AN ABSTRACT OF THE THESIS OF

Inga L. Gelford  for the degree of  Masters of Science  in  Microbiology
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Agrobacterium tumefaciens

Abstract

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Dr. Lyle Brown

Agrobacterium tumefaciens, the causative agent of crown gall
disease, does not readily infect commercial varieties of alfalfa (Medicago
sativa). Cloned virulence genes from the megaplasmid pTiBo542 have
been shown to enhance Agrobacterium host range and infectivity on
dicotyledonous plants. To obtain a gene transfer system for commercial
alfalfa cultivars, an Agrobacterium strain containing a disarmed T-DNA, a
cloned virG gene from pTiBo542, and an octapine Ti plasmid TiA6NC was
constructed. This strain, A348 (pToK9, pGA472), was used to inoculate
excised petioles of the alfalfa cultivar "Gladiator". Neomycin resistant callus
tissue grew readily on 100 μl/ml kanamycin and on appropriate media, leaf
and embryo development was induced. DNA hybridization analysis of the
transformed plant genomic DNA isolated from callus tissue confirmed the
presence of the npt gene. VirG enhanced A. tumefaciens strains can thus
be used in foreign gene transfer and expression in commercial cultivars of
alfalfa.
Transformation of Alfalfa using
Agrobacterium tumefaciens

by

Inga L. Gelford

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Typed by Inga Gelford for Inga Gelford
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TRANSFORMATION OF ALFALFA USING AGROBACTERIUM TUMEFACIENS

INTRODUCTION

Genetic engineering of plants has the potential to uncover some of the mysteries of plant physiology and genetics and may provide for more efficient plant production in agriculture. If systems are developed for the stable incorporation of foreign genes into higher plants, the conferring of characteristics such as herbicide or pesticide resistance becomes possible. Further, reporter genes with a product easy to assay in plants could be used to study gene regulation and protein function. One of the major obstacles in developing gene transfer systems for plants has been finding a system that could be used with facility for dicotyledonous crop plants. This thesis describes a gene transfer system for Medicago sativa (alfalfa) that takes advantage of the natural gene transfer system employed by the bacterium Agrobacterium tumefaciens. This system utilizes an easily selectable trait of neomycin/kanamycin resistance to detect gene transfer into alfalfa plant cells.
LITERATURE REVIEW

General background of *A. tumefaciens*

*Agrobacterium tumefaciens*, a soil bacterium that incites tumors at plant wound sites (for reviews see 6, 90, 98), harbors a large, tumor-inducing (Ti) plasmid (Figure 1) which codes for the transfer and integration of a specific segment of Ti plasmid DNA (T-DNA) into the plant genome (1, 17, 18, 67, 74, 82). Two major regions of the Ti plasmid have been studied in detail; the T-region and the virulence region.

The T-region includes the T-DNA, the border repeats and a cis-active DNA sequence called "overdrive". Carried within the T-DNA border repeats are genes coding for auxin, cytokinin and opine biosynthesis. When the genes catalyzing the production of the phytohormones auxin and cytokinin are transcribed and translated in the plant cell, the hormonal imbalance resulting from their expression leads to tumorous growth.

The second well documented region on the Ti plasmid is the virulence region which is located to the left of the T-DNA as drawn in Figure 1. Within this region are several loci responsible for T-DNA transfer, host range specificity, and for enhanced virulence.

The opines are modified amino acids secreted by the tumor cells which serve as a nitrogen and carbon source for bacteria harboring the corresponding Ti plasmid. Usually only one type of opine is encoded by a Ti plasmid and thus Ti plasmids are conveniently classified by the type of opine they produce. Octapine, nopaline, and L,L-succinamopine are three
Figure 1: Diagram showing the T-region and virulence region of the different types of Ti plasmids. From top to bottom: octapine Ti plasmid pTiA6, L,L succinamopine Ti plasmid pTiBo542, and nopaline Ti plasmid pTiC58. Definition of symbols: BL, border left; BR, border right; TL, T-DNA left; TC, T-DNA center; TR, T-DNA right.
examples of opines produced by Ti plasmid containing Agrobacterium strains. The Ti plasmids of these strains are thus referred to as octapine, nopaline, and L,L-succinamopine Ti plasmids, respectively. Genes encoding enzymes for the uptake and catabolism of the specific opine are on the Ti plasmid but separate from the T-DNA (14, 35, 36, 48, 64, 65).

A. tumefaciens causes tumors on a wide range of dicotyledonous angiosperms, such as tobacco, petunia, pea, sunflower, and Kalanchoe, and on some gymnosperms (27, 67, 84). There is evidence that several monocots such as Dioscorea bulbifera (yam), Asparagus officinalis and Zea mays can be infected with A. tumefaciens but generally without production of tumorous growth (reviewed in 72).

