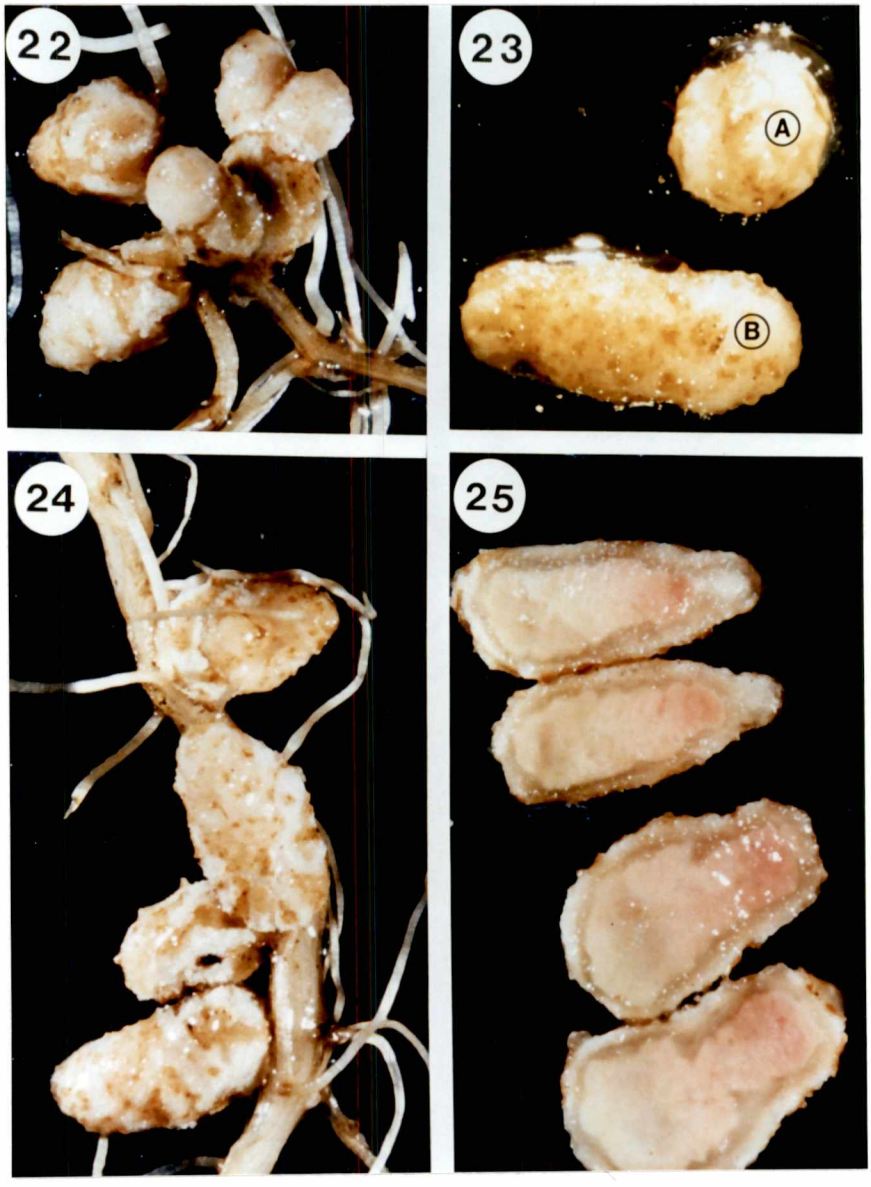
Plate 17. Nodules with an altered morphology

Figure 22. A budding and elongated nodules from 'rhizogenes' induced roots of soybean cv. 'Peking.'

Figure 23. Comparison of normal and altered nodule phenotypes derived form 'rhizogenes' induced roots. On top, (a) a transgenic nodule with a normal phenotype from 'rhizogenes' induced roots of soybean cv. Peking. On bottom (b) a transgenic nodule with an altered, more determinate nodule structure.

Figure 24. Elongated nodules from 'rhizogenes' induced roots of soybean cv. 'Peking.'

Figure 25. A cross section of the elongated nodules from 'rhizogenes' induced roots of soybean cv. Peking. These nodules appeared to have an active meristematic region at the nodule tip.


22
23
A
B
24
25

The frequency of alteration of nodule phenotype with *Agrobacterium rhizogenes* induced roots was investigated (Table 3). These abnormal nodules were seen only in cv. Peking plants. The altered nodule phenotype was seen in plants with roots transformed with the *A. rhizogenes* Ri T-DNA alone, and plants transformed with *A. rhizogenes* Ri T-DNA and a binary expression cassette encoding anti-sense chalcone synthase (CHS). Table 4 compared abnormal nodule numbers on transgenic *A. rhizogenes* derived roots to nodule numbers on non-transgenic plants. It appears that these plants with abnormal nodules also have significantly higher nodule numbers.

Table 3. Frequency of plants displaying an altered of nodule phenotype with *Agrobacterium rhizogenes* induced roots from cv. Peking.

Nodule phenotype	**Transgene treatment**				
	Control WT roots	K599 roots	K599 GUS I roots	K599 CHS sense roots	K599 CHS anti-sense roots
Plants with normal nodules.	20(Nif+)	17(Nif+)	20(Nif+)	25(Nif+)	20(Nif+)
Plants with abnormal nodules	0	3(Nif-)	0	5(Nif-)	0

Degree of nodule abnormality and age of the plants

An initial group of five plants exhibiting abnormal nodule phenotypes were closely examined. The frequency of abnormal nodules on each plant was assessed thirty days after inoculation. In four out of five of these plants approximately 95% of the nodules were abnormal. The fifth plant showed a mixture of abnormal and normal nodules (approximately 70% abnormal, 30% normal). The normal nodules appeared on separate root branches. Nodules were harvested from these plants at regular monthly intervals. The degree of abnormality was maintained for the first three months. However, the degree of abnormality decreased with the age of the plant over the following four to six months. These nodules, instead of appearing branched or elongated, appeared cone shaped. It appeared that the nodules developed with an active meristem, but their growth was much more limited. Transverse sections of these nodules exhibited infection zones following a meristematic region.

Table 4. Comparison of abnormal nodule frequency on transgenic *A. rhizogenes* roots to nodule fequency on non-transgenic plants. Five plants per treatment were counted (± standard error).

Genotype	Nodule number	
	non-transgenic root system	transgenic root system
Peking	12±3	33±9

Comparison of the acetylene reduction activity of normal and transgenic nodules

Roots and nodules from control non-transgenic plants and plants with transgenic roots and nodules of normal phenotype and abnormal nodule phenotype were assayed by acetylene reduction to evaluate their nitrogen fixation activity. The transgenic nodules with a normal phenotype produced 38.4 μM of ethylene per gram of nodule fresh weight in a 30 minute period. This compared to 44.6 μM ethylene per gram nodule fresh weight over the same time period for the non-transgenic control nodules. The transgenic nodules with an abnormal phenotype did not show any nitrogen fixing ability when assayed for acetylene reductase activity. This assay showed that the normal transgenic nodules possessed nitrogenase activity and were active in fixing atmospheric nitrogen. Also, the activity of the nodules was of a comparable level to that of the normal nodules. The abnormal nodules appeared not to be able to fix nitrogen. This suggested that the alteration in nodule structure may prevent the nitrogen fixing ability. After the acetylene reduction assay the transgenic roots and nodules were confirmed GUS positive.

Cucumopine assay of transgenic soybean roots

For the cucumopine assay, *in vitro* grown 'rhizogenes' roots transformed with and without the p35 GUS intron plasmid and control non-transgenic roots were assayed for their cucumopine content. Non-transgenic and transgenic 'rhizogenes' induced roots of tobacco were also included as controls. All of the putative transgenic root cultures tested expressed cucumopine activity (nine transgenic root cultures; Figure 26, Plate 18).

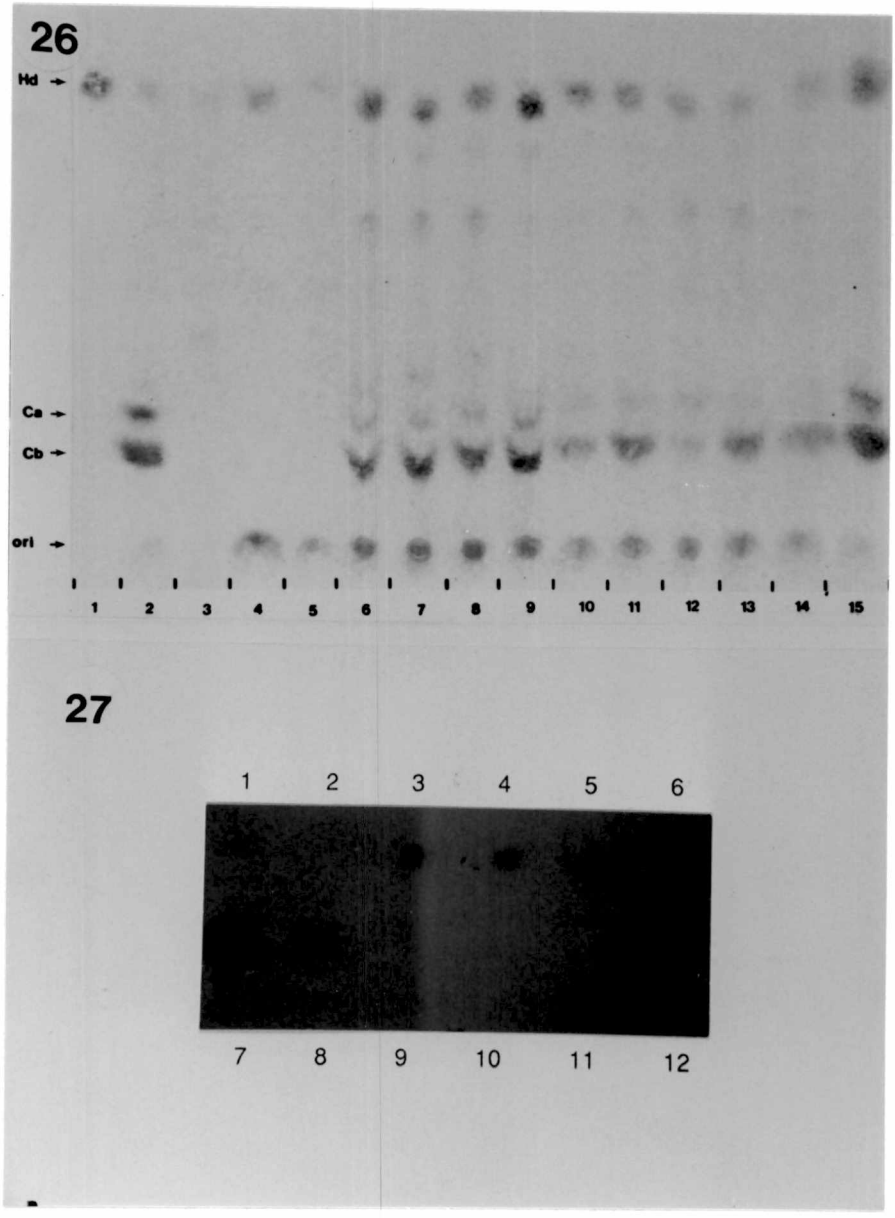
NPT II assay of transgenic soybean roots

Roots and nodules were taken from plants (GUS positive/kanamycin resistant roots) growing in the greenhouse 6 weeks after transfer to soil and inoculation with USDA 110. This tissue was used to perform a radioactive analysis for NPT II activity.

Plate 18. Cucumopine and NPT II assays.

Figure 26. Cucumopine assay of transgenic *A. rhizogenes* induced root tissue. The root tissue was assayed for cucumopine by high voltage electrophoresis. Lane 1 is a histidine standard. Lanes 2 and 15 are extracts of hairy roots induced by *A. rhizogenes* K599. Lane 3 is non-transgenic tobacco root tissue. Lane 4 and 5 are non-transgenic soybean cv. Peking roots. Lanes 6, 7, 8, 9 and 10 are extracts of hairy roots induced by *A. rhizogenes* K599 harboring the p35S GUS I plasmid (GUS positive roots) on soybean cv. Peking. Lanes 11, 12, 13 and 14 are extracts of hairy roots induced by *A. rhizogenes* K599 alone. The labels are as follows Hd, histidine; Ca, cucumopine; Cb, acid degradation product of cucumopine; ori, origin. All of the 'hairy root' cultures tested positive for cucumopine.

Figure 27. Dot blot showing NPT II activity in *A. rhizogenes* transformed soybean roots from one plant cv. Peking, blots 1 and 2: non-transgenic roots, blots 3 to 6: soybean transgenic roots (NPT II and GUS I positive), blot 7: transgenic nodule, blot 8: transgenic root tips, blot 9: non-transgenic nodules, blot 10: *Bradyrhizobium* USDA110 , blots 11: non-transgenic nodules and blot 12 is of non-transgenic tobacco leaves.


26
Hd
Ca
Cb
ori
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
27
1 2 3 4 5 6
7 8 9 10 11 12

The NPT II dot blot showed weak expression of NPT II activity in the roots and slightly higher activity in the nodules. However, the control tissue was clearly negative (Figure 27). This contrast in the low NPT II gene expression compared to the high GUS activity seen in the same tissue could be explained in the difference in the strength of the promoters 'driving' each gene. The 35S CaMV promoter was significantly stronger than the nopaline synthetase (NOS) promoter 'driving' the NPT II gene (Sanders *et al.*, 1987). Sanders *et al.* (1987) showed that NPT II transcript levels were 30 times higher and NPT II enzyme levels were 110 times higher with the 35S CaMV promoter as compared to the NOS promoter.

Southern analysis of transgenic soybean roots

The Southern blot analysis of the putative transgenic roots and nodules clearly demonstrated a classical integration pattern for the integration of the T-DNA of the Ri plasmid (Figure 28, Plate 19). This blot was probed with a right border probe for the Ri plasmid pRi2695. Lanes 1 to 5 are transgenic root systems, lane eight shows non-transgenic plant DNA 'spiked' with *Agrobacterium* harboring the Ri plasmid and the p35S GUS I plasmid. The same blot was stripped and reprobed with the p35S GUS I plasmid (Figure 29). This blot showed that the transgenic roots (lanes 1, 2, 3, 5, and 6) have plasmid sized restriction fragments (lanes 11 and 12 contain 10 and 100 pg of GUS I plasmid DNA). These fragments are from inside and outside the T-DNA borders (see Figure 1). There appeared to be no unique plasmid-sized bands. For example, the 1.7 kb fragment present in all lanes was an external fragment to the T-DNA borders. Lane 5 of figure 29 was from transgenic (Ri) roots harboring the KYLX p3-1 GUS plasmid. This hybridization pattern was also identical to that obtained with the whole KYLX p3-1 GUS plasmid. The DNA used for the blot in Figure 30 was obtained from 8 independent transgenic GUS positive roots cultures grown *in vitro* for 3 months without antibiotics. These roots were seemingly bacteria-free, any contaminating bacteria would have quickly contaminated the liquid culture medium. Histological analysis of these root cultures was performed to search for contaminating bacteria in the plant tissue. The DNA hybridized in the blot was digested with the restriction enzyme *Pvu*II and probed with the 0.8 kb 35S CaMV promoter fragment. Only a 5 kb plasmid sized fragment was observed. The DNA in the blot shown Figure 31 was digested with *Eco*RI (this enzyme only cuts the GUS I plasmid once) and probed with a 3 kb 35S GUS fragment. Only a 14 kb fragment was seen. These results indicated that the Ri T-DNA was classically integrated but the GUS I plasmid was not classically integrated.

Plate 19. Southern hybridization analysis of transgenic roots.

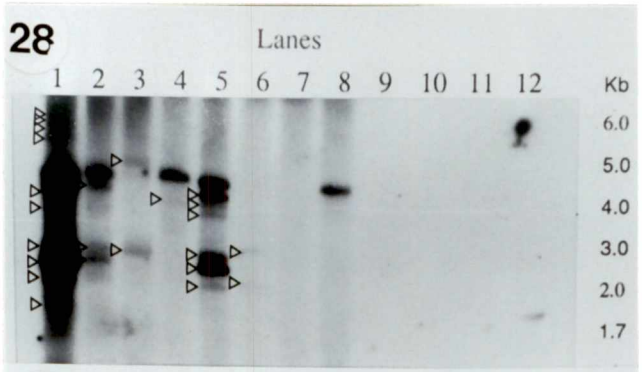
Figure 28. The Southern blot for *Agrobacterium rhizogenes* Ri T-DNA integration. A 4 kb *Hin*dIII pRi2659 T-DNA border fragment (right T-DNA border and part of the cucumopine gene)was used as the probe. Lane 1: DNA isolated from *A. rhizogenes* GUS (GUS I) positive transformed roots of a Bragg plant. Lane 2: DNA isolated from isolated from *A. rhizogenes* GUS (GUS I) positive transformed roots and nodules of an nts382 plant. Lane 3: DNA isolated from *A. rhizogenes* GUS (GUS I) positive transformed roots and nodules of a Bragg plant. Lane 4: DNA isolated from *A. rhizogenes* (Ri T-DNA) only transformed roots and nodules of a Peking plant. Lane 5: DNA isolated from *A. rhizogenes* GUS (KYLX GUS) positive transformed roots. Lane 6: DNA isolated from *A. rhizogenes* GUS (GUS I) positive transformed roots of a cv. Peking plant. Lane 7: control non-transgenic root DNA. Lane 8: control non-transgenic root DNA spiked with *Agrobacterium* harboring the GUS I plasmid. Lane 9 and 10: blank. Lanes 11 and 12: contain 10 and 100 pg respectively of GUS I plasmid DNA. The plants DNA in lanes 1 to 6 show classical integration patterns for the Ri T-DNA region (see arrows for unique bands indicating T-DNA/plant DNA boundaries). The bacterial plasmid hybridization pattern is seen in lane 8.

Figure 29. Southern blot from Figure 29 stripped and reprobed (i.e. lanes as before) for integration of the GUS I plasmid T-DNA. The whole GUS intron plasmid was used as the probe. The DNA from transgenic roots and nodules in lanes 1, 2, 3 and 6 showed non-classical plasmid (GUS I) hybridization patterns. All the plasmid bands (see arrows) are present for these lanes (1, 2, 3 and 6), however, some are too faint to see on the photograph (hybridization pattern identical to the restriction pattern of the GUS I plasmid; see Figure 1 for *Pvu*II digestion pattern). This indicates that the GUS I plasmid is not integrated into the plant genome (no unique plant, plasmid border fragments). Lanes 8, 11 and 12: also show a GUS I plasmid pattern. These lanes either contain *Agrobacterium* DNA (lane 8) or GUS I plasmid DNA (lanes 11 and 12). Lane 4: (control) DNA isolated from *A. rhizogenes* (Ri T-DNA) only transformed roots does not show hybridization to the p35SGUS I plasmid fragments. Lanes 5 DNA isolated from *A. rhizogenes* GUS (KYLX GUS) positive transformed roots also shows a hybridization pattern identical to the restriction pattern of the KYLX GUS plasmid (restriction pattern not shown). This also indicates that this plasmid is not classicaly integrated.

Figure 30. Southern blot of soybean *A. rhizogenes* roots transformed with the GUS intron gene and the NPT II gene. Root DNA was digested with *Pvu*II and probed with a 0.8kb *Hin*dIII *Bam*HI fragment of the 35S promoter. Integration of the T-DNA into the plant genome will be indicated by different sized border fragments (plant/plasmid border fragments). If the plasmid was non-integrated, a 5 kb border fragment will hybridize. Lanes 1-3: *Agrobacterium* DNA harboring the GUS intron gene. Lanes 5 to 8 and 10 to 13: DNA from GUS positive root cultures. All the bands were 5 kb in size (see arrow). This hybridization pattern

indicates that the GUS I plasmid was not integrated into the plant genome (no unique plant/plasmid border fragments are seen).

Figure 31. Southern blot of soybean 'rhizogenes' roots transformed with the GUS intron gene and the NPT II gene. Root DNA was digested with *Eco*RI and probed with the *Hin*dIII fragment of the 35SCaMV promoter and GUS I gene. Lanes 1 to 3: DNA from 'rhizogenes' GUS positive root cultures. Lanes 4 and 5: control non-transgenic root DNA. Lane 6: plasmid DNA. Lanes 7 and 8: Ri T-DNA transformed roots only. Hybridization in all lanes is at the 14 kb level. This indicated that the whole GUS I plasmid was not classsically integrated.