Properties of the Ti plasmid

T-region

The T-region includes the T-DNA, a DNA strand that is transferred into plant cells, border repeats, and a conserved sequence to the right of the T-DNA (Figure 1). Genes within the virulence region encode proteins involved with T-DNA transfer. No transfer functions are coded for on the T-DNA itself. The only cis-acting elements required for T-DNA transfer are the 25 base pair imperfect direct repeats which border and functionally define the T-DNA (8). Deletion of the left border repeat does not abolish virulence whereas deletion of the right border repeat totally abolishes pathogenicity (42, 49). The right border repeat must be in the correct orientation for T-DNA transfer. A conserved sequence to the right of the right border repeat, called
"overdrive", greatly stimulates right border function when placed in either orientation (70).

The T-DNA of the Ti plasmid also contains the loci responsible for tumor maintenance, \textit{tms, tmr, tml} and the opine synthetic locus (33, 92). Mutations in the \textit{tms} loci results in the overproduction of shoots; in \textit{tmr}, the overproduction of roots; and in \textit{tml}, an increase in tumor size. The \textit{tms} locus produces two transcripts, \textit{tms 1} and \textit{tms 2}, that encode auxin biosynthetic enzymes, tryptophan monooxygenase and indole acetamide hydrolase. The \textit{tmr} locus encodes a cytokinin biosynthetic enzyme, isopententyl transferase. The \textit{tml} loci encodes two transcripts, the function of which is uncertain. The opine synthetic locus and additional regions of Ti plasmids are not required for tumor maintenance.

The structures of the T-regions of the different types of Ti plasmids have been shown to be non-identical (Figure 1). T-DNA from nopaline Ti plasmids is a continuous segment which integrates into the plant genome as a single unit, whereas T-DNA from octapine and L,L succinamopine Ti plasmids is found as three T-DNA segments; T-DNA left (\textit{TL}), T-DNA center (\textit{TC}), and T-DNA right (\textit{TR}) (45, 88). All three segments are potentially transferred and integrated into the plant genome. The spatial arrangement of genes also differs between the two T-DNA types. Nopaline plasmids have the tumor maintenance genes (\textit{tms, tmr, tml}) and the nopaline biosynthetic locus, \textit{nos} (nopaline synthetase), arranged in a continuous manner while the octapine and L,L succinamopine plasmids have the tumor maintenance genes and the octapine biosynthetic locus, \textit{ocs} (octapine synthetase), located within the \textit{TL} borders. \textit{TC} and \textit{TR} do not contain genes required for tumor maintenance and are not always found integrated next to the \textit{TL}-DNA.
Other opine biosynthetic genes have been found within the TR-DNA borders, however, a genetic marker has not been found for the TC-DNA (23, 24).

The mechanism of T-DNA transfer involves border-specific nicks which prepare the T-DNA for transfer to the plant cell. The transfer of the T-DNA may involve a circular T-DNA molecule (3, 58) or a linear, single-stranded T-DNA molecule (2) as the molecular intermediate. However, the proposed mechanism of unidirectional replication (2) directed by each border sequence is most consistent with the linear, single-stranded model. This model offers an explanation for the requirement of correctly oriented Ti plasmid border repeats, since replication would proceed in only one direction. It would also explain the ability of any single border to direct transfer of binary vector DNA into plant cells independent of its orientation (31, 49, 51).

Virulence region

The genes of the virulence region are involved in host specificity and transfer of the T-DNA structure, but the virulence genes are not stably integrated into the plant genome (5). The virulence region is composed of several complementation groups (47, 54, 59). Octapine Ti plasmids studied so far have 7 virulence loci designated *virA, virB, virC, virD, virE, and virF* (54, 71). The described nopaline and L,L-succinamopine plasmids contain all the analogous virulence loci except *virF* (39). The virulence regions of the various Ti plasmids studied have been shown to be conserved in sequence and function, and this conservation also extends to the virulence regions of *Agrobacterium rhizogenes* Ri plasmids (30, 47, 50, 53). Any differences in homology seem to fall outside the six complementation groups.
The virulence region products are necessary for T-DNA processing, transfer, integration and host range specificity. The structural organization of a L,L succinamopine virulence region is shown in Figure 2.