28
Lanes
1 2 3 4 5 6 7 8 9 10 11 12
Kb
6.0
5.0
4.0
3.0
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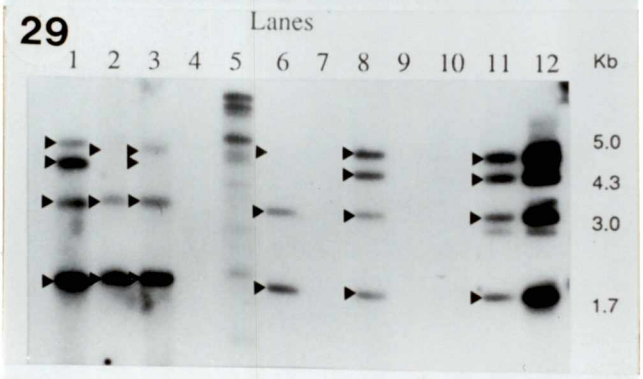
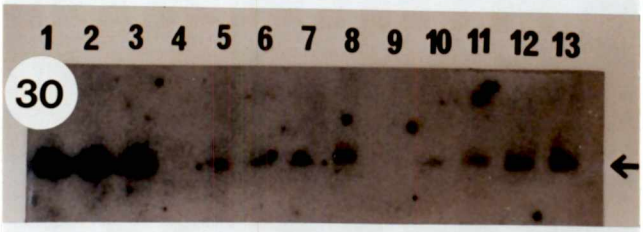
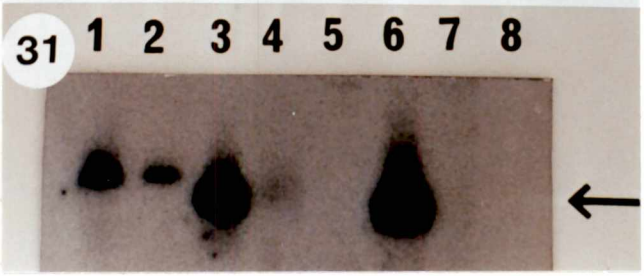

29
Lanes
1 2 3 4 5 6 7 8 9 10 11 12
Kb
5.0
4.3
3.0
1.7


1 2 3 4 5 6 7 8 9 10 11 12 13
30


31
1 2 3 4 5 6 7 8

Histological analysis of transgenic nodules with an altered phenotype

The abnormal transgenic nodules were sectioned and then examined under the microscope for normal and abnormal nodule structures. Figure 32 (Plate 20) shows the cross section of an transgenic nodule with an abnormal macroscopic morphology. However, all the microscopic structures seen in this figure are normal. This figure shows (1) the outer nodule cortex, (2) the sclerenchyma layer, (3) the inner cortex, (4) a vascular bundle, (each arrow shows a separate vascular bundles) and (5) the infected region. Close-up views of these structures are seen in the following figures, the sclerenchyma layer (Figure 33), vascular bundle (Figure 34 and 35), and the infection zone (Figure 36).

Further histological sections revealed that the abnormal transgenic nodules (30 days old nodules) had some abnormal structures. Figure 37 (Plate 21) shows the boundary of two nodules which have budded from a mother nodule (see Figure 22 for an external view of such a nodule). Figures 38 and 39 reveal meristematic regions in the abnormal (for macroscopic views of these regions see Figure 25). These meristematic regions are at the tip of the elongated nodule and appear to break (disturb) through the sclerenchyma layer. In normal spherical determinate soybean nodules meristematic activity ceases early in the nodule's development. It appeared that the meristematic activity in these nodules did not cease.

Histological proof of meristematic region in abnormal mature transgenic soybean nodules

The histological sections (shown in Figures 40 to 43, Plate 22) showed areas of active meristematic activity in the mature abnormal (30 days old) transgenic soybean nodules. These figures (40 to 42) show that infection threads followed these newly developed cells and the cells quickly became colonized with bacteroids. The mitotic events shown in Figure 43 proved that these regions are meristematic.

Histological analysis of transgenic root tissue

The Southern blot results which indicated that the GUS I plasmid was not classically integrated prompted us to look for possible bacterial contamination of the GUS positive roots. Figures 44 to 47 (Plate 23) show transgenic root tissue magnified X10, X40, X60, X100.

Plate 20. Histological sections of normal structures in abnormal nodules.

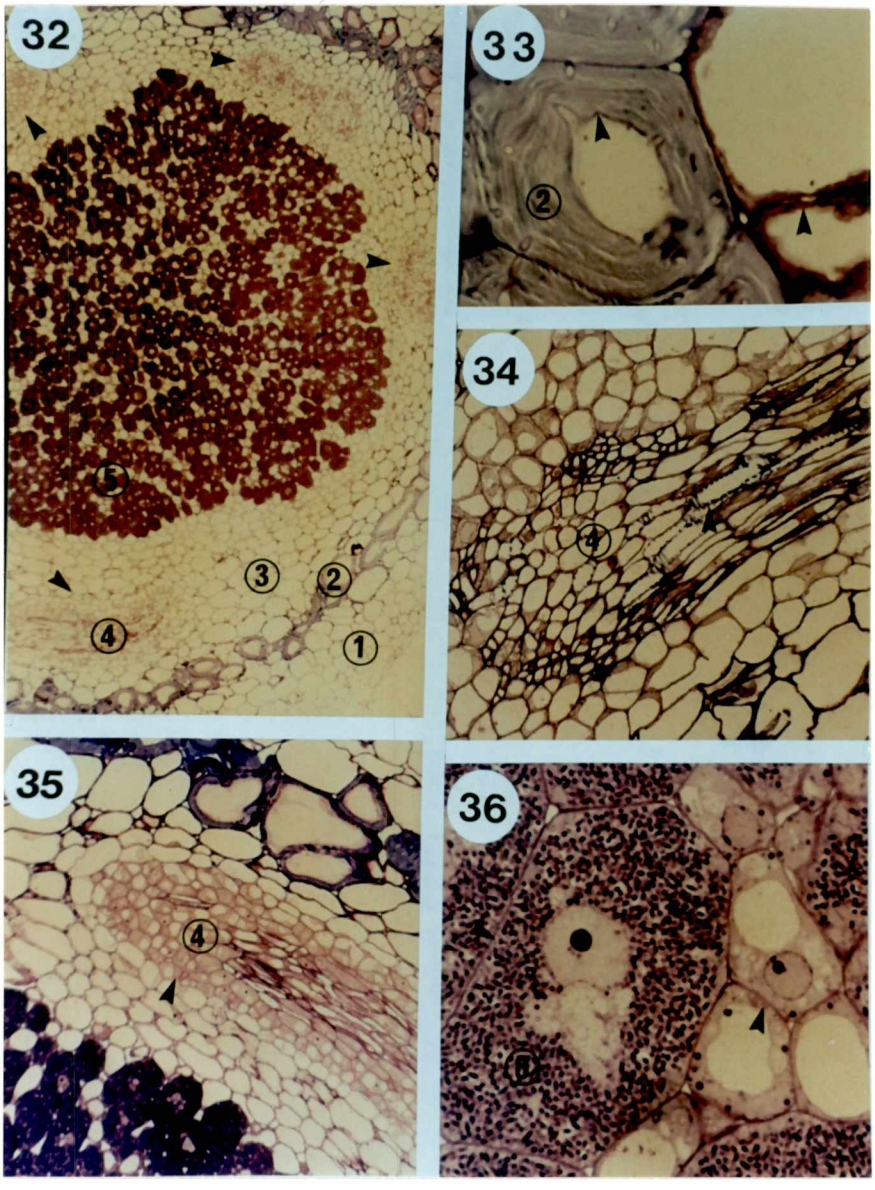
Figure 32. A transverse section of a transgenic soybean nodule of cv. Peking. This nodule had an abnormal elongated phenotype. However, all the structures seen in this figure are normal. Label 1 shows the outer nodule cortex region, label 2 the sclerenchyma layer, this layer gives structural support to the nodule, label 3 the inner cortex, label 4 a vascular bundle, each arrow shows a separate vascular bundle.and label 5 is the infected region (magnification X10).

Figure 33. A close-up view of the sclerenchyma tissue seen in Figure 32. This tissue is formed from lignification of plant cells (2). The arrows indicate the lumen of the lignified cell compared to the normal cellulose cell wall of the inner cortex (magnification X60).

Figure 34. A view of a vascular bundle (4) in the nodule inner cortex. The arrow indicates secondary thickening in the xylem vessels (magnification X20).

Figure 35. A view of a vascular bundle (4) showing its position in the nodule inner cortex. The arrow indicates the vascular bundle support/transfer cells. The phloem brings sugar metabolites for the nitrogen-fixing bacteria in the infected zone. The xylem takes fixed nitrogen to the rest of the plant in the form of ureides and amides (magnification X20).

Figure 36. A close-view of the infection zone. The infected cell (6) indicated is full of bacteroids. Bacteroids are membrane bound pockets for the nitrogen fixing bacteria. This cell is approximately six times larger than the un-infected neighbor cell also marked with an arrow. These smaller un-infected cells act as transport cells exporting ureides out of the nodule and sugars into the nodule. The nucleus and nucleolus of the infected cell is large indicating that the cell is highly active in transcriptional processes (magnification X60).


32
5
3
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1
33
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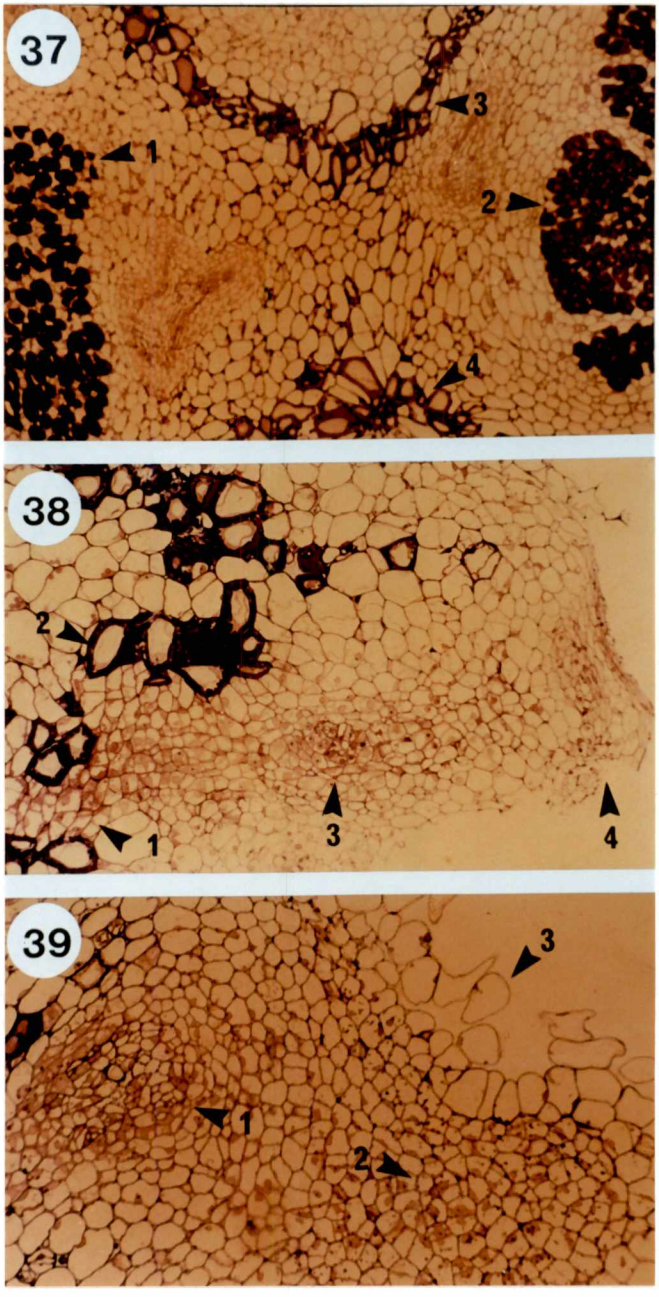
34
4
35
4
36
5

Plate 21. Meristematic activity in abnormal nodules.

Figure 37. The boundary of two nodules which have budded from a mother nodule (transverse section). These nodules share a sclerenchyma layer (3) and an inner cortical layer. The vascular bundles appeared to be discrete for each nodule. The infected regions of each nodule were separate (arrows 1 and 2) (magnification X10).

Figure 38. A meristematic tip of an abnormal transgenic nodule (transverse section). Arrow 4 shows the very tip of this elongated nodule, arrow 3 shows an actively meristematic region, arrow 1 shows a break in the sclerenchyma layer with the meristematic tissue breaking through this region (Magnification x 20).

Figure 39. A meristematic tip of an abnormal transgenic nodule (transverse section). This nodule appears to have two meristematic regions and may be the beginning of a budding nodule. Arrows 1 and 2 show two separate meristematic regions. Arrow 3 shows the outer cortical cells. These cells appear to be very loosely connected to the nodule and even sloughing off (see figure 24 for a macro-view)(magnification X20).


37
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2
3
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38
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39
1
2
3

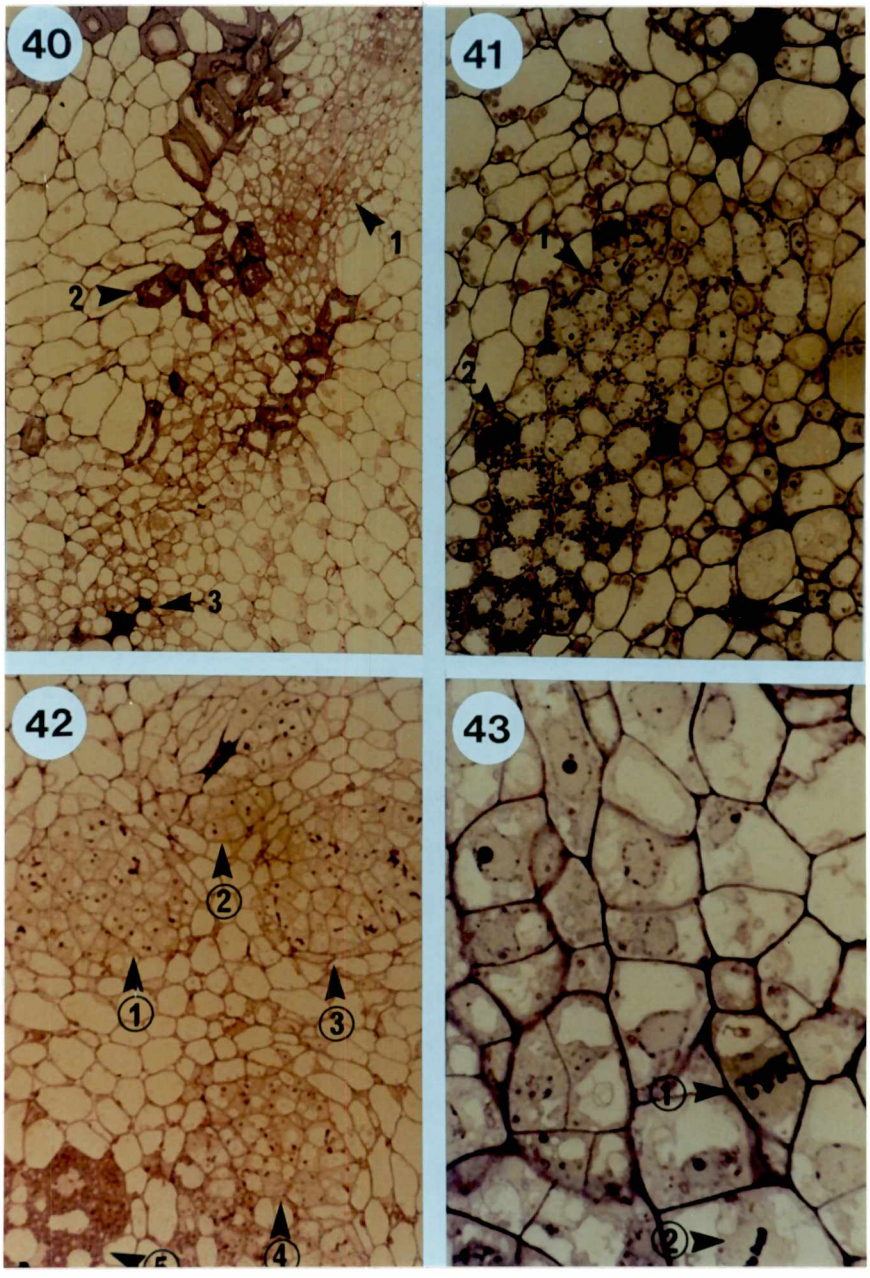
Plate 22. Active infection of meristematic cells in abnormal nodules.

Figure 40. Meristematic regions (longitudinal section) (1) of an abnormal transgenic nodule seen to actively progress through the sclerenchyma (2) layer and closely followed by bacterial infection threads (3). These infection threads release bacteroids into the newly formed cells behind the nodule meristem (magnification X20).

Figure 41. Meristematic regions (longitudinal section) of an abnormal transgenic followed by active bacterial infection. Arrow 1 shows cells that have only a few bacteroids per cell, arrow 2 shows cells with more bacteroids, and arrow 3 shows a bacterial infection thread (magnification X40).

Figure 42. Four meristematic regions (transverse section of nodule tip)(arrows 1,2,3 and 4) of an abnormal transgenic nodule (transverse view). Closely following these meristematic regions are infection threads and infected cells (magnification X20).

Figure 43. Cells in the meristematic region of an abnormal transgenic nodule. This figure (see arrows 1 and 2) proved that these regions were meristematic in that it shows mitotic events. The chromosomes indicated by arrows 1 and 2 were aligned in the metaphase configuration ready to be pulled apart to separate poles (magnification X60).


40
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2
3
41
1
2
42
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2
3
4
5
43
1
2

Plate 23. Sections of transgenic GUS positive root cells

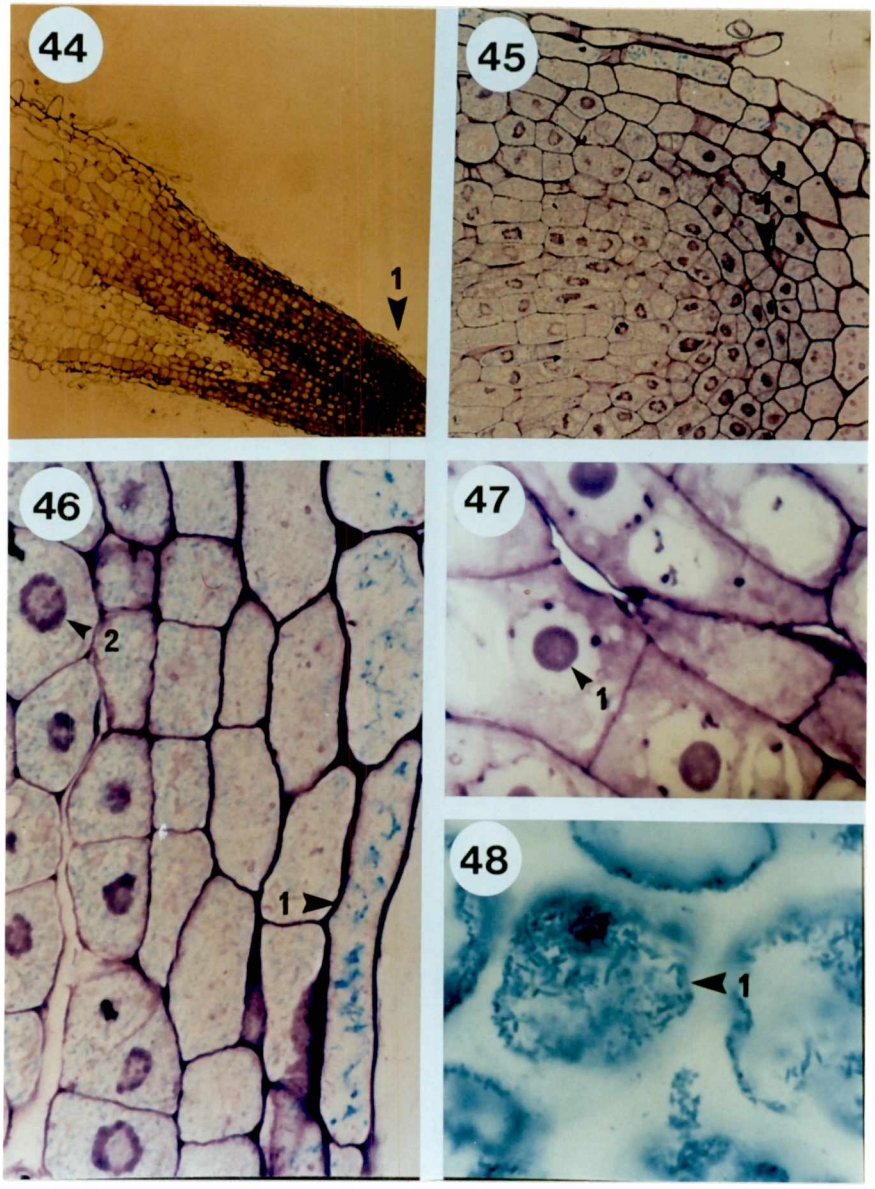
Figure 44. Transgenic (GUS positive) root tissue, magnification X4.

Figure 45. Transgenic (GUS positive) root tissue, magnification X10.

Figure 46. Transgenic root tissue magnified X60. This root tissue was transformed with the GUS I plasmid and assayed for GUS activity before sectioning and staining with toluidine blue. Some areas of GUS activity can clearly be seen in the epidermal cells (arrow 1).

Figure 47. Transgenic root tissue magnified X100. This photo clearly shows the cell nucleus (arrow 1).

Figure 48. Bacterial contamination of soybean cotyledonary tissue with *Agrobacterium*. This *Agrobacterium* harbors the KYLX p3-1 GUS plasmid which can be transcribed by the bacteria. The bacteria typically contaminate the cell surfaces or intercellular spaces. No GUS activity is seen within the cell cytoplasm (magnification X100).


44
1
45
1
46
2
1
47
1
48
1

No bacterial contamination was seen. This root tissue was transformed with the GUS I plasmid and assayed for GUS activity before sectioning and staining with toluidine blue. Some areas of GUS activity can be seen in figure 46 (arrow 1), this activity is clearly within the cell cytoplasm. Figure 48 shows typical bacterial contamination of soybean tissue with *Agrobacterium*. This *Agrobacterium* harbors the KYLX p3-1 GUS plasmid which can be transcribed by the bacteria. The bacteria typically contaminate the cell surfaces or intercellular spaces. No GUS activity is seen within the cell cytoplasm.

DISCUSSION

Efficient production of 'rhizogenes' roots and their nodulation in a range of soybean genotypes

By using the *Agrobacterium rhizogenes* strain K599 with the pRi2659 plasmid transgenic roots were produced in all the soybean nodulation mutants very efficiently (20 to 95% of the inoculations gave hairy roots, depending on genotype). This correlated with the results obtained by Savka *et al.* (1990) and showed that the cucumopine strain K599 pRi2659 bacterium was more virulent on soybean than agropine strain A4. Simpson *et al.* (1986) showed only 2% of A4 inoculations on soybean gave hairy roots. This is the first report of nodulated 'rhizogenes' roots in soybean. The production of transgenic roots to check the nodulation ability has been a problem with other *A. rhizogenes* strains. The ability of transgenic soybean roots to nodulate with this *A. rhizogenes* must be a characteristic of the *Agrobacterium* strain as these results are contrary to the interference of nodulation seen in red clover (*Trifolium pratense*), siratro (*Macroptilium atropurpureum*), and alfalfa (*Medicago sativa*) with transgenic roots induced by *A. rhizogenes* strain 8196 (Beach and Gresshoff 1985). Many of the transgenic roots produced did not appear extremely 'hairy' in their phenotype but still expressed GUS activity (GUS intron gene) and showed classical integration of the Ri T-DNA by Southern blot analysis.

The use of the GUS intron gene to accurately report transformation events

By using the p35S GUS intron plasmid (Vancanneyt *et al.*, 1990) as a binary we were able to easily select and clearly assay for transgenic roots. The ß-glucuronidase (GUS) gene (Jefferson, 1987a,b) has long been used as a reporter gene in transformation systems. However, the GUS gene, along with other reporter genes has suffered from the

ability of *Agrobacterium* to transcribe and translate it (Vancanneyt *et al.*, 1990). Thus, *Agrobacterium* expressing GUS activity while being harbored within the intercellular spaces can compromise the correct detection of transformation events and therefore lead to false positives. After identifying these false positives in other systems, it was felt necessary to use the GUS intron gene. This gene construct utilizes a plant intron (the second intron of the ST-LS1 alcohol dehydrogenase gene) in the coding region of the GUS gene. The lack of splicing apparatus in prokaryotes results in no GUS activity in *Agrobacterium*. Only until the T-DNA harboring the GUS gene is transferred to the eukaryotic plant cell will the GUS gene function. By using the intron containing GUS gene very elegantly distinguished between bacterial contamination and true transformation events. To date no reports have shown bacterial expression of a gene containing a eukaryotic intron.

High frequency of binary plasmid transfer

All of the roots (100%, 9 out of 9) initiating from the wound site tested positive for cucumopine activity. This suggests that all of these roots were 'rhizogenes' roots and not contaminating non-transgenic roots. Between 70 and 100% of the hairy roots initiated from the wound site tested positive for GUS activity. This indicated that the binary p35S GUS I plasmid was co-transfered with the Ri T-DNA at a very high rate. Efficient selection of kanamycin resistant roots in liquid media enabled us to select for the GUS positive roots. Transgenic roots grew rapidly in culture, however, observations showed marked differences in the degree of the 'hairy root phenotype ' and speed of root growth between plants and isolated root cultures.

Tissue specific expression of the GUS gene, promoted by 35S CaMV constitutive promoter

The 35S CaMV promoter is considered a constitutive promoter. However, roots showed the highest GUS activity in the meristematic and xylem/phloem areas. Tissue specificity should be seriously considered when using GUS as a marker gene in promoter analysis studies. For example, Bogusz *et al.*, (1990) used the *Parasponia* hemoglobin promoter and GUS reporter gene fusions in transgenic *Lotus* and tobacco. Miao *et al.* (1992 submitted) used the same promoter reporter gene fusion technique in *L. corniculatus* to study the tissue-specific expression of nodulin 26. High GUS expression was reported in root meristems with such a construct (Miao *et al* ., 1992 submitted). Such studies should

consider the potential for tissue specific expression regulated by elements other than the promoter specificity.

Transgenic GUS positive roots gave GUS positive nodules. When complete nodules were incubated in the GUS substrate, most of the GUS activity was situated in the nodule cortex. This was thought to be due to the lower amount of plant cytoplasm in this area and higher level of *Bradyrhizobium* cytoplasm. However, when the nodules were dissected and incubated in the GUS substrate, GUS activity was seen throughout the nodule. This could be explained by the fact that diffusion of the GUS substrate into the nodules infection zone was poor. Alternatively, nodules possessed an oxygen diffusion barrier and the last stage of the chemical reaction catalyzed by the ß-glucuronidase reaction required the dimerization by atmospheric oxygen of the free (colorless) indoxyl group to a highly colored blue dye.

Autonomous plasmid replication in plants

Transgenic GUS positive nodules and GUS positive aseptically grown root cultures were analyzed with Southern hybridization. The root cultures were treated with antibiotics (cefotaxime, amoxicillin, carbenicllin and clavulanic acid) to kill contaminating *Agrobacterium* after root induction but then grown in liquid Mon Mor medium without antibiotics for 3-5 months. Analysis of the Southern blots from these roots indicate that the pRi2659 T-DNA shows classical integration. However, Southern hybridization of the same roots (reprobing the same blot) indicated that the p35S GUS intron plasmid did not show a typical integration pattern. The plant hybridization patterns were identical to plasmid digestion patterns (5^b, 4.3^b, 3^i, 1.7^e kb. fragments: b: border, i: internal, e: external. See plasmid map Figure 1). Only plasmid sized fragments were seen. If integration had occurred, one would expect at least some hybridization to fragment of different sizes indicating plant DNA plasmid DNA boundaries (as seen for the Ri plasmid). Some larger bands from the transgenic plants were present on the original film but too faint (lane 4B, 5 and 6) to visualize on the photograph. This was probably due to poor DNA transfer during blotting. Also a 1.7 kb fragment which was external to the T-DNA borders was present in all lanes with transgenic (GUS positive) root DNA. This result was reproducible when probing with border fragments *Hin*dIII 35S GUS fragment or the 35S fragment alone and with *Pvu*II digests, and *Eco*RI digests. With *Eco*RI digest the plasmid was cut just once and the hybridization band was plasmid size (i.e. 14 kb).

Aseptic culture of the GUS Ri roots for 5 months, the expression of the GUS intron gene and the absence of bacteria in histological analysis of transgenic GUS positive root tips are evidence against bacterial contamination. This indicated that this plasmid transfer did not represent a classical transformation event. It appeared that the p35S GUS intron plasmid was not integrated but may actually existed as an extra-chromosomal element.

Such extra-chromosomal elements have been demonstrated with transposable circular DNA in maize (Weiner *et al.*, 1986). In the animal kingdom extra-chromosomal maintenance of transgenic plasmid DNA has been demonstrated in *Caenorhabditis elegans* (Mello *et al.*, 1991), *Drosophila* (Steller and Pirrotta, 1985), *Xenopus* (Marini *et al.*, 1988) and sea urchin (McMahon *et al.*, 1985).

Mello *et al.* (1991) found that chromosome integration of plasmid DNA injected into gonad cells was relatively rare. These plasmid sequences were replicated and inherited in a non-Mendelian manner as large arrays formed by homologous recombination between homologous sequences in the plasmids. This result suggested that either the plasmid origins of replication function in the eukaryote or that the plant initiates DNA replication of this extra chromosomal DNA at some other non-identified region. It is not yet proven that there is an absolute requirement for sequence-specific origins in eukaryotes (Marini *et al.*, 1988).

Stinchcomb *et al.* (1985a) found that once these extra-chromosomal structures are established they undergo very little further rearrangement. In Xenopus oocyte cytoplasm, foreign DNA sequences are rapidly condensed into chromatin and are assembled into structures resembling typical eukaryotic nuclei (Forbes *et al.*, 1983). Stellar and Pirrotta (1985) found that plasmid DNA injected into *Drosophila* embryos becomes enclosed in nuclei or nucleus-like structures where it remains throughout embryonic development.

The most convincing evidence for the replication of defined circular plasmids comes from Marini *et al.* (1988). *Xenopus laevis* embryos were injected with plasmid DNA and in some embryos the circular plasmid DNA was replicated through blastulation. Autonomous plasmid array formation was also seen in other embryos.

Integration patterns of plasmid DNA introduced into soybean suspension cultures by particle bombardment have indicated head to tail concatemers with strong hybridization signals to unit plasmid length DNA (Finer and McMullen 1991). The non-unit length

fragments also observed could either be partial plasmid pieces or plant-plasmid DNA borders (Finer and McMullen, 1991). Extra-chromosomal plasmid arrays give plasmid patterns identical to this. Much remains to be learned about the correlation of the structure of introduced DNA and the resulting integration pattern and gene expression. (Finer and McMullen 1991). Inheritance studies need to be performed on this tissue. These extra-chromosomal elements were inherited (Mello *et al.*, 1991), but in a non-Mendelian fashion. Since autonomous plasmid arrays have been shown to replicate in eukaryotic cells (Mello *et al.*, 1991) it is indeed plausible that individual plasmids can replicate autonomously in transgenic plant tissue.

Evidence for such autonomous replication of plasmids also exists in plants. Chee *et al.* (1989) used *Agrobacterium* to transform soybean cv. A0949. Southern blot results from subsequent R0 plants and a non-Mendelian inheritance to the R1 generation of offspring indicated that the transgene was either maintained extrachromosomally or that the R0 and R1 plants were harboring *Agrobacterium*. This earlier work with transgenes lacking introns always led to speculation of bacterial contamination because of the bacterium's ability to express such genes. By using the GUS intron gene in our study we have avoided such speculation.

Attempted regeneration of soybean 'rhizogenes' roots

Rech *et al.*(1988) reported regeneration from *A. rhizogenes* transformed roots of plants from the wild soybean *Glycine canescens*. An attempt was made to repeat this work with G.*max*, however, only green callus could be induced from roots. Such lack of cross-species adaptability of regeneration regimes were common and thus the result was not unexpected.

Normal nodulation phenotype in the majority of plants with rhizogenes roots

This study has not only demonstrated that *A. rhizogenes* transformed roots offer a rapid and efficient route for the introduction of foreign genes into soybean roots, but the ability to nodulate the roots, in the majority of plants the nodule phenotype was unaffected. It was found that 85% of the plants with transgenic root systems had normal nodule phenotypes. This was assessed by the nodule number of the various nodulation mutants which was not significantly different from the control non-transgenic roots of the same mutants. Also the nodule phenotype and nitrogen-fixing ability were not affected.

A small sub-group of these plants (15%) appeared to have an altered nodule phenotype and greater nodule numbers. This can be best described as more indeterminate in structure (soybean nodules normally being determinate, see Figure 49). These nodules varied in the extent of modification. They all appeared elongated and possessed an active meristem as seen in the determinate nodule structures. Some were rod-shaped, cone-shaped or rod-shaped budding nodules. Histological analysis revealed that the infection zone followed the actively growing nodule meristem. The nodule possessed seemingly normal infected cells (*Bradyrhizobium japonicum*) but the nodules did not fix nitrogen. Thus, the nodule was non-functional.

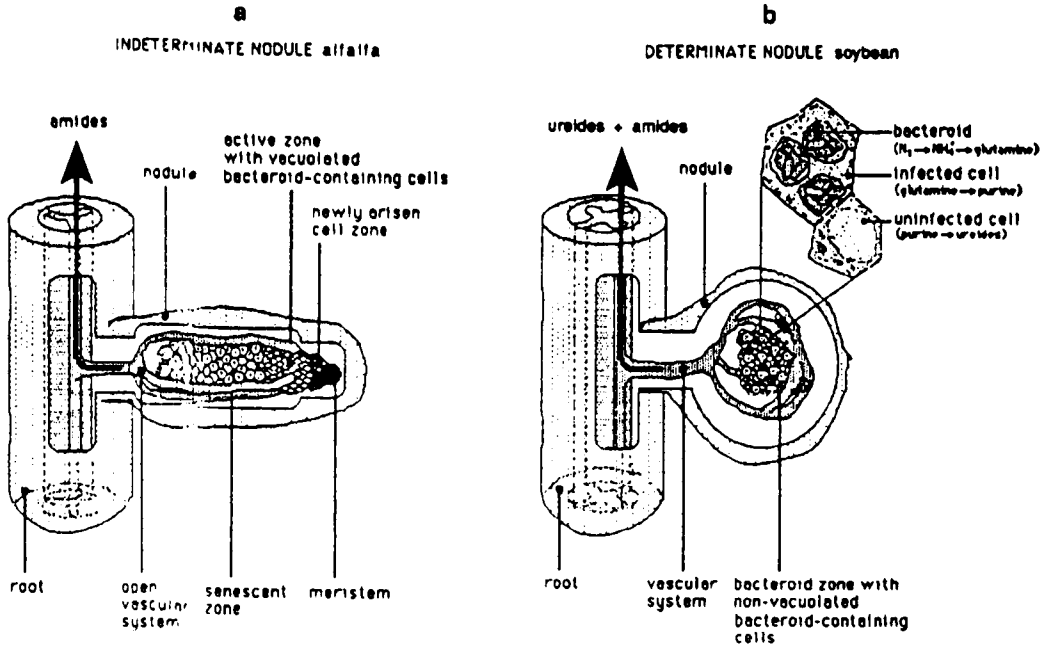


Figure 49. Examples of indeterminate and determinate nodule types. Indeterminate nodules such as those found on alfalfa are characterized by a persistent active meristem and cylindrical shaped nodules. Cells produced from the meristematic region are colonized by nitrogen-fixing bacteria. These cells develop into mature nitrogen-fixing cells. The older nitrogen-fixing cells in eventually senesce. In general the main export molecules from indeterminate nodules are amides. Determinate nodules such as those found on soybean are spherical due to the early cessation of meristematic activity. Cells within the nitrogen fixing zone persist throughout the lifetime of the nodule without senescing. The main export molecules of determinate nodules are ureides and amides. (reproduced from Gerahty 1990).

The effect of rol genes on nodulation phenotype

Stougaard *et al.* (1987b) used 'rhizogenes' roots (induced by strain A4) or regenerated plants to study nodulation genetics in *Lotus corniculatus*. Out of approximately 1000 plants and root systems studied in this system, no nodules with abnormal phenotypes were found (pers. comm. Dr. Jens Stougaard). This suggested that either the soybean/'*rhizogenes*' association was different to the Lotus/'*rhizogenes*' association, or the *rol* genes in the cucumopine *A. rhizogenes* strain may function differently to those in the agropine A4 strain.

Plant growth and morphogenesis is altered by the rol proteins which modify endogenous plant hormones (Estruch *et al.*, 1991a). Estruch *et al.* (1991b) found that the rolC protein releases cytokinins from glucoside conjugates. Also the rolB protien was found to hydrolyses indole glucosides thus releasing auxins from its conjugate (Estruch *et al.*, 1991c). The combined action of these two genes is thought to modulate the intracellular concentration of auxins and cytokinins to produce 'rhizogenes' roots. Plant developmental and physiological processes including nodulation can be influenced by enzymatic systems leading to conjugation and deconjugation of auxin and cytokinin phytohormones.

The reason for the altered nodule phenotype in such a small percentage of the plants is not known. However, it can be hypothesized that those plants with normal nodules had *rol* gene integration in regions of heterochomatin and *rol* expression was suppressed. Those plants where the *rol* genes were integrated in euchromatin may have high enough *rol* gene expression to modify the nodule phenotype. Similar variation in the expression levels of inserted genes has previously been reported. Jones *et al.* (1985) found greater than 200 fold variation in the expression of an octopine synthase gene promoted by a chlorophyll a/b binding protein gene promoter in transgenic plants. Positional effects on transgene expression have also been seen in transgenic mice (Stewart *et al.*, 1982) and transgenic fruit flies (Hazelrigg *et al.*, 1984).

The alteration of nodule phenotype from a 'determinate' to an 'indeterminate type' phenotype by the addition of a limited number of transgenes indicated that these nodule types are variants of the same developmental program, which may be switched by developmentally pleiotrophic genes such as the *rol* genes of *A. rhizogenes*.

Application of this gene transfer system in soybean

The *A. rhizogenes* transformation system is ideally suited to the study of the genetics of nodulation in *G. max,* similar to that employed in *Lotus corniculatus* (Petit *et al.*, 1987; Stougaard *et al.*, 1987a). Other possible applications include the screening developmentally or environmentally regulated promoters. The sceening of transgenes in the control of soybean cyst nematode resistance, mycorhizal associations, fungal and bacterial root pathogens. More fundamental work can be broached with the molecular investigation into what factors stimulate cell division and differentiation in plant tissues.

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CHAPTER 6

INVESTIGATION OF THE ROLE OF CHALCONE SYNTHASE EXPRESSION ON NODULATION IN TRANSGENIC ROOTS OF SOYBEAN

ABSTRACT

Chalcone Synthase (CHS) is the key enzyme in the flavonoid pathway whose products are involved in plant defense, pigmentation and micro-symbiont signalling during nodulation. This important enzyme is encoded by a multi-gene family in plants. To study the role of CHS expression in the nodulation process, sense and antisense CHS (gene 4) constructs were introduced into soybean roots using *Agrobacterium rhizogenes*. Gene transfer was confirmed by PCR analysis and Southern hybridization. In nodulation studies no difference was noticed in nodule number, morphology or competency of the nodules to fix nitrogen in a limited number of plants with 'rhizogenes' roots transgenic for sense or antisense CHS. Expression of CHS was monitored in roots by Northern analysis. No alteration was noticed in nodule-specific CHS (gene 7) expression. It is thought that the CHS genes 4 and 7 exibited too great a sequence divergence to act specifically as antisense molecules for each other. The majority of the control transgenic roots possessed normal nodule phenotypes when transformed with *Agrobacterium rhizogenes*. However analysis of the transgene effect on nodulation was complicated by a rare alteration in the nodule phenotype. This alteration in nodule phenotype is thought to be due to high expression of the *rol* transgenes from the Ri plasmid of *Agrobacterium rhizogenes*.

INTRODUCTION

CHS and its functions

There are seven genes encoding CHS in *G. max* (Akada *et al.*, 1990; 1991a; b). It is thought that each gene is expressed in different ways (spacial and temporal) in response to environmental and developmental signals. Environmental signals that stimulate CHS expression include UV light (Kreuzaler *et al.*, 1983) phytopathogens and elicitors (Ryder *et al.*, 1984; Bell *et al.*,1986; Ryder *et al.*,1987), wounding (Bell *et al.*,1986; Ryder *et al.*,1987) and nodulation (Estabrook and Gopalan 1991). In petunia the expression of

different CHS family members were found to be flower specific, light dependent and developmentally controlled (Koes *et al.*, 1989).