Some of the virulence genes (virA, virB, virD, and virG) are essential for tumor induction, while others (virC, virE, and virF) are only necessary for infection of certain plant species. The virA locus contains one open reading frame (ORF) which encodes a cytoplasmic membrane protein that recognizes plant wound extract signals (63). The exact function of the virB operon gene products is not entirely known but these gene products are involved with the T-DNA transfer structure (54). The virC complementation group encodes two polypeptides which are involved with host range specificity (22, 96). The virD locus codes for several proteins, and two of these are part of an endonuclease complex specific for the T-DNA borders (2, 97). The virE locus is responsible for enhanced efficiency of infection and has three potential ORFs arranged as an operon. A hydrophilic protein encoded by ORF3 may be the diffusible protein responsible for enhanced efficiency (39, 93). The virG locus encodes a regulatory protein that activates virulence gene transcription (71, 94).

Two systems of regulation have been established for the virulence genes: positive regulation of virB, virC, virD, virE, and virG in response to acetosyringone or plant extracts (71, 94); and negative regulation of virC and virD which was abolished by a chromosomal mutation in the ros loci (21). The positive regulatory system involved the protein products of virA and virG, which were required for induction of the remaining virulence region genes. The cytoplasmic membrane protein virA recognized a plant wound signal molecule, such as acetosyringone (63), and upon binding of the plant
Figure 2. The Bam HI and Sal I restriction map of the virulence region of pTiBo542. The letters A, B, G, C, D, and E represent the virulence loci vir A, vir B, vir G, vir C, vir D, and vir E, respectively. The vir region loci included in the plasmids pTVK178, pTVK291, and pToK9 are indicated by the bars below the plasmid designation.
signal molecule, the virA protein activated the virG positive regulator protein. This resulted in the enhanced transcription of the highly inducible virB, virC, virD, and virE loci and the weakly inducible virA and virG loci (71). Under laboratory conditions, acetosyringone induction resulted in a 13-120 fold increase in virB, virC, virD, and virE gene expression, while virA and virG gene expression increased 0.9-2.6 fold and 3.6 fold, respectively (71, 79). The virA and virG gene products are homologous to the E. coli regulatory proteins EnvZ/OmpR (14, 69, 94). These proteins respond to environmental signals by activating gene expression. A study done by Rogowsky, et al. (1987) showed that an increase in the virG gene dosage alone resulted in a proportional, acetosyringone-independent increase in virulence gene expression. This implied that virG protein was the activator of virulence region transcription, not virA, and that even inactivated virG protein was able to stimulate some transcription of virulence genes (71). The presence of a plant inducer molecule and virA resulted in a further increase of virulence gene expression (71).

Negative regulation mediated by the gene products of the ros locus is directed against a subset of the virulence genes in the nopaline and octapine plasmids that have been studied (20). A mutation in the ros locus increases the expression of virC and virD without the presence of plant exudates and Ti plasmid encoded factors. The ros mutation also eliminated the major A. tumefaciens exopolysaccharide resulting in a dry, florette colony morphology (21). Other evidence suggesting that ros regulation is negative comes from the observations that virC and virD are expressed at low basal levels in A. tumefaciens but are expressed at greatly elevated levels in E. coli (21), and in vitro assays of virC and virD promoters show DNA can be
transcribed efficiently and accurately by RNA polymerase isolated from *A. tumefaciens* (R. Tait and C. Kado, unpublished data).

Chromosomal virulence genes

There are two chromosomal virulence loci *chvA* and *chvB* that are involved with *Agrobacterium* plant cell binding. The *chv* loci genes are nonregulated and expressed constitutively, conferring cell surface characteristics necessary for virulence. Mutations in either of the *chvA* or *chvB* loci lead to defective bacterial-plant cell attachment and avirulence (29).

Pathogenicity

The host range of *A. tumefaciens* has been shown to be determined by the type of Ti plasmid present in the bacterium (60, 83). Determinants of host range specificity have been mapped to loci both within and outside the T-DNA (16, 37, 40, 47, 53). The loci within the T-DNA involved the cytokinin biosynthetic gene of the different Ti plasmids (95) and the non-T-DNA loci included *virA*, *virC* and *virE* of the Ti plasmid virulence region (22). Some *A. tumefaciens* isolates, such as strains A348 and A281, have a broad host range, infecting many species of plants. Other isolates are limited and may infect mainly one group of plants such as strain 1D1109 on grapevine (*Vitis* spp.) or AB2/73 on *Lippia canescens* (60, 86). *Agrobacterium tumefaciens* strain A281, harboring the Ti plasmid pTiBo542, has an unusually wide host range and induces large, fast appearing tumors (46, 57). The supervirulent
phenotype resides on the Ti plasmid within a 2.5 kb region containing \textit{virG} and the 3' end of \textit{virB} \((52)\). A plasmid containing this 2.5 kb fragment introduced into other less virulent \textit{A. tumefaciens} strains has increased their virulence and host range \((44, 45)\).