CHS expression and flavonoid induction: its possible involvement in the nodulation process

Both enzymes, CHS and phenylalanine ammonia lyase (PAL) transiently increased in expression in *G. max* in the 1 to 3 day period following infection with *Bradyrhizobium japonicum* (Estabrook and Sengupta-Gopalan, 1991). This increase in expression did not happen when the plants were inoculated with *Rhizobium meliloti*. Estabrook and Sengupta-Gopalan (1991) studied the differential expression of PAL and CHS during nodule development. They demonstrated that subsets of both gene families were induced after specific infection by *Bradyrhizobium japonicum*. This subset induced early in the symbiosis was different from gene members induced as host defense or stress response during the latter stages of ineffective symbiosis. CHS and PAL expression was shown to increase from 1 day post infection to 4 days post infection. When comparing the change in expression over the same period between the supernodulating mutant nts382 (Carroll *et al.*, 1985a; b) and its wild-type parent cv. Bragg, the nts382 mutant showed a much more dramatic increase in CHS and PAL levels. The nts382 mutant differs from its parent cv. Bragg in the total number of infections per nodules formed (3 to 40 times the nodule number). Thus, it appeared that flavonoid pathway enzymes were more active post-inoculation in the supernodulating mutant nts382 than in the Bragg parent. Because of the complexity of this pathway, this could mean an increase in one or many possible final compounds. Kapulnik *et al.* (1987) showed a direct correlation between the amount of flavonoids exuded from the root of alfalfa and the number of nodules subsequently formed. They also showed that direct application of luteolin (flavonoid inducer of *R. meliloti nod* genes) to the rhizosphere of alfalfa resulted in increased nodulation. With these results in mind, it might be logical to think that flavonoid biosynthesis was a component of autoregulation in soybeans.

In contrast, Sutherland *et al.* (1990) showed no significant variation in flavonoid exudates or content of inoculated and uninoculated roots of cv. Bragg and the supernodulating mutant nts382. Mathews *et al.* (1989b) showed that three day old seedlings of soybean cultivar Williams had a 10 times greater ability to induce *Bradyrhizobium nod* genes than did three day old cultivar Bragg seedlings, although mature plants had the same nodulation frequency. Van Brussel *et al.* (1990) proposed a model for the *Vicia sativa*-

Rhizobium. leguminosarum symbiosis, in which, after the bacterial *nod* genes were induced, the bacteria produce factors which signaled the plant to make more flavonoids. The results from Estabrook and Sengupta-Gopalan (1991) were in agreement with this model and they postulated that additional flavonoids were involved in maintaining *nod* gene expression while the infection progressed.

Flavonoids are known to perform other functions in plants. Jacobs and Rubery (1988) proposed that flavonoids act as natural auxin transport inhibitors. Auxin transport inhibitors have been shown to elicit nodule-like structures on alfalfa roots (Hirsch *et al.*, 1989). Conversely some phenyl-propanoid derivatives have been shown to have cytokinin-like activities (Binns *et al.*, 1987) and cytokinins have also been implicated in nodule initiation (Long and Cooper, 1988). It is possible that the secondary flavonoids produced as a result of infection function by affecting hormone distribution and are involved in cortical cell proliferation.

CHS expression and phytoalexin production: its possible involvement in the nodulation process

An additional level of regulation may exist in the phenyl-propanoid pathway in the way of phytoalexin involvement in nodule development. Phytoalexin (antimicrobial) compounds are end products in this pathway. The important phytoalexin in soybean is glyceollin I. Work by Parniske *et al.* (1991a; b) has shown that the glyceollin I is important in limiting the expansion of bacterial infection zone during nodule development. Limiting the CHS levels in the nodulation process may have various effects depending on which step is most critical to nodule initiation and development. It is clear that the phenyl-propanoid pathway is important in the legume-*Rhizobium* symbiosis but to date we are a long way from understanding the role of these genes or their biochemical function.

Homology in the CHS multi-gene family of soybean

The differential expression of the family of CHS genes to different stimuli is due to variation in the promoter sequence. Thus signalling and transcription response of each gene is different (pers.comm., S. Dube, Center for Agricultural Biotechnology, University of Maryland). There is at least 90% homology (97% between genes 4 and 6) at the protein level between the CHS genes, while gene 7 (nodule-specific CHS) shows the

greatest diversity (85% homology at the DNA level) (pers. comm., S.Dube). However, this sequence diversity does not appear to have been a problem in anti sense studies where parsley CHS was used as an antisense construct for transformation of petunia and tobacco (85% homology at the DNA level; pers. comm., Alexander van der Krol, Plant Molecular Biology, Rockefeller University, New York)

Chalcone synthase catalyses the first step in the flavonoid pathway as seen in Figure 1. This pathway is specific to the formation of anthocyanins, phytoalexins, flavones, flavonols, flavonoid pigments and isoflavonoids which are responsible for flower color, phytopathogen defense, and '*rhizobial*' signaling (Hahlbrock,1989; Dixon *et al.*, 1990).

Regulation of gene expression by antisense technology

The ability to regulate gene expression via antisense technology has been demonstrated in a wide variety of bacterial, animal and plant systems. This technique involves blocking the flow of information from DNA to protein by the introduction of a complementary (- strand) RNA sequence specific to the target (+ strand) mRNA. These RNA molecules match and pair via Watson and Crick base pairing (Izant and Weintraub, 1985). The duplex then either is rapidly degraded by RNAase' which specifically attack double stranded RNA molecules. Alternatively, nuclear processing of the RNA is blocked, or transport from the nuclear membrane in eukaryotes is blocked or the translation to protein is blocked (van der Krol A, *et al.*, 1990; see Figure 2).

Naturally occurring antisense regulation

Although artificial antisense regulation has been demonstrated in a wide variety of bacterial, animal and plant systems, it also exists naturally in prokaryotes and eukaryotes. In prokaryotes osmoregulation, plasmid replication, transposition and phage reproduction are all regulated by the interaction of a sense and a short antisense transcript (Ease *et al.*,1989; Green *et al.*, 1986; Inouye, 1989; Pines and Inouye, 1986; Simons, 1988). The first regulation of gene expression by artificial antisense technology was first shown by Pestka *et al.* (1984) with antisense constructs of the *ompC* protein and Coleman *et al.* (1984) with ß-galactosidase antisense constructs.

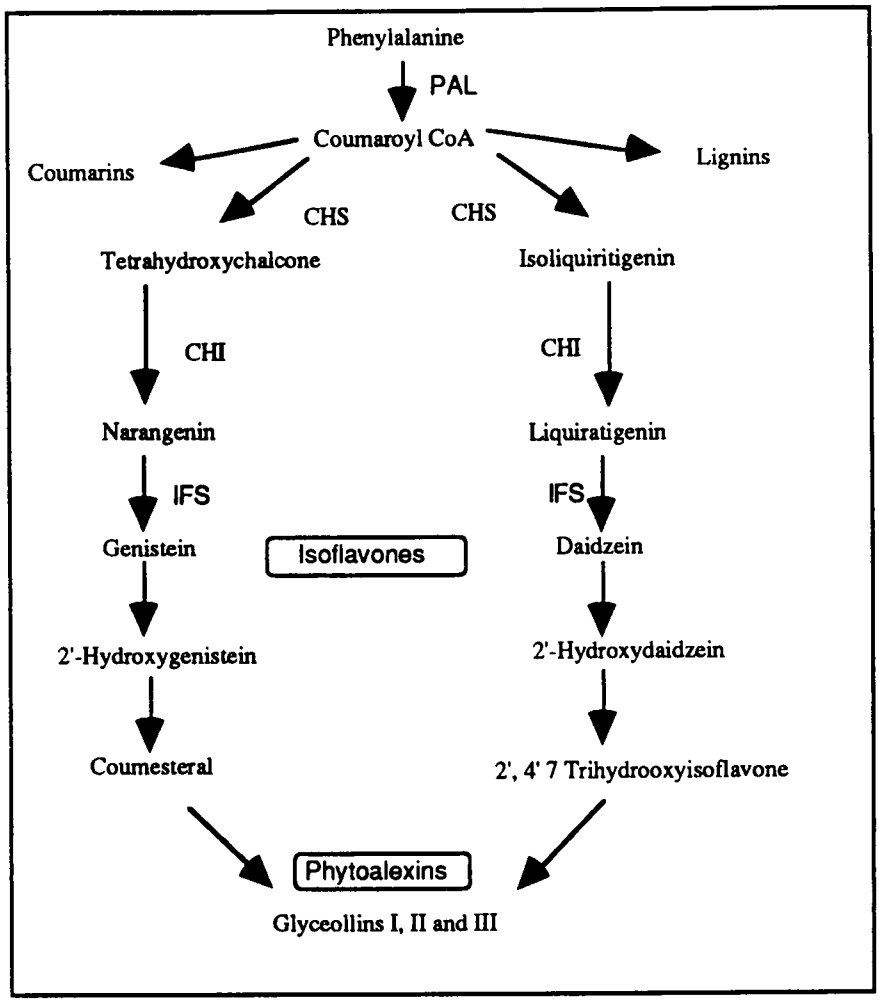


Figure 1. The flavonoid pathway in *G. max*. The enzyme phenylalanine ammonia lyase (PAL) catalyses the production of *p*-coumaroyl CoA from phenylalanine. Chalcone synthase (CHS) a key enzyme in the flavonoid pathway catalyzes the production of tetrahydroxychalcone and isoliquiritigenin from *p*-coumaroyl CoA. Chalcone isomerase (CHI) is the next enzyme in the pathway. CHI catalyzes the production of narangenin and liquiratigenin. Isoflavone synthase (IFS) synthesizes the production of diadzein and genistein. In soybean these isoflavones act as chemical attractants for the microsymbiont *Bradyrhizobium japonicum* to the soybean roots. The end products of the flavonoid pathway are phytoalexins. These act as antimicrobial/plant defense compounds. In soybean the important phytoalexins are glyceollin I, II and III.

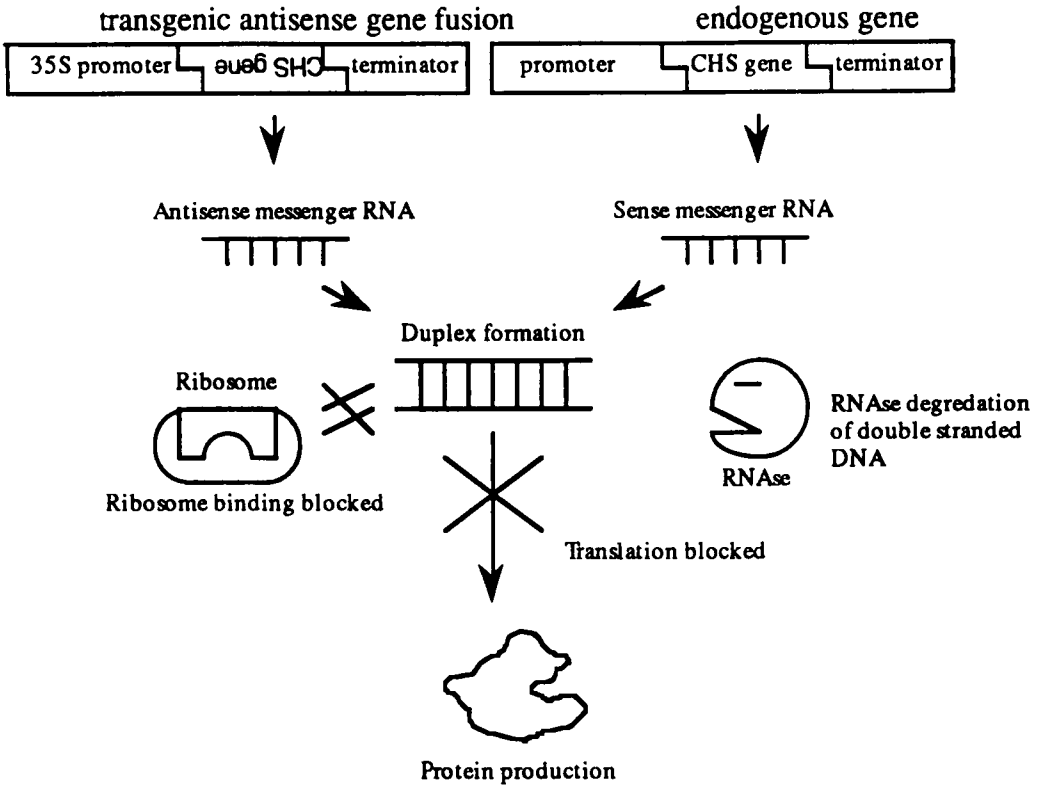


Figure 2. Possible interference with eukaryotic gene expression by antisense RNA. At present it is unclear which stages are significant in eukaryotes, however, evidence exists for the rapid degradation of RNA/RNA duplexes (van der Krol A, *et al.*, 1990). Complementary RNA molecules which inactivate gene expression have been named antisense (Izant and Weintraub, 1985).

In eukaryotes transcripts with naturally occurring complementary sequences have been shown in chicken myosin heavy chain (MHC) (Heywood, 1986; Lagrutta *et al.*, 1988; McCarthy *et al.*, 1983), barley alpha-amylase (Rogers, 1988) mouse dihydrofolate reductase (Farnham *et al.*, 1985) *Drosophila* dopa decarboxylase (Spencer *et al.*, 1986), and the satellite RNA of cucumber mosaic virus (Rezaian and Symons, 1986). The *in vivo* regulation of gene expression by these sequences remains undefined. However, translation has been shown to be regulated with the MHC system *in vitro* (Heywood, 1986; Lagrutta *et al.*, 1988).

In prokaryotes the antisense sequence is usually a short stretch of bases complementary to the 5' region of the target mRNA (van der Krol, 1988). However, in eukarotes there seems to be a much wider variation in the length of the antisense transcript and the region

of complementation on the target mRNA. For example, barley alpha-amylase antisense RNA covers the full length of the target mRNA (Rogers, 1988), whereas MHC antisense mRNA shows complementation at the 3' and 5' ends only (Heywood, 1986; Lagrutta *et al.*, 1988).

Artificial antisense regulation

Artificial antisense constructs have been constructed and used in a wide range transgenic plant and animal hosts to regulate specific gene expression (Table 1). The first report of regulation of a gene in plants by antisense technology was by Ecker and Davis (1986), who showed the inhibition of transiently expressed chloramphenicol acetyl transferase (CAT) activity in carrot protoplasts. Antisense regulation was demonstrated on whole plants by suppressing the expression of the marker genes nopaline synthase (Rothstein *et al.*, 1987: Sandler *et al.*, 1988) and CAT (Delauney *et al.*, 1988). The first endogenous plant gene regulated by antisense RNA was the CHS gene from petunia which was re-introduced by transformation into petunia and tobacco (van der Krol *et al.*, 1988). The first plants obtained were transformed with a full length cDNA copy of petunia CHS in antisense orientation. Later it was found that the same activity of gene suppression could be obtained with a shorter half or quarter length fragment homologous to the 3' end of the gene (van der Krol *et al.*, 1990). Regenerated plants showed a high frequency of altered (less) pigmentation in their flowers. In petunia this meant purple flowers engineered to white flowers and for tobacco pink to white. This reduction in flower pigmentation was also associated with a reduction approaching 1% of the normal wild-type CHS mRNA levels. The petunia CHS antisense also worked efficiently in tobacco even though the sequence divergence was estimated to be around 20% (van der Krol *et al.*, 1988).

Legume transformation as a tool to identify the role of specific genes involved in the nodulation process

The isolation of several nodulation cDNA clones and the comparison of sequence homology and amino acid composition with other genes has defined the biochemical function of only a few nodulins (Nap and Bisseling 1990). Many like Enod2 elude precise assignment, although similarity to other proteins exist. The nodulin Enod2 was found to be similar to hydroxyproline rich cell wall proteins. Plant transformation will help to elucidate the role of these symbiotic plant genes (Delauney *et al.*, 1988).

Antisense gene	antisense:sense gene-ratio [1]	inhibition[2]	host cell/ organism	antisense promoter	Target gene	Reference
β-galactosidase	1 : 1	95e	mouse 3T3	SV-40 early	transformed	102
HSV-TK	100 : 1	95te	mouse L	HSV-TK	transformed	56,57
	n.d.	90e	mouse L	MMTV-LTR Mouse Metallothionein I	transformed	63
CAT	5 : 1	88e	Mouse L	MMTV-LTR	transformed	57
	n.d.	0e	Monkey VC	SV-40 early	transformed	61
	3 : 1	90e	*N. tabacum*	CaMV 35-S	transformed	31
	n.d.	0e	*N. tabacum*	RubiscoSS	transformed	31
	100 : 1	95e	Carrot protoplasts	NOS CaMV 35-S PAL	co-electroporated	34
RSV env	12 : 1	80e	R-Q cells	RSV-LTR	transformed	20
Drosophila hsp-26	50-100 : 1	90p	*Drosophila* cells	*Drosophila* hsp-70	endogenous	82
mouse β-actin	n.d.	phenotypic	Mouse L	MMSV-LTR	endogenous	57
Dictyostelium discoidin I	50 : 1	90e,p phenotypic	*Dictyostelium* discoideum	*Dictyostelium* disc I-a	endogenous	28
Dictyostelium MHC	n.d.	phenotypic	*Dictyostelium* discoideum	*Dictyostelium* actin-6	endogenous	64,65,92
Dictyostelium D2	n.d.	phenotypic	*Dictyostelium* discoideum	*Dictyostelium* actin-6	endogenous	103
mouse *c-fos*	50 : 1 1/2-30 : 1 2 : 1	95%/phenotypic phenotypic phenotypic	Mouse 3T3 SSV-NIH3T3 F9	MMTV-LTR SV-40 early SV-40 early	endogenous endogenous endogenous	90 84,85 36
human *c-fos*	n.d.	phenotypic	mouse 3T3	MMTV-LTR	endogenous	54
human *c-myc*	120-20	70%/phenotypic	human HL-60	SV-40 early	endogenous	129
rat *c-src*	n.d.	phenotypic	Rat 3T3	mouse metallothionein I	endogenous	4
A. tumefaciens NOS	n.d.	90e 85e	*N. tabacum* *N. tabacum*	CaMV 35-S Pet. Cab 22R	transformed transformed	100 104
PVX coat protein	n.d.	crossprotection	*N. tabacum*	CaMV 35-S	inoculated	50
CMV coat protein	n.d.	crossprotection	N.tabacum	CaMV 35-S	inoculated	29
P. hybrida CHS	1 : 2 5 : >1(?)	phenotypic phenotypic	*P. hybrida* *N. tabacum*	CaMV 35-S CaMV 35-S	endogenous endogenous	68 68
tomato poly-galacturonase	1 : 1	90e	*L. esculentum*	CaMV 35-S	endogenous	111
tobacco RubiscoSS	1-4 : 1	70e,p	*N. tabacum*	CaMV 35-S	endogenous	98

Table 1. Effects on eukaryotic gene expression by artificial antisense genes: [1] the copy number of the antisense gene(s) per genome and the copy number of (active) target gene(s) per haploid genome is given. [2] the level of inhibition is expressed as a reduction (in percentage) of enzyme activity (e), protein steady state level (p), RNA steady state level (r), viral proliferation (v) or was scored by phenotypic changes. Reproduced from van der Krol (Ph.D Thesis, 1989. The University of Amsterdam, Holland).

Stougaard *et al.* (1987b) were the first researchers to use transgenic plants to study the genetics of nodulation. They demonstrated nodule specific expression of a complete soybean leghemoglobin gene in the root nodules of transgenic *Lotus corniculatus*. Later work focused on using the 5' promoter sequence from the leghemoglobin gene fused to the CAT reporter gene in order to show tissue specific expression in transgenic nodules of *Lotus corniculatus* (de Bruijn *et al.*,1989, 1990; Jensen *et al.*, 1988; Szabados *et al.*, 1990). Similar work was carried out using the *Parasponia* hemoglobin promoter and GUS reporter gene fusions in transgenic *Lotus* and tobacco (Bogusz *et al.*, 1990). Miao *et al.* (1992) used the same promoter reporter gene fusion technique to study the tissue specific expression of nodulin 26. Nodulin 26 is a major protein of the peribacteroid membrane. GUS expression was seen at emerging nodules and root primordia. Roots expressing antisense N-26 showed retarded hairy root growth (*A. rhizogenes*) in *Vigna aconitifolia*. Antisense studies were also conducted with nodulin 35. Soybean nodulin-35 (N-35) encodes a subunit of uricase localized in the peroxisomes of uninfected nodule cells (Nguyen *et al.*, 1985). Lee *et al.* (1992) introduced an antisense cDNA clone into *Vigna* behind a CaMV-35S promoter. Nodules that formed on the resultant transgenic roots were smaller in size and plants exhibited a yellow leaf phenotype. Uricase activity was reduced by 50-60% as shown by enzymatic assays. Electron microscopy also showed that the size of the peroxisomes was reduced.

Purpose of this study

In this study the purpose was to investigated the effect on nodulation of increasing and reducing CHS production by the transformation of CHS sense and antisense constructs driven by a constitutive 35S CaMV promoter into soybean roots. Antisense CHS work in other systems indicated that this should change the level of the phenylpropanoid pathway products in the transgenic root system. The resultant transgenic roots were assayed for CHS expression. The root systems were nodulated and studied for subsequent changes in nodule morphology, number and efficiency in fixing nitrogen.

MATERIALS AND METHODS

Construction of an antisense CHS clone

At the start of this study a full length genomic *G. max* CHS clone was obtained from Dr. Lila Vodkin, University of Illinois at Urbana-Champaigne. This gene was 2 kb in length and carried one intron of 122 bp. Its coding region base sequence was compared to a partial 3' nodule specific cDNA clone kindly provided later by Elizabeth Estabrook, New Mexico State University, New Mexico (data not shown). This sequence comparison showed 85% homology between the cDNA and the genomic clone. The gene was also compared to the sequence of the CHS genes 1 through 4 out of the 6 total in the multi-gene family (Akada *et al.*,1990,1991a; b). This gene shared 98% homology with the exon region of these genes. Genomic clones had been shown to successfully work as antisense constructs (R.Visser, Ph.D Thesis, 1990), therefore it was decided to clone the full length genomic CHS clone from *G.max* into a plant expression vector. To enable the study of over-expression and under-expression of the CHS gene, the sequence was cloned in the sense and antisense orientations into a plant expression cassette behind a 35S CaMV promoter. This construct was suitable for both particle gun transformation as purified DNA and *Agrobacterium* transformation as a binary plasmid.

Cloning of the CHS gene into a plant expression cassette

The plant expression cassette KYLX 7-1 (Schardl *et al.*, 1987) shown in figure 3 was kindly donated by Dr. Arthur Hunt, University of Kentucky, Lexington, Kentucky. It was chosen for a number of reasons: (a) because of the ability to use it in particle gun or *Agrobacterium* transformation systems; (b) the presence of the selectable marker gene NPT II driven by the NOS promoter which could be used to select for transformed tissue; (c) the presence of a high expression enhanced 35S promoter to drive transcription of the CHS construct and the ribulose bisphosphate carboxylase 3' terminator sequence (*rbcS*3') to terminate transcription; and (d) the presence of T DNA borders, enabling *Agrobacterium* transfer of these genes.

The KYLX 7-1 plasmid and the CHS gene (4) cloned in the pUC 19 plasmid were grown in *E. coli* strain JM83 in liquid LB medium under the appropriate antibiotic selection. The plasmids were isolated by an alkaline lysis protocol (Bassam *et al.*, 1987).

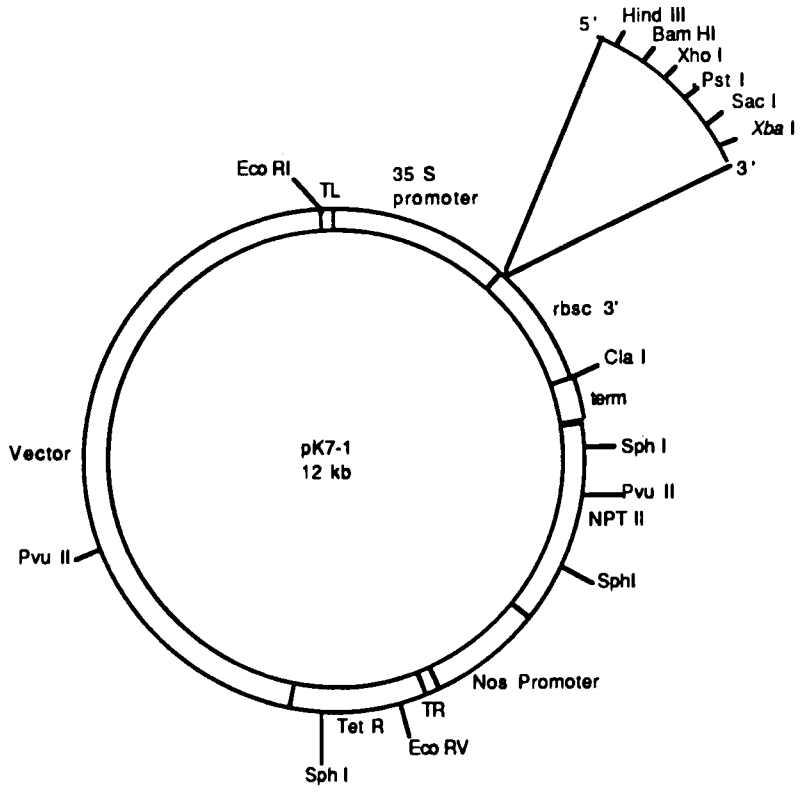


Figure 3. Plant binary vector KYLX 7-1. This vector is suitable for plant expression studies. A multiple cloning site exists between an enhanced (duplicated enhancer region) 35S CaMV promoter and a *rbcS* 3' terminator. Selection in *E. coli* was with 12.5 mg/l tetracycline and in *Agrobacterium* 25 mg/l tetracycline. Selection of transgenic plant tissue was performed with kanamycin 50-250 mg/l (dependent on the plant species and tissue type).

Both plasmids were digested with the *Hin*dIII restriction enzyme and electrophoresed in a 1% agarose gel for 2 hours at 100 volts. The smaller CHS fragment (2kb) and the linearised KYLX 7-1(12 kb) were excised from the gel and the DNA was isolated using a Geneclean^{TM} kit (BIO 101 Inc., La Jolla, CA). The DNA fragments were then quantified by gel electrophoresis and the amounts estimated by comparison to lambda phage DNA standards. Ligation of the fragments was performed according to Maniatis *et al.* (1989).

Competent *E.coli* cells (JM83) were prepared and used for transformation of the ligated DNA according to Maniatis *et al.* (1989). Antibiotic resistant colonies appeared after 12-24 hrs. These colonies were grown in liquid LB medium with the appropriate selection for 12 hrs. DNA was isolated from these cells according to Bassam *et al.* (1987). Larger plasmids (14 kb) containing the 2 kb CHS sequence were screened for by gel electrophoresis. Any colonies appearing to harbor ligated constructs were verified by

restriction digest of the CHS fragment back out of the KYLX 7-1 plasmid with *Hin*dIII. The orientation of the fragment in the multiple cloning site was verified by using *Pvu*II. This restriction enzyme was found to possess an asymmetric restriction site in the CHS sequence and two other sites in KYLX 7-1 and could be used to differentiate between sense and antisense orientations of the CHS gene. Plasmid maps of sense and antisense constructs were constructed.

Triparental mating of the sense/antisense constructs into Agrobacterium rhizogenes

This vector was transformed into the *A. rhizogenes* strain K599 by the triparental mating technique (Van Haute *et al.*,1983), using the mobilizing plasmid pRK2013. The transformed *Agrobacterium rhizogenes* was selected on minimal BMM medium supplemented with tetracycline 25 μg/ml. The transformation was confirmed by isolation of DNA from the *Agrobacterium* and re-transformation of *E. coli* and subsequent restriction analysis of plasmid DNA isolated from the *E. coli*.

Bacterial culture

Agrobacterium rhizogenes cucumopine strain K599 was kindly supplied by Dr. Steven Farrand, Department of Plant Pathology, University of Illinois, Urbana, Illinois. The bacteria was grown in minimal liquid (constant agitation at 150 rpm) Bergersen's Modified Medium (BMM; Bergersen, 1969) at 28°C. *Agrobacterium rhizogenes* harboring the KYLX 7-1 CHS sense and antisense contructs were grown with 25 μg/ml tetracycline in the medium.

Bradyrhizobium japonicum strain USDA110 was used for all plant inoculations. The bacteria was grown for 4-5 days in liquid BMM medium under constant agitation at 150 rpm at 28°C, prior to plant inoculation. The inoculations were performed with 20ml of approximately $2x10^6$ bacteria per ml added to each plant.

Culture and inoculation of soybean seedlings

Seeds of cv. Peking were surface-sterilized. This was performed by first soaking the seeds in a aqueous soap solution containing 100 μg/ml Captan™ (a fungicide) for 10 minutes (constant agitation). This solution was then decanted and a 40% solution of Chlorox added with 1-2 drops of Tween 20. After constant agitation for 10 minutes this

solution was also decanted and the seeds washed 5 times with sterile distilled water. Finally the seeds were dusted with Captan and Banrot fungicides and left to stand for 1 hr in a laminar flow hood. All manipulations hereafter were performed asepticaly in a flow hood. Seeds were then plated on a medium composed of 1/10th Gamborg's B_5 salts (Gamborg *et al.*, 1968), 30 g/l sucrose, 8 g/l Difco Bacto agar, pH 5.7, autoclaved for 20 mins 120°C (1/10th B_5). Approximately 10 seeds were plated on each 20x100 mm Petri dish and incubated at 25°C under a constant fluorescent light regime of 300 $\mu M\ s^{-1}\ m^{-2}$ for four days prior to inoculation with *Agrobacterium.*. Seedlings of cv. Peking, were then lightly wounded with a sterile scalpel on the hypocotyl just beneath the cotyledons and approximately 10 µl of the *Agrobacterium* were used to inoculate these wounds. The inoculated seedlings were cultured in Magenta jars (a 10x7x7 cm autoclavable clear plastic vessel, Magenta Corp Chicago, USA.) on 1/10th B_5 medium under identical conditions used for seed germination.

Selection and subsequent culture of transgenic roots

As soon as the transgenic roots appeared from the wound site (2-4 weeks), the seedlings were removed from the Magneta jars and the hypocotyl cut below the original wound site. The manipulated seedling was then transferred to a new Magneta jar containing approximately 20 mls of Mon Mor (MM) medium (Savka *et al.*, 1990). The medium was supplemented with 100 µg/ml kanamycin to select for transgenic roots and 100 µg/ml cefotaxime, 500 µg/ml carbenicillin, 75 µg/ml amoxicillin and 37.5 µg/ml clavulanic acid to kill all residual *Agrobacterium* cells. After 1-2 weeks of culture in the liquid MM medium under conditions identical as for seed germination enough root material was produced to: a) check for residual bacterial contamination, b) propagate the roots independently of the explant shoot, and c) transfer the explant with the transgenic roots to soil.

Evaluating root tissue for residual Agrobacterium contamination

Small (5 mm) roots sections were asepticaly ground in an Eppendorf tube using a mini-pestle and 300 µl of 0.8% NaCl solution. A 100 µl aliquot of this slurry was plated on BMM medium supplemented with 100 µg/ml kanamycin. Alternatively the 5 mm root sections were directly placed on this selective medium and left to culture for 4-7 days at 28°C. Bacterial contamination was assessed by the presence or absence of colony growth.

Culture of the transgenic roots independent of the explant

Transgenic roots growing from the explant shoot which had been cleared of residual bacterial contamination could be subcultured as independent root cultures. The root tissue was rapidly multiplied by inoculation of 2 cm root fragments into 16 ml of liquid MM media in a 100x20 mm sealed Petri dish (Parafilm^{TM}) supplemented with kanamycin 100 µg/l (kanamycin was only included if the roots contained the binary plasmid encoding the KYLX7-1 CHS sense or antisense constructs). The cultures were maintained in 100x25 mm Petri dishes in an illuminated incubator at 25°C, with a constant fluorescent light regime of 50 µM s^{-1} m^{-2}.

PCR analysis for gene transfer

All putative transgenic plants were screened for transfer of the desired sequences by polymerase chain reaction (PCR) amplification (with designed primers from National Biosciences, 3650 Annapolis Lane, Plymouth MN) of specific sequences (Mullis and Faloona, 1987). Ri plasmid transfer was confirmed by amplification of either a 500 base pair fragment of the cucumopine gene located in the T-DNA from the Ri plasmid (primers: 5' CTA ACT GGT GGG CTC CAT CT 3' and 5' CAT GCT GCG CGA CCA GTC GA 3'). KYLX 7-1 CHS sense and antisense plasmid transfer was confirmed by amplification of a 500 and 150 bp fragment from the enhanced CaMV 35 S promoter (primers: 5' CTA CAA ATG CCA TCA TTG CG 3' and 5' ATC ACA TCA ATC CAC TTG CT 3'). The CaMV 35 S promoter located on the KYLX 7-1 plasmid had a duplicated enhancer region. This had the effect of producing two PCR amplification products as one primer site was duplicated. This PCR assay showed the presence of the transgene but did not show the incorporation status.

Nodulation of the Agrobacterium rhizogenes rooted explants

Explants with roots testing positive for gene transfer by PCR analysis were transferred to the greenhouse. Control plants of all genotypes were propagated at this time by sowing seeds and culturing them under identical conditions to the putitive transgenic plants. All plants were potted in 3 inch pots in a 50/50 mixture of fine Vermiculite^{TM} and Faffard^{TM} soil-less compost. The greenhouse temperature ranged between 15 and 28°C, and light intensity between 300 and 500 µM s^{-1} m^{-2}. The plants were slowly acclimated to greenhouse humidity over a seven day period with the aid of a plastic propagator.

Inoculation of the plants with *Bradyrhizobium japonicum* USDA110 at an inoculum density of approximately $2x10^6$ cells was performed at transfer to soil and again seven days later. Care was taken to keep the soil as dry as possible without letting the plants wilt as the plants with transgenic roots seemed more susceptible to rotting than non-transgenic roots. Plants were removed from the soil four weeks after transfer to soil. The roots were washed under water, and the nodule number counted and nodule phenotype examined. The roots and nodules of these transgenic plants were compared to control plants of all the plant genotypes. The transgenic nature of the nodulated roots was confirmed by PCR and Southern blot analyses.

Acetylene reduction activity of transformed nodules

Transgenic and non-transgenic control nodules were tested for their acetylene reduction activity (ARA) according to Bergersen (1979) on a gas chromatograph (G.C.).

Confirmation of transgenic nature of the nodulated *root systems using PCR and Southern analyses.*

PCR analysis was repeated on the DNA from root and nodule tissue collected from the greenhouse grown plants to check for the transfer of the KYLX 7-1 CHS sense and antisense sequences and the Ri T-DNA sequences. Southern hybridization analysis was performed on the nodulating root systems essentially according to Southern *et al.* (1975). Genomic DNA from transgenic and non-transgenic control roots grown *in vitro* and roots and nodules grown *in vivo* was isolated according to Dellaporta *et al.* (1983). Purified DNA was digested with *Pvu*II restriction endonuclease and separated by agarose gel electrophoresis (10 μg /lane, 0.9% agarose) and transferred onto Zeta-Probe^R Nylon membrane by vacuum blotting (LKB). Blots were probed for the transfer of the T-DNA from the Ri plasmid. Probing for Ri T-DNA transfer was performed with a 4 kb *Hind* III right border T-DNA probe for the pRi2659 plasmid. Probes were prepared by a random priming reaction using [^{32}P]dCTP-label according to the Boehringer Mannheim Random Primer Labeling Kit. Hybridization and washing were performed at 60^{o}C according to the Zeta Probe^R, BioRad hybridization instruction manual. Exposure of the Kodak X-Omatic AR film was for 24 to 72 hrs at -70^{o}C.

Northern analysis of transgenic roots and nodules

RNA was isolated from root and nodule tissue confirmed to be transformed by Southern hybridization and PCR analysis. The RNA was isolated according to Manniatis (1989)by cesium chloride centrifugation. Northern blots and slot blots were performed according to Maniatis *et al.* (1989). The slot bolts were initially probed with a double stranded CHS DNA probe (gene 7 nodule-specific CHS) and then stripped and reprobed with a ubiquitin gene probe. The ubiquitin gene is expressed in all known plant and animal tissues, its expression between the RNA samples should be equal. Thus this probe enabled us to check the quantity of RNA loaded in each slot bolt.

RESULTS

Construction of sense and antisense CHS constructs

Larger plasmids (14 kb) containing the 2 kb CHS sequence were identified by gel electrophoresis (Figure 4, Plate 24). The orientation of the fragment in the multiple cloning site was verified by using *Pvu*II. This restriction enzyme was found to possess an asymmetric restriction site in the CHS sequence and two other sites in KYLX 7-1 and could be used to differentiate between sense and antisense orientations of the CHS gene (Figure 5). Plasmid maps of sense and antisense constructs are shown in Figure 6.

Production of transgenic roots

Roots from all the transgenic plants ready for transfer to soil (Plate 25, Figure 7) were analyzed for gene transfer by PCR amplification of transgenic sequences. By PCR analysis all of the roots (100%) produced from the wound site showed the transfer of the Ri T-DNA. This was tested by amplification of a 700 bp region from the cucumopine gene (Figure 8). The roots were also tested for transfer of the KYLX 7-1, CHS sense/antisense plasmid sequences. This was tested by amplification of a 150 and 500 bp region from the enhanced (duplicated region in the promoter) 35S CaMV. Of the plants tested 90% transfer of this transgenic sequence.

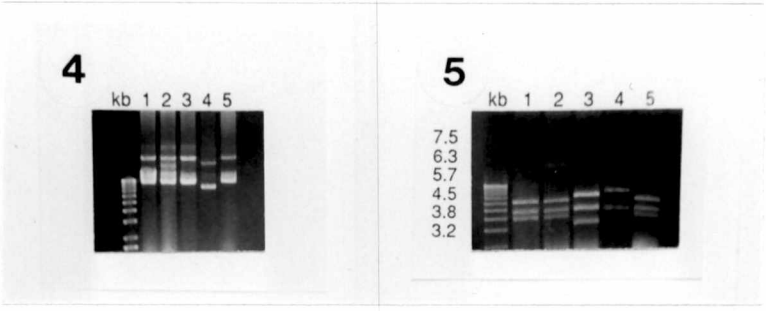


Plate 24. Construction of CHS sense and antisense constructs.

Figure 4. Plasmids with the CHS gene insertion were detected by agarose gel electrophoresis. Any plasmids larger than the KYLX 7-1 plasmid (12 kb) were further analyzed by restriction mapping to confirm the correct size insert (*Hin*dIII digest gave a 2 kb fragment) and the orientation of the sequence.

Figure 5. Analysis of sense or antisense orientation of the CHS sequence.. The plasmids were restricted with the restriction endonuclease *Pvu*II. This enzyme enabled identification of the sense and antisense constructs by the fact that *Pvu*II has an asymmetric restriction site in the CHS gene sequence. Lane 4 is KYLX 7-1 (no insert) restricted with *Pvu*II, this yields 7.5 and 4.5 kb restriction fragments. Lanes 1, 2 and 5 are antisense CHS constructs, restriction with *Pvu*II yields 4.5, 3.8 and 5.7 kb. Lane 3 is a sense construct, restriction with *Pvu II* yields 4.5, 3.2 and 6.3 kb fragments.