\textbf{Methods of gene transfer into plants using \textit{A. tumefaciens}}

Methods of gene transfer into plants include electroporation of DNA into plant cells, plant protoplast fusions, and \textit{Agrobacterium}-derived transfer systems \((34)\). Each method has its own advantages for different systems. The natural gene transfer system of \textit{A. tumefaciens} provides an effective method of transformation for dicotyledonous plants and the tumor producing T-DNA can be disarmed by deletion of the oncogenes \((tms, \textit{tmr}, \textit{tml} \text{ loci})\) preventing tumor formation and allowing regeneration of whole plants.

Hoekema, et al. \((41)\) showed that the T-DNA can be placed on a separate plasmid from \((\text{in trans})\) the Ti plasmid in \textit{A. tumefaciens} and still retain normal tumor-inducing capacity. T-DNA cloned into a small plasmid vector can easily be manipulated for construction of in frame chimaeric gene fusions containing regulatory sequences recognized by the plant. These can serve as reporter genes to examine control of plant gene expression \((11, 78)\).

The modified T-DNA can either be transferred back to the Ti plasmid by homologous recombination with wild-type T-DNA or used in a binary vector system for transfer of foreign DNA into plant genomes \((5, 10, 55)\). Chimaeric genes that code for antibiotic resistance allow for easy selection of transformed cells and regeneration of whole plants.
An et al. (5) constructed Ti plasmid-based vectors, including pGA472 and pGA471, that consisted of a chimaeric gene made up of the transcriptional and translational control signals from the nopaline synthetase (nos) gene in frame with the coding sequence of the neomycin phosphotransferase (npt) gene of Tn5, the border repeats from the T-DNA of pTiT37, and a wide host range replicon (5). The nos promoter and termination sequences are recognized by the plant and needed for efficient translation of the npt protein. Plant cells expressing the npt gene can then be selected with either neomycin or kanamycin. The T-DNA border repeats are recognized by the virulence genes for transfer of the chimaeric gene into the plant genome (41, 54). A wide host range replicon enabled the plasmid to replicate in E. coli and A. tumefaciens. DNA cloned into this vector was efficiently transferred into plants when in trans with the virulence region contained on a Ti plasmid in A. tumefaciens.

Transformation of higher plants using a binary vector system

Several approaches involving a binary vector system in Agrobacteria have been used to transform higher plants. The binary system consists of a Ti plasmid and a small vector such as pGA472 in an A. tumefaciens background. Agrobacterium tumefaciens harboring pGA472 has been used to transform plant cell suspensions and protoplasts (4, 7, 61). However, both of these systems are limited because of the difficulty of obtaining regenerated plants from either plant cell suspensions or plant protoplasts. Agrobacterium rhizogenes is considered closely related to A. tumefaciens and it transforms
plant cells in similar manner. However, instead of producing tumors it induces "hairy-root" formation at wound sites. The endogenous plasmid is called Ri for "root-inducing". Unlike transformation with *A. tumefaciens* wild-type T-DNA, several species of plants that have been transformed with intact Ri T-DNA (including the hormone biosynthetic genes) have been regenerated into whole plants (19, 25). Also unlike *A. tumefaciens*, where the Ti plasmid T-DNA does not tend to cointegrate with the disarmed vector DNA in a binary system, *A. rhizogenes* Ti plasmid T-DNA frequently cointegrates with the disarmed vector T-DNA. *Agrobacterium rhizogenes* containing pARC4, a vector similar to pGA472 with the nopaline synthetase gene as a transformation marker, has been used to successfully transform plants (78, 81). *Agrobacterium rhizogenes* efficiently transfers both the vector T-DNA and the Ri plasmid T-DNA producing transformed "hairy-roots". These roots can be regenerated into whole plants containing full length Ri plasmid T-DNA along with the desired vector DNA (81).

A particularly successful method of plant transformation uses the binary vector system in *A. tumefaciens* to infect explants. Tobacco, white clover, and *Arabidopsis thaliana* as well as others have been successfully transformed with the *npt* gene by cocultivation of explants with *A. tumefaciens* harboring pGA472 or a similar vector, selection for *npt*-transformed plants cells and regeneration of those cells on the appropriate media (9, 77, 91). This method avoids the establishment of cell suspensions, protoplasts, or "hairy-roots" and eliminates the need for wild-type T-DNA incorporation.
Transformation of *Medicago sativa*

Genetic engineering of *Medicago