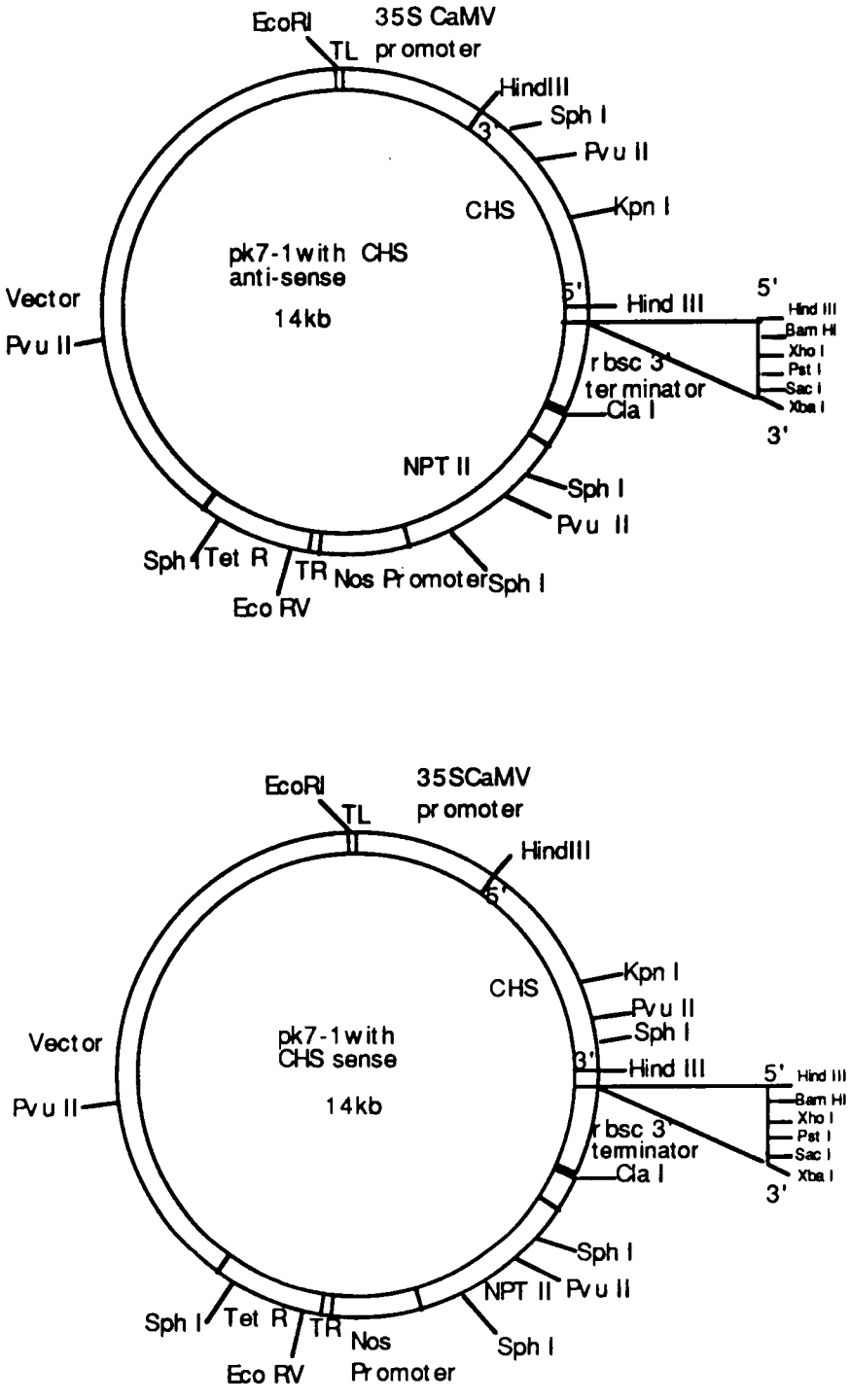


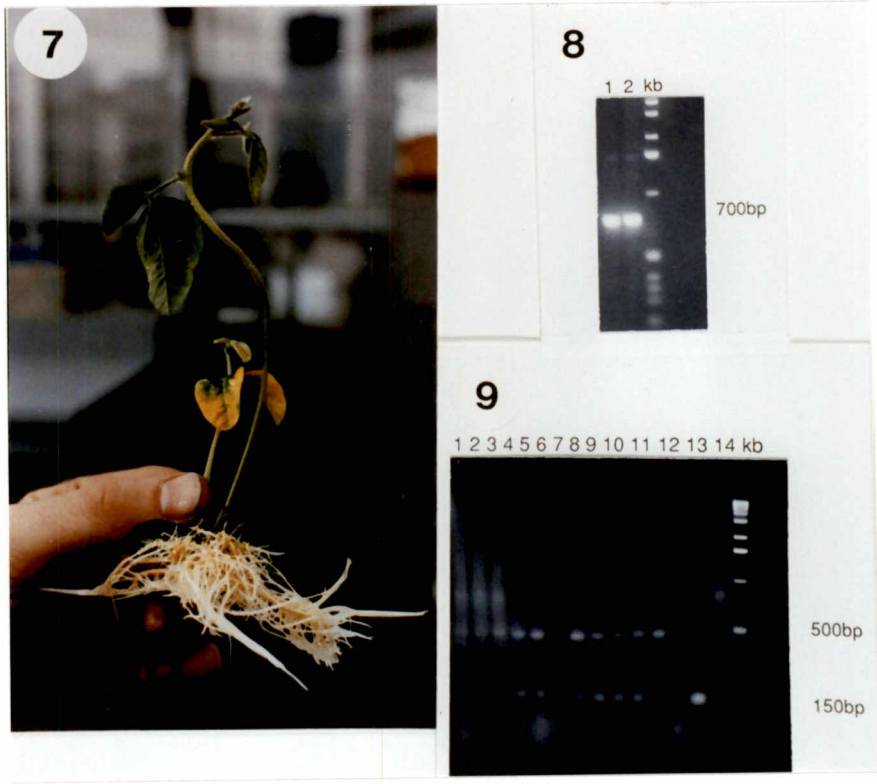
Figure. 6. Plasmid map of CHS (gene 4) cloned into KYLX 7-1 in the antisense (top) and sense (bottom) orientations.

Plate 25. Production of transgenicaly rooted plants.

Figure 7. A transgenic (*Agrobacterium rhizogenes* induced) root system of soybean cv. Peking. This plant's root system showed the presence of both the Ri and engineered T-DNA containing an antisense CHS construct (confirmed by Southern hybridization and PCR analysis).

Figure 8. PCR amplification of a 700 bp fragment from the Ri-T-DNA cucumopine gene. DNA from *A. rhizogenes* induced roots on soybean cv. Peking. The DNA was analyzed for transfer of transgenic sequences for the Ri plasmid by amplification of this 700 bp fragment. Lane 1 and 2 are DNA from two separate plants. Lane 1 shows amplification of a cucumopine gene fragment from DNA isolated from a plant with normal nodules. Lane 2 shows amplification of a cucumopine gene fragment from DNA isolated from a plant with abnormal elongated nodules.

Figure 9. PCR amplification of a 150 and 500 bp fragment from the 35SCaMV promoter. DNA from *A.r hizogenes* induced roots on soybean cv. Peking was analysized for transfer of the KYLX 7-1, CHS sense/antisense construct by amplification of these 150 and 500 bp fragments. Two fragments were amplified because the 35S CaMV promoter used in this study was enhanced by the duplication of a region of the promoter. Thus one of the primer sites was duplicated. Lanes 1 to 5: DNA isolated from abnormal transgenic nodules and root tissue transgenic for CHS sense and the Ri T-DNA. Lanes 6, 7 and 8: DNA isolated from normal transgenic nodules and root tissue transformed with CHS sense and the Ri T-DNA. Lanes 9 and 10: DNA isolated from normal transgenic nodules and root tissue transformed with CHS antisense and the Ri T-DNA. Lane 11: KYLX 7-1, CHS sense plasmid. Lane 12 and 13 DNA isolated from the pUC 35S GUS plasmid. This plasmid has a 35S CaMV promoter, however, the promoter is not enhanced. Thus there is only one amplification fragment. Lane 14: DNA from *A. rhizogenes* harboring the KYLX 7-1, CHS antisense plasmid. All template DNA was loaded at 100 pg per reaction.


7
8
1 2 kb
700bp
9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 kb
500bp
150bp

Transgenic roots were sub-cultured from the transgenic plants ready for transfer to soil. These roots were cultured *in vitro* in liquid MonMor medium as well as *in vivo* on the rooted plants. These *in vitro* root cultures and *in vivo* roots and nodules later were analyzed for CHS gene expression.

Confirmation of transgenic nature of the roots by Southern hybridization

The Southern blot analysis of the putative transgenic roots and nodules clearly demonstrates a classical integration pattern for the integration of the T-DNA of the Ri plasmid (Plate 26, Figure 10). This blot was probed with a right border probe for the Ri plasmid pRi2695. Southern hybridization for the KYLX 7-1, CHS sense/antisense constructs did not show classical hybridization patterns (classical hybridization: hybridization to fragments different to that expected from plasmid digestion alone). Only plasmid sized fragments were seen (data not shown). Therefore, PCR analysis was used to assess the transfer of the KYLX 7-1, CHS sense/antisense plasmid.

Analysis of CHS expression in transgenic roots and nodules

The RNA was isolated from transgenic roots and nodules harboring the CHS sense and antisense construct (Figure 11). A Northern dot blot assay was performed on this RNA. The blot was initially probed with the double stranded DNA nodule-specific CHS probe (gene 7) (Figure 12). This was performed to see if there was any difference in the nodule-specific CHS expression between the control plants and the CHS sense and antisense transformed plants. The one blot for antisense CHS (lane 2) showed greater hybridization than the sense and control blots.

However a control re-probe for the same blot with the gene ubiqutin also showed a higher level of hybridization in the antisense blot (lane 2, Figure 13). Thus, the higher hybridization in level in this lane was probably due to inaccuracy in loading the RNA. The ubiqutin gene transcripts are present in all known RNA extractions. If an equal amount of RNA was loaded, an equal signal from each slot blot should have resulted. It appeared that there was not an alteration in the nodule specific CHS level between the control non-transgenic and the transgenic roots and nodules of the sense and antisense plants.

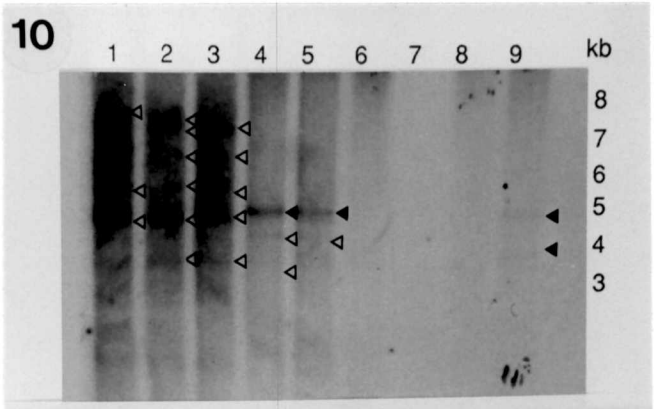
Plate 26. Molecular analysis of gene integration and gene expression.

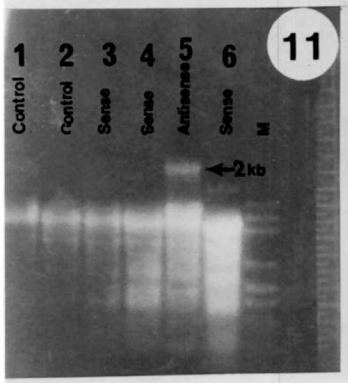
Figure 10. The Southern blot for *Agrobacterium rhizogenes* Ri T-DNA integration. A 4 kb *Hin*dIII pRi2659 T-DNA border fragment (right T-DNA border and part of the cucumopine gene) was used as the probe. Lanes 1 to 5 were DNA from transgenic roots and nodules. Lane 6 was DNA from non-transgenic root tissue. Lane 7 was blank. Lane 8 was control non-transgenic tobacco leaf DNA. Lane 9 shows non-transgenic root DNA spiked with *Agrobacterium rhizogenes* DNA. The black arrows show plasmid-sized fragments. The white arrows show unique fragment sizes.

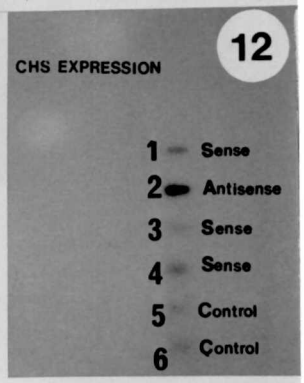
Figure 11. RNA profile on a gel. The gel was a 1.5% agarose denaturing (formamide) gel. The RNA was collected from root cultures grown under selection *in vitro* nodules from plants grown *in vivo*. Lane 1: RNA from control non-transgenic roots of cv. Peking. Lane 2: RNA from *A.rhizogenes* induced roots of cv. Peking. Lanes 3 and 4: RNA from 'rhizogenes' roots also transformed with the KYLX 7-1 CHS sense construct. Lane 5: RNA from 'rhizogenes'roots also transformed with the KYLX 7-1 CHS antisense construct. Lane 6: RNA from abnormal nodules derived from 'rhizogenes' roots transformed with the KYLX 7-1 CHS sense construct. The RNA in lane 5 appears to have a large amount of RNA at the 2 kb size range. This may be ribosomal RNA.

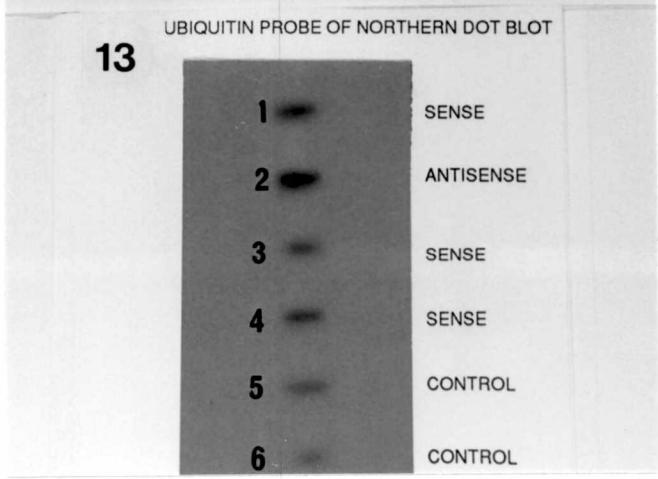
Figure 12. Northern dot blot of transgenic root tissue probed for transcripts of the nodule-specific CHS (7) gene. Lane 1: RNA from abnormal nodules derived from 'rhizogenes' roots transformed with the KYLX 7-1 CHS sense construct. Lane 2: RNA from 'rhizogenes' roots also transformed with the KYLX 7-1 CHS anti sense construct. Lanes 3 and 4: RNA from 'rhizogenes' roots also transformed with the KYLX 7-1 CHS sense construct. Lane 5: *A. rhizogenes* induced roots of cv. Peking. Lane 6: RNA from control non-transgenic roots of cv. Peking.

Figure 13. Northern dot blot of transgenic root tissue probed with ubiquitin gene. This blot is from Figure 11, stripped and reprobed. Two months passed between probing and reprobing. Lane 1: RNA from abnormal nodules derived from 'rhizogenes' roots transformed with the KYLX 7-1 CHS sense construct. Lane 2: RNA from 'rhizogenes' roots also transformed with the KYLX 7-1 CHS antisense construct. Lanes 3 and 4: RNA from 'rhizogenes' roots also transformed with the KYLX 7-1 CHS sense construct. Lane 5: *A. rhizogenes* induced roots of cv. Peking. Lane 6: RNA from control non-transgenic roots of cv. Peking. This result shows that lane 2 probably has more RNA in it. Thus the signal obtained in Figure 11 is due to excess RNA and not a greater amount of CHS transcript.


10
1 2 3 4 5 6 7 8 9
kb
8
7
6
5
4
3


11
1 Control
2 Control
3 Sense
4 Sense
5 Antisense
6 Sense
M
2kb


12
CHS EXPRESSION
1 Sense
2 Antisense
3 Sense
4 Sense
5 Control
6 Control


13
UBIQUITIN PROBE OF NORTHERN DOT BLOT
1 SENSE
2 ANTISENSE
3 SENSE
4 SENSE
5 CONTROL
6 CONTROL

Analysis of nodule phenotype in transgenic plants

The majority (85%) of the plants with transgenic root systems had normal nodulation frequencies and phenotypes (Figure 14, Plate 27). However, approximately 15% of the plants produced abnormal nodule phenotypes (Figures 15 and 16). These abnormal nodules also failed to fix nitrogen (Fix-) when assayed for acetylene reductase activity. This change in nodule phenotype was seen in plants transformed with 'rhizogenes' roots only and 'rhizogenes' roots transformed with KYLX 7-1 CHS sense constructs (Table 2). The nodule number of these plants with abnormal nodules was significantly higher than that seen with control non-transgenic plants and transgenic plants with normal nodule phenotypes (Table 3). Thus this rare altered nodule phenotype was interpreted to be due to the introduction and high expression of *rol* genes from the Ri plasmid of *A. rhizogenes*.

Table 2. Frequency of plants showing an alteration of nodule phenotype with *Agrobacterium rhizogenes* induced roots.

	Control WT roots	K599 roots	K599 GUS I roots	K599 CHS sense roots	K599 CHS anti sense roots
Plants with normal nodules.	20(Nif+)	17(Nif+)	20(Nif+)	25(Nif+)	20(Nif+)
Plants with abnormal nodules	0	3(Nif-)	0	5(Nif-)	0

Table 3. Comparison of abnormal nodule numbers on transgenic *A. rhizogenes* roots to nodule numbers on non-transgenic plants. Five plants per treatment were counted (± standard error).

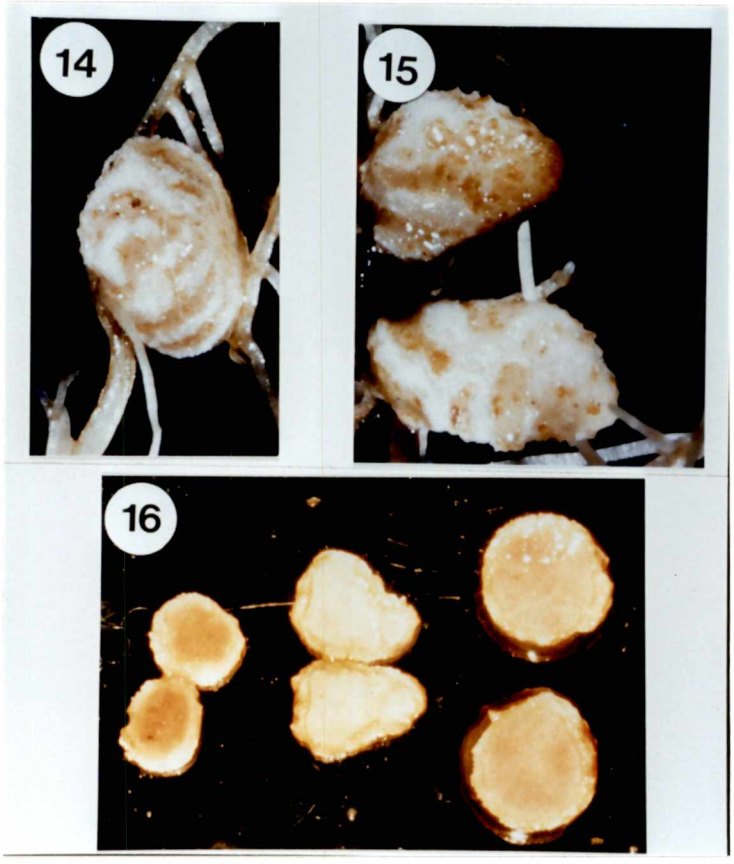
Genotype	**Nodule number**	
	non-transgenic root system	transgenic root system
Peking	12±3	33±9

Plate 27. Trangenic nodules with normal and altered phenotypes.

Figure 14. A nodule with a normal spherical phenotype from transgenic 'rhizogenes' roots harboring the KYLX 7-1 CHS sense construct. Neither the *rol* genes from the Ri plasmid or the introduced CHS (gene 4) gene altered the nodule phenotype, number or function.

Figure 15. Trangenic nodules exhibiting an altered nodule phenotype. These nodules were produced from 'rhizogenes' roots harboring the KYLX 7-1 CHS sense construct. However, identical roots were also found on root systems transgenic only for the Ri plasmid. The nodules typically exhibited an elongated nodule phenotype more characteristic of an indeterminate nodule. Histological analysis (data seen in chapter 5) showed that the nodules had active meristematic regions at the apex.

Figure 16. Transgenic nodules exhibiting normal and abnormal nodule phenotypes. The nodule on the left is from 'rhizogenes' roots harboring the KYLX 7-1 CHS antisense construct. This nodule was spherical (normal); it was transversely sectioned to show its normal non-meristematic infection zone. The nodule in the middle is from 'rhizogenes' Ri transformed roots. This nodule was elongated (abnormal); it was transversely sectioned to show its elongated meristematic infection zone (see chapter 5 for histological proof of meristematic activity). The nodule on the right was from 'rhizogenes' roots harboring the KYLX 7-1 CHS sense construct. This nodule was spherical (normal); it was transversely sectioned to show its normal non-meristematic infection zone.


14
15
16

DISCUSSION

Transgenic roots of soybean harboring CHS sense and antisense constructs were produced and nodulated with relative ease. However, no alteration in the nodule phenotype, number or distribution of the nodules in the transgenic root systems was found to be specifically due to transfer of the CHS sense and antisense genes. The CHS sense and antisense construct (gene 4) used in this study appears not to affect the nodule specific (gene 7) CHS expression as measured by Northern blot analysis. It would appear that the CHS gene used in this study either did not work or did not posses enough sequence homology to the nodule-specific CHS gene to act effectively in an antisense fashion. CHS gene 7 (nodule-specific CHS) shows the greatest diversity in sequence to the other CHS (1 to 6) genes. There was at least 97% homology between CHS genes 4 and 6. However, there was only approximately 85% homology between gene 7 (nodule specific CHS gene) and the other CHS genes in soybean. This sequence diversity was not a problem in antisense studies where parsley CHS was used as an antisense construct for transfromation of petunia and tobacco (85% homology at the DNA level; pers.comm., A. van der Krol)

Many antisense CHS plants died on transfer to soil. One could hypothesize that CHS gene 4 reduction could make the root systems of these plants more susceptible to bacterial and fungal attack. However, the specific action of CHS (gene 4) is not known. CHS has been shown to be expressed in response to phytopathogens and elicitors (Ryder *et al.*, 1984; Bell *et al.*, 1986; Ryder *et al.*, 1987), UV light (Kreuzaler *et al.*, 1983), wounding (Bell *et al.*, 1986; Ryder *et al.*, 1987), and nodulation (Estabrook and Sengupta-Gopalan, 1991). Further work is required to determine whether the CHS 4 gene antisense construct did not affect nodule-specific CHS 7 expression but affected the other CHS genes and possibly changed plant defense ability.

Only a limited number of plants and root systems were analyzed for expression levels of the CHS gene. It would be interesting to perform Northern hybridizations on this root tissue with a single stranded probe for sense or antisense CHS gene 4 to look for regulation of this gene. The results were complicated by the fact that the nodule morphology was altered in some plants presumably due to unusually high expression levels from the Ri transferred *rol* genes. This altered phenotype was relatively uncommon and it indicated that the majority of the *rol* genes transferred were integrated into an area of the genome where low expression resulted and the nodule phenotype was not affected.

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CHAPTER 7

CONCLUDING REMARKS

INTRODUCTION

Progress in using transformation as a tool to study the genetics of nodulation in Glycine max

Much of this thesis has focused on developing methods for efficient transformation in soybean. This was a prerequisite for using transformation as a tool to study the genetics of nodulation. Soybean has classically been considered a difficult plant to culture and transform. Although transformation of soybean was first demonstrated in 1988 by *Agrobacterium*-mediated transformation (Hinchee *et al.*, 1988) and particle gun transformation (Christou *et al.*, 1988), success with these systems has not been widely reported in other laboratories. This work attempted transformation of the nodulation mutants described in this thesis using these two systems. Regeneration protocols were developed and transformation procedures tested, only to find neither system was ideal. Selection or development of an efficient transformation system was paramount to progress in this project. The development of a new (revised) transformation procedure allowed rapid and efficient analysis of nodulation in transgenic roots of all the soybean mutants. This is the first time that soybean nodulation has been studied in transgenic systems. In developing this system, this research produced data supporting classical and non-classical transformation events in the same plants. Alteration of nodule phenotype was also demonstrated through the introduction of transgenic sequences.

This chapter of concluding remarks attempts to review and integrate the research completed in each chapter. The experiments followed a logical progression (Figure 1). In discussing each chapter, an attempt will be made to highlight the achievements and pitfalls, and then discuss possible improvements, new experiments and ideas under a future experiment section.

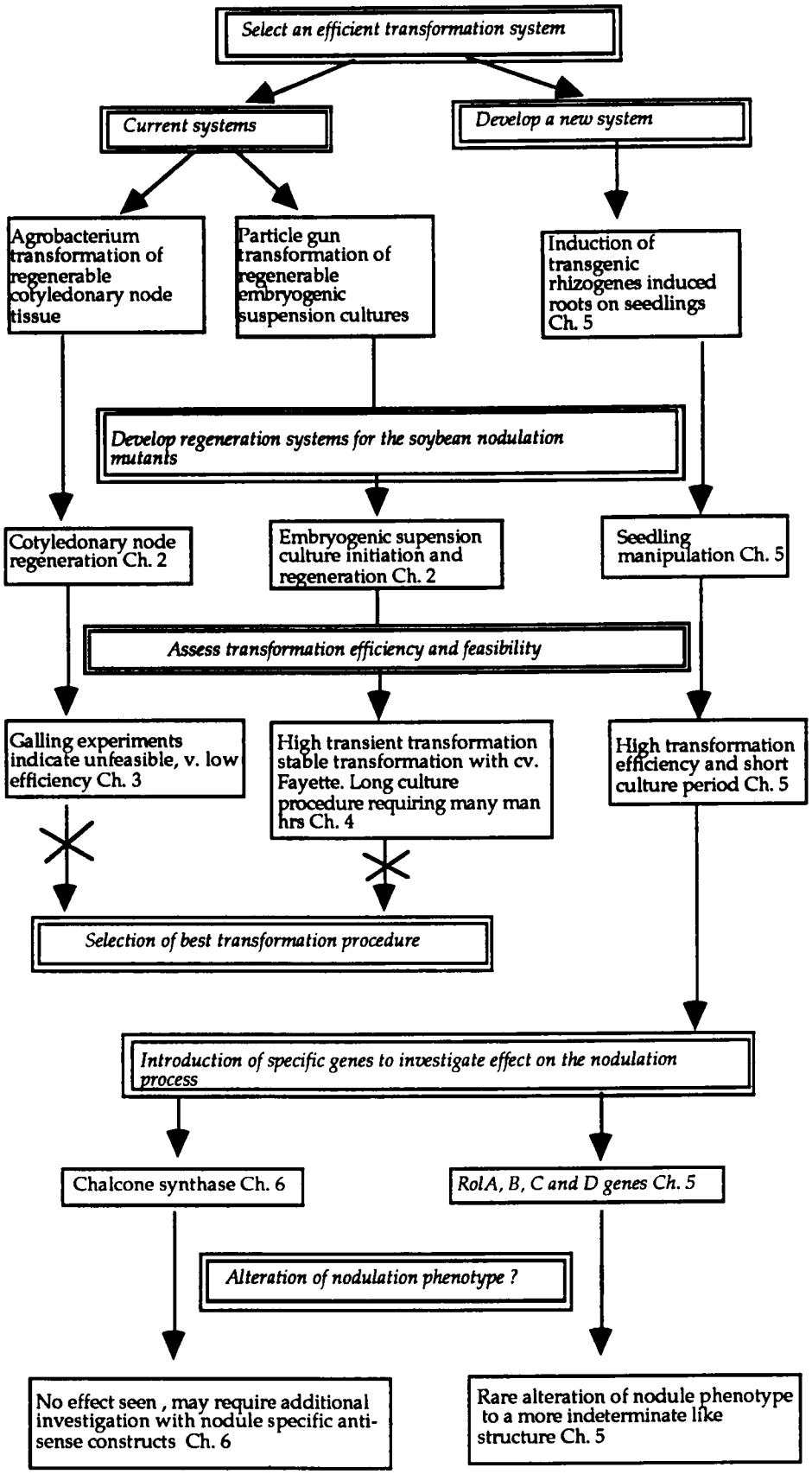

Figure 1. Flow chart of research pathway

Chapter 2

The direction of this work was to first develop efficient regeneration systems for the soybean nodulation mutants nts382, nts1007, nod49 and nod139 and the parental cv. Bragg. Two published transformation systems were based on *Agrobacterium tumefaciens* (Hinchee *et al.*, 1988) and particle gene gun transformation (Christou *et al.*, 1988; 1989). However, other labs had trouble in reproducing whole plant transformation with these systems. Even to date only one other laboratory (Townsend *et al.*, 1992) has shown proven success with the *Agrobacterium*-based system and a limited number of labs have succeeded with particle gun transformation of soybean (Parrot *et al* 1992; Finer and McMullin 1991). This reflects the technical problems seen in soybean tissue culture and transformation. This research developed regeneration systems which would be suitable for *Agrobacterium* based transformation (shoot regeneration from cotyledonary nodes, adapted from Wright *et al.*, 1986; Hinchee *et al.*, 1988) and particle gun transformation (plant regeneration from embryogenic suspension cultures, adapted from Finer and Nagasawa 1988). This study demonstrated that with media modifications it was possible to successfully regenerate all of the nodulation mutants from cotyledonary petiole derived shoots. Embryogenic suspensions of Bragg and nts1007 were initiated and regeneration of plants was demonstrated from cultures of nts1007. The nodulation phenotypes were unaffected by these regeneration systems suggesting that they would be suitable for transformation projects based on the analysis of nodulation.

Future experiments

Soybean suspension cultures are slow growing (2 week doubling time). Consequently antibiotic selection of transgenic embryogenic clumps can take 10 to 20 weeks. Regeneration of plants from the embryogenic suspensions also takes 3-4 months. This means that either soybean is intrinsically slow in culture or the growth conditions are not optimal. As initiation of embryogenic clumps was twice as rapid at pH 7 than at pH 5.6, it would be valuable to see what effect pH 7 would have on the suspension growth.

Chapter 3

The next stage was to test the susceptibility of the nodulation mutants to two *Agrobacterium tumefaciens* strains (A208 and A281) which had been reported to

successfully transform soybean (Hinchee *et al.*, 1988; Zhou and Atherly 1990). The bench mark for comparison was the susceptible cultivar Peking. Although transgenic galls could be induced on all of the nodulation mutants, their susceptibility was much lower than that seen in cv. Peking. Interestingly the supernodulating mutant nts382 which is very susceptible to the microsymbiont *Bradyrhizobium japonicum* infection did not show the same susceptibility to the pathogen *Agrobacterium tumefaciens*. This fact is interesting given that the overall biology of these two organisms is similar. Attempts to increase *A. tumefaciens* virulence to soybean by the addition of *vir* genes *virG*, *virB* from strain A281 failed. When bacteria were stimulated with specific media and then used to infect cv. Peking, gall size was increased. This is not seen with the unsusceptible nodulation mutants. Hinchee *et al.* (1988) reported that transformation efficiencies of cv. Peking with a disarmed A208 strain were significantly lower that seen with the wild-type strain. This suggested the transformation efficiency using a disarmed A208 strain to infect the nodulation mutants would be so low that the system would not be feasible. Attempts to transform the nodulation mutants using a disarmed A208 strain (data not shown) and a cotyledonary petiole regeneration system did not produce any transgenic plants. This lead to the investigation of other transformation systems including particle gun transformation and *Agrobacterium rhizogenes* transformation.

Future experiments

The low susceptibility of *Agrobacterium*-induced galls of A208 and A281 does not exclude the possibility that there may be other *Agrobacterium* stains which are very virulent on these soybean varieties. Owens and Cress (1985) tested three *Agrobacterium* strains on 24 soybean cultivars. Byrne *et al.* (1987) tested three different *Agrobacterium* strains on 17 soybean cultivars. However, these were not exhaustive screenings. If specific cultivars are used, the test pool is narrowed. Even if a wild-type virulent *Agrobacterium* strain were found for the soybean nodulation mutants, it would still require disarming. It may be that there is not a naturally occurring virulent *A. tumefaciens* strain for the soybean mutants. Yet this research has shown that suitable virulence genes do exist for the soybean nodulation mutants in the *A. rhizogenes* cucumopine strain K599. Thus it may be possible to increase the virulence of a disarmed *A. tumefaciens* strain by the introduction of virulence genes from *A. rhizogenes* K599. These genes could be transfered on plasmids much in the same way that Jin *et al.* (1987) increased the virulence of *Agrobacterium tumefaciens* strain A281 by the addition of virulence genes. It is more

likely that the *virC* gene determining host specificity would be the gene of choice from *A. rhizogenes* strain K599.

This study demonstrated the ability to introduce and express genes harbored on an engineered Ti plasmid (p35S GUS intron) in *Agrobacterium tumefaciens* galls of all the soybean mutants. This ability could be utilized to introduce and express specific DNA sequences which could affect the nodulation phenotype of the soybean nodulation mutants.

Miranda *et al.* (1991) demonstrated that the inversion of the right T-DNA border of the Ti plasmid could promote transfer of extremely long pieces of DNA (up to 200kb). This ability to transfer such large pieces of DNA could be utilized in plant transformation for the identification of biologically significant regions of the plant genome. These two systems could be coupled to make a rapid and easy gene transfer system. For example, after using a molecular marker to map a locus such as the supernodulating mutant nts382, the next stage is to walk towards the locus using overlapping yeast artificial chromosomes (YAC). YACs from this region would also be produced from the wild-type parent Bragg. If we assume that one of the YAC's contained the locus for nts382, a YAC from the homologous region in Bragg would contain the wild-type allele (non-mutated sequence). It might be possible to transform the part of the Bragg YAC (approximately 200kb) into a plant using a modified *Agrobacterium* plasmid vector system. The plasmid would consist of a pBS(+) plasmid modified by the incorporation of a right T-DNA border (Figure 2). The large closely linked but unidentified piece of DNA would be ligated into the multiple cloning site (colony screening by color selection) and the resultant plasmid cloned into *Agrobacterium*. The *Agrobacterium* would then be used for inoculation of nts382 plants. Those plants which produced galls would be inoculated with *Bradyrhizobium.japonicum* and screened for diminished nodule numbers.

It is assumed that the translated gene products from the gall would be translocated to the root system and enough shoot-derived signal could be produced by a gall. This hypothesis could be checked initially by a gall grafting experiment. A gall from a Bragg plant could be grafted onto an nts382; plant cultured for 2 weeks, then the resultant plant inoculated with *B. japonicum* and subsequently screened for nodule phenotype. This kind of grafting experiment is similar to that described by Delves *et al.* (1986; 1987a; b) where grafting of Bragg and nts382 shoots onto their counterpart root stocks showed that the supernodulating phenotype is determined by the upper part of the plant.

Although only 15% of nts382 inoculation sites produced galls the frequency per plant could be increased by inoculating at 10 sites per plant. This frequency is actually good when one considers the ease and the speed at which 200 or 300 seedlings could be grown, inoculated, screened for galls and then for altered nodulation phenotype. This system offers a quick, easy and practical way of proving functionality to a gene sequence (Figure 3).

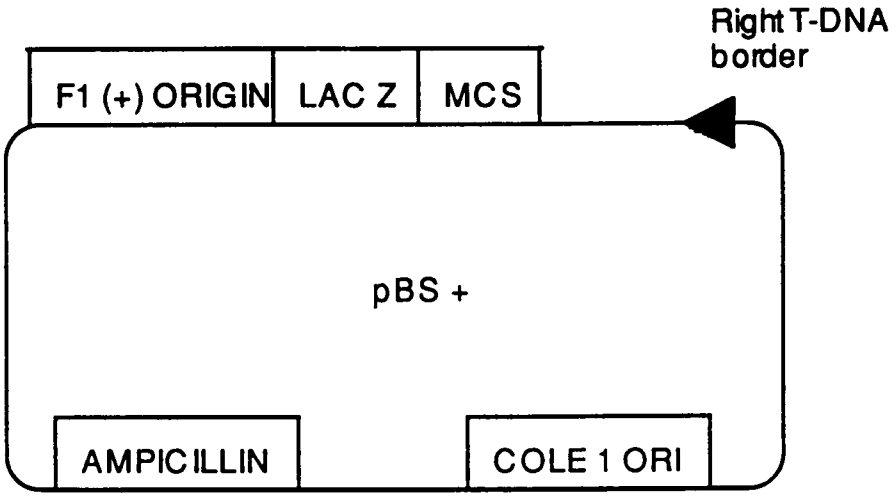

Figure 2. Plasmid T_R 200 would consist of a pBS+ plasmid modified by the incorporation of a right T-DNA border. The large functionally unidentified piece of DNA would be ligated into the multiple cloning site (MCS; colony screening by color selection) and the resultant plasmid cloned into *Agrobacterium*. Alteration or correction of the soybean nodulation mutant phenotype would prove functionality to the introduced sequence.

Chapter 4

Particle gun transformation of soybean had been reported by Christou *et al.* (1988). However, the system used was not described in detail and access to the electric particle gun was not permitted. This study pursued efforts to reproduce these results using a home-made electric particle gun with no success. Later the construction of a helium particle gun enabled us to optimize transient and stable transformation parameters.

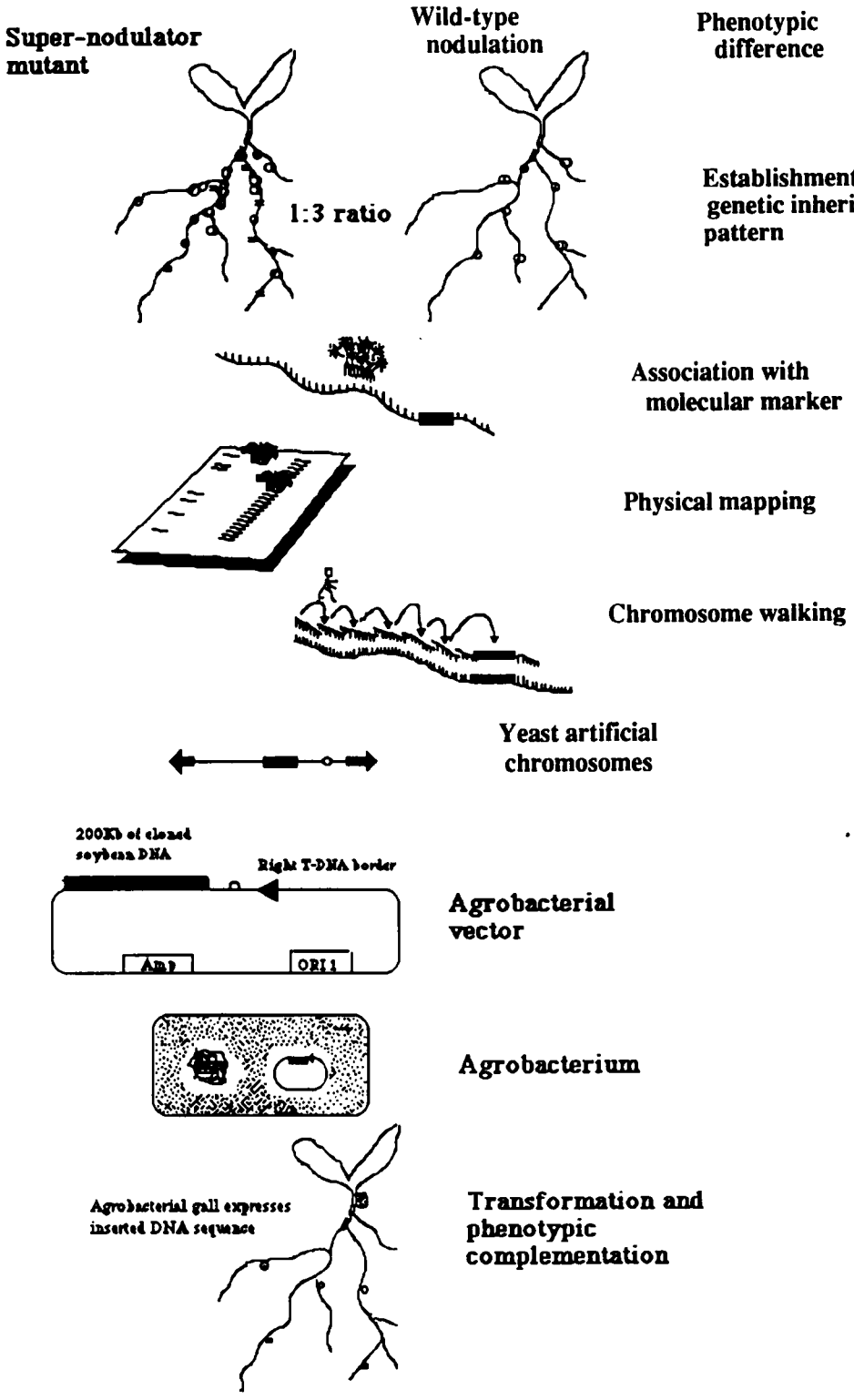

Figure 3. Positional cloning strategies in plants using *Agrobacterium tumefaciens* mediated gene transfer. The advantage of this system as compared to a 'shot gun' cloning type of experiment is that less time is required in culturing transgenic plants.

Although it was demonstrated that this transformation process worked for soybean (Bond *et al.*, 1992) and whole transgenic plants can be produced (Christou *et al.* 1988; Finer and McMullen 1991), it suffers from the fact that many hours are required in the production of transgenic plants and maintenance of the embryogenic suspension cultures. A conservative estimate would be 15 months; 6 months for initiating embryogenic suspension cultures, one day to shoot the cultures, 5 months of antibiotic selection of transgenic suspensions (2.5 months before transgenic clumps are seen and 2.5 months to multiply them to a significant quantity), 2 months for embryo development and 2 months for desiccation, germination and establishment in soil. This also includes monthly sub-culture of suspension cultures or weekly sub-culture during the selection period. The major problem with this system is the slow growth of the embryogenic suspension cultures (a two week doubling time which is rather slow compared to other plant suspension systems). There is also the added threat of contamination of the cultures at any time during this 15 months period. The careful use of antibiotics such as cefotaxine, clavulanic acid and amoxicillin during this period can control any bacterial contamination. However, yeast or fungal contamination cannot be controlled. The use of fungicides in the suspension media is usually detrimental to the cultures. There is also the threat of producing sterile plants from long term suspension cultures.

Future experiments

This system could be drastically improved if the speed of suspension culture growth could be increased. The media requirements for suspension culture growth may need to be modified. When the pH of the initiation medium was adjusted from pH 5.6 to pH 7, embryo initiation was greatly enhanced. It is possible that culturing the suspension cultures in liquid medium at pH 7 would also increase the speed of growth. Overall this transformation technique is fairly good, but it does not lend itself to the rapid production of rooted transgenic plants. At this system's present state of development it is much more suited to the type of research where transient expression could be useful (promoter analysis i.e. GUS fusions with a cell cyclin promoter), or where gene expression in embryo development is to be studied or regulated.

John Finer (pers. comm) demonstrated the ability to mix multiple plasmid types before bombardment and obtain multiple types of plasmids integrated into regenerant plants (20 plasmids). This finding raises the possibility of shot gun transformation experiments (bombardment of a mixture of DNA sequences at one time). Sequences of DNA whose

function is unknown but are closely linked to the mutated loci in the soybean nodulation mutants could be transformed into mutant embryogenic suspensions via particle bombardment. The resultant plants would be screened for correction of the mutated phenotype (Figure 4). Eventually the wild-type sequence homologous to the mutated locus could be found by complementation of a nodulation mutant. The function of the sequence could be investigated by sequence analysis. If the sequence is part of an open reading frame and not a promoter or intron sequence, expression studies could be made.

This transformation system could be greatly improved and with culture improvements will probably be the finite transformation system for soybean in the future.

Chapter 5

After attempting to transform the soybean nodulation mutants with *A. tumefaciens* and particle gun based transformation systems, a major concern existed that we still did not have an efficient and rapid transformation system. Without this we could not analyze the effect of specific gene constructs on nodulation in the soybean nodulation mutants.

The most direct and simplest way to study nodulation in a transgenic environment was to produce transgenic roots with *Agrobacterium rhizogenes* and to nodulate those roots. A similar system had been employed with *Lotus corniculatus* and the *Agrobacterium rhizogenes* strain A4 (Petit *et al.*, 1987; Stougaard *et al.*, 1987). However, previous work indicated that soybean was not susceptible to the commonly used *A. rhizogenes* strains. A publication by Sakava *et al* . (1990) introduced us to a strain that was virulent on soybean *Agrobacterium rhizogenes* strain, cucumopine K599 pRi 2695. Sakava *et al.* (1990) used the unmodified K599 strain to produce transgenic roots *in vitro* for the culture of soybean cyst nematode larve. This bacterium was obtained and the binary mini Ti p35S GUS intron plasmid was introduced into it. This plasmid enabled this study to effectively follow the development of transgenic roots with a very quick and easy assay. Also, the intron in the GUS gene removed the possibility of false positives through bacterial expression of the gene.

Previous reports indicated that the "rhizogenes" nature of the roots would interfered with nodulation (Beach and Gresshoff, 1985). It was not known if these roots could be nodulated. Indeed, the first *in vitro* attempts at nodulating these roots failed. Thus nodulation in soil was attempted.

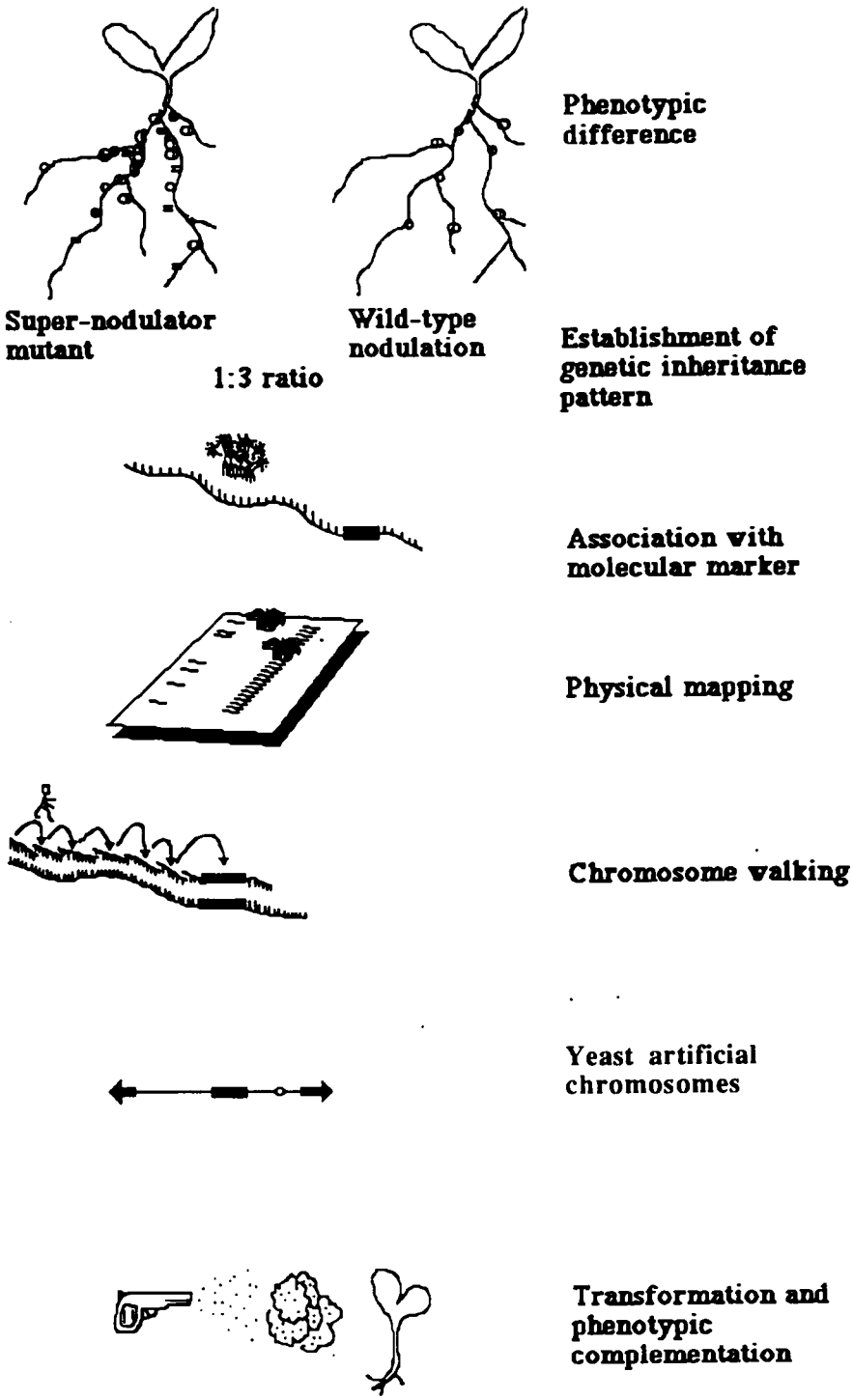

Figure 4. Positional cloning strategies in plants, using particle gun gene transfer. This figure represents the main steps from the observation of a phenotypic difference to the isolation and functional transfer of the gene (taken from Bond *et al.*, 1992)

The majority (90%) of the plants with transgenic root systems produced normal (Fix^+) nodulation profiles and phenotypes. Supernodulating mutants and non-nodulating mutants with transgenic root systems were 'faithful' to their non-transgenic phenotype. A small number of plants with transgenic root systems (10%) produced abnormal (Fix^-) nodules. The nodule phenotype was typically more indeterminate in that all of the nodules possessed an active meristem. The nodules were also elongated (tubular or cone shaped) and sometimes 'budding'. This altered nodule phenotype is unique to this system.

The alteration of nodule phenotype is most likely due to the expression of the *rol* genes and their subsequent alteration of 'perceived' auxin levels in the root and nodule. It is not clear if the *rol* genes modify auxins, thus making them more active or make the plant cell more sensitive to auxin.

This rare alteration in nodule phenotype to a more indeterminate nodule phenotype is significant. Determinate and indeterminate nodules were thought to be fundamentally different. We have shown that by the addition of a limited number of genes (*rol* genes *A*,*B*,*C* and *D*) we can change the nodule phenotype significantly.

The speed and ease of producing transgenic root systems in this way has a great advantage over the particle gun embryogenic suspension transformation system. The disadvantage is that the whole plant is not transformed, and thus no germ line transfer of these traits is possible. If germ line transfer were possible, mutant lines of plants with altered nodule morphology could be established for genetic, molecular and biochemical analysis much in the same way that the supernodulating (nts) and non-nodulating (nod) mutant lines have been produced.

The alteration in nodule phenotype seen in 15% of the transgenic root systems could limit the future analysis of genes that are thought to be involved in the nodulation process. In these cases careful analysis of the plants would be required to assure that the alterations in phenotype are due to the desired gene sequence and not the *rol* genes.

Another interesting result from the GUS positive 'rhizogenes' roots is the integration status of the transgenes. The T-DNA from the Ri plasmid appears classically integrated when analyzed by Southern blot analysis. However, the p35S GUS I plasmid (Vancanneyt *et al.*, 1990) did not appear classically integrated. The results obtained from

Southern hybridization suggest that the plasmid is autonomously replicating in the plant cell.

Such extra-chromosomal elements have been demonstrated with transposable circular DNA in maize (Weiner *et al.*, 1986). In the animal kingdom extra-chromosomal maintenance of transgenic plasmid DNA has been demonstrated in *Caenorhabditis elegans* (Mello *et al.*, 1991), *Drosophila* (Steller and Pirrotta, 1985), *Xenopus* (Marini *et al.*, 1988), and sea urchin (McMahon *et al.*, 1985).

Mello *et al.* (1991) found that chromosome integration of plasmid DNA injected into gonad cells was relatively rare. These plasmid sequences are replicated and inherited in a non-Mendelian manner as large arrays formed by homologous recombination between homologous sequences in the plasmids. This result suggests that either the plasmid origins of replication function in the eukaryote or that the plant initiates DNA replication of this extra-chromosomal DNA at some other unidentified region. It is not yet proven that there is an absolute requirement for sequence-specific origins in eukaryotes (Marini *et al.*, 1988).

Stinchcomb *et al.* (1985a) found that once these extra chromosomal structures are established they undergo very little further rearrangement. In Xenopus oocyte cytoplasm, foreign DNA sequences are rapidly condensed into chromatin and are assembled into structures resembling typical eukaryotic nuclei (Forbes *et al.*, 1983). Stellar and Pirrotta (1985) found that plasmid DNA injected into *Drosophila* embryos becomes enclosed in nuclei or 'nucleus like' structures, where it remains throughout embryonic development.

The most convincing evidence for the replication of defined circular plasmids comes from Marini *et al.* (1988). *Xenopus* laevis embryos were injected with plasmid DNA and in some embryos the circular plasmid DNA was replicated through blastulation. Autonomous plasmid array formation was also seen in other embryos.

Integration patterns of plasmid DNA introduced into soybean suspension cultures by particle bombardment have indicated head to tail concatemers with strong hybridization signals to unit plasmid length DNA (Finer and McMullen, 1991). The non-unit length fragments also observed could either be partial plasmid pieces or plant-plasmid DNA borders (Finer and McMullen 1991). Extra-chromosomal plasmid arrays give plasmid patterns identical to thos observed by Finer and McMullen, (1991). Much remains to be

learned about the correlation of the structure of introduced DNA and the resulting integration pattern and gene expression (Finer and McMullen, 1991). Inheritance studies need to be performed on this tissue. These extra-chromosomal elements are inherited (Mello *et al.*, 1991) but in a non-Mendelian fashion. As autonomous plasmid arrays have been shown to replicate in eukaryotic cells (Mello *et al.*, 1991), it is indeed plausible that individual plasmids can replicate autonomously in transgenic plant tissue.

Evidence for such autonomous replication of plasmids also exists in plants. Chee *et al.* (1989) used *Agrobacterium* to transform soybean cv. A0949. Southern blot results from subsequent R0 plants and a non-Mendelian inheritance to the R1 generation of plants indicated that the transgene was either maintained extra-chromosomally or that the R0 and R1 plants were harboring *Agrobacterium*. This earlier work with intron-less transgenes always lead to speculation of bacterial contamination because of the bacteria's ability to express such genes. By using the GUS intron gene in our study we have avoided such speculation.

Future experiments

Further experiments are required to prove definitively that the introduced plasmid is not integrated into the genome. Inverse PCR could be used prove or disprove the existance of plant / plasmid DNA borders. *In situ* hybridization would show the locality, segregation and possible number of plasmids in the plant cell. DNA preparations from long term aseptically grown transgenic roots could be used to transform bacteria or yeast and check for plasmid transfer and glucuronidase activity (in the case of the yeast cells).

The involvement of the *rol* genes in the alteration of nodule morphology should be examined. The rare phenotypic difference could be due to a positional integration effect, incomplete T-DNA transfer, or T-DNA instability. The incomplete transfer theory could be tested by probing blots with the left non-essential border of T-DNA. If the left border were absent, it would show incomplete T-DNA transfer occurred. If the left border were always present, it would indicate that the difference in phenotype was due to a positional effect. The effect of individual *rol* genes on nodulation could be examined by transposon mutagenesis of each gene in the *Agrobacterium*. Transformation of the soybean plants with each mutated strain would follow. It could be that any one single gene, or all of the genes cumulatively, are responsible for the modified phenotype. Examination of cellular compounds by HPLC could elucidate how the *rol* genes work and how they modify the

plant cell to produce the hairy root phenotype and modify the nodule phenotype. It is not known whether the *rol* genes increase the cellular sensitivity to auxins or that they modify them (conjugate, hydrolyse, methylate, etc.) to a more biologically active form. It is very likely that the biochemical change induced by the *rol* gene is what induces meristematic activity in the altered nodules.

This system would be ideal to help find the mutated loci in the non-nodulating (nod49 and nod139) mutants. Indeed, it could also be used to prove functionality to a sequence thought to be mutated in the supernodulating mutants (nts382). First, the isolated DNA would need to be sequenced and the promoter removed and replaced with a constitutive promoter such as 35SCaMV. Transgenic roots could then express the wild-type sequence homologous to the *nts382* locus and either regulate or completely suppress nodulation. Complete nodulation suppression could be due to overexpression of the coding sequence when promoted by the strong 35S promoter. The regulatory aspects (shoot and root, etc.) of the promoter region could be analyzed by production of a GUS fusion construct. This construct could be transferred by *Agrobacterium rhizogenes*.

Transformed root cultures have been used for environmental studies such as the decontamination of polluted soil, uptake of metals from sewage (review see Potter, 1991). There is possible scope for industrial and pharmaceutical applications such as the production of animal and plant enzymes. Also more direct plant studies of mycorrhizal fungi, parasitic nematodes, herbicide and nematicide action have involved rhizogenes roots (review see Potter, 1991). Rhizogenes roots could also be useful for fundamental studies on gene transfer via T-DNA or transposable elements .

Chapter 6

I intended to apply this efficient root transformation system to elucidating the role of an important gene in the nodulation process. The gene I chose was chalcone synthase (CHS). This gene was considered important for its key position toward the beginning of the flavonoid pathway. This pathway produces many products which are deemed important in the nodulation process (Recourt *et al.*, 1992). Regulation of CHS by antisense technology in petunia and tobacco transformation systems had shown striking modifications in flower color. This indicated that it was feasible to decrease CHS levels in soybean plants with antisense technology and possibly see a phenotypic change in nodulation. The flavonoid pathway is known to produce a number of compounds which

act as specific chemical attractants for *Rhizobium* during the infection stage. The important signal molecules in soybean are genestein and diadzein. Phytoalexin (antimicrobial) compounds are also produced in this pathway. The important phytoalexin in soybean is glyceollin I. Work by Parniske *et al.* (1991a; b) has shown that the glyceollin I is important in limiting the expansion of bacterial infection zone during nodule development. Limiting the CHS levels in the nodulation process may have various effects depending on which step is most critical to nodule initiation and development.

The CHS multi-gene family has seven members in soybean (Akada *et al.*, 1990a). It is thought that each gene is expressed in different ways in response to environmental and developmental signals. Environmental stresses include UV light (Kreuzaler *et al.*, 1987) phytopathogens and elicitors (Ryder *et al.*, 1984; Bell *et al.*,1986; Ryder *et al.*,1987), wounding (Bell *et al.*,1986; Ryder *et al.*,1987) and nodulation (Estabrook and Sengupta-Gopalan 1991). The differential expression of the family of CHS genes to different stimuli is due to variation in the promoter sequence and therefore signaling and transcription response of the gene. At the protein level there is at least 90% homology (97% between genes 1 and 6) between the CHS genes. Gene 7, the nodule-specific CHS shows the greatest diversity to the other CHS genes with 85% homology at the DNA level.

This sequence diversity was not a problem in antisense studies where Parley CHS was used as an antisense construct for transformation of petunia and tobacco (85% homology at the DNA level).

At the beginning of this project we obtained a genomic clone of CHS gene 4 (complete coding region) to make a sense and antisense construct. Ideally, the regulation of CHS levels in nodules by antisense technology would be best accomplished with a complete nodule specific cDNA clone of CHS (gene 7). At the time we started this project, little was known about the role of specific CHS genes in soybean. Estabrook and Sengupta-Gopalan published the isolation of the soybean nodule-specific CHS late in 1991. We introduced sense and antisense CHS contructs into soybean rhizogenes induced roots. Northern blots of these transgenic roots were probed with CHS gene 7, no differences were noticed in the CHS expression levels. It would appear that the CHS gene used in this study either did not work or did not possess enough sequence homology to the nodule specific CHS gene to act effectively as antisense constructs. We did not observe any phenotypic changes in nodule phenotype or number that were due to the introduction of

the CHS sense or antisense genes. However, the antisense root systems seemed more susceptible to rooting (data not shown). It could be possible that the antisense construct may affect a CHS gene related to plant defense. Obviously the plants were still able to signal the bacterium to induce nodulation. Further experiments are needed to elucidate the role of CHS in nodulation.

Future experiments

If time permitted, more sophisticated analysis of the CHS levels in our transgenic roots and nodules would be undertaken via single stranded probing of Northern blots. Also the full length nodule-specific cDNA clone of CHS (gene 7) would be used to make an antisense construct. It would also be much more elegant to demonstrate transformation by either producing a GUS gene fusion with the CHS antisense construct or incorporating an extra expression cassette containing the CHS gene and a 35S promoter into a non invasive coloning site in the p35S GUS I plasmid (*Eco*RI or *Hinc* II restriction sites). In experiments of this kind where there exists the chance of a chimera of transgenic and non-transgenic roots and either high or low gene expression in the same root system, incorporation of the GUS gene would be invaluable marker. Most engineered Ti plasmids only transfer 10-15kb. *Agrobacterium rhizogenes* cucumopine and mannopine strains transfer 35-40 kb of T-DNA. Thus, the incorporation of an extra gene promoter cassette within the T-DNA borders of the GUS I plasmid should not cause technical problems with T-DNA transfer.

Summary

This study has demonstrated ability to analyze nodulation in soybean through a rapid and efficient *Agrobacterium rhizogenes* transformation system. This is a significant advance in the area of soybean nodulation genetics. The modification of nodule phenotype in soybean by the addition of specific transgenes raises fundamental questions as to the difference between indeterminate and determinate nodules.

The observed non-classical integration events in 'rhizogenes' induced roots also raises another fundamental question as to how transgenic sequences are maintained in plants. This research may have raised more questions and proposed more hypotheses than it has answered. However, it has still made a significant contribution to our scientific

knowledge in this field. It is hoped that the future will see these observations and hypotheses tested.

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VITA

James Edward Bond was born in Maldon, Essex, England on December 9th, 1964. He was the fifth and last child to Lilian May Bond a teacher, and Phillip Charles George Bond a maintenance fitter. He started school at the age of 3 and attended a number of Primary Schools in the Maldon area. His main interests were frogs, gardening and science. In 1983 at the age of 18 he graduated from the The Plume Comprehensive School with nine '0' levels and three 'A' levels in Biology, Chemistry and Physics.

After spending the summer inspecting the sites of Europe he visited and fell in love with the town and University of Aberystwyth, Wales. That autumn he enrolled at the University of Aberystwyth to read for an honors degree in Genetics and Plant Breeding. During his study James found a passion for research and in the summer of 1985 he spent four months at the University of Bangkok, Thailand learning techniques such as orchid tissue culture. In September of the same year he entered an industrial training period with Shell Oil Research. In 1987 James completed his undergraduate degree and traveled to Mexico and Canada before starting his Ph.D. at the University of Tennessee in Knoxville.

Four and a half years, or five football seasons later he graduated with a Ph.D. in Plant Molecular Genetics. James is pursuing his career in science with a post-doctoral position at CSIRO, Adelaide, Australia. He will be working on the production of seedless tangerines through genetic manipulation and improving his wind surfing skills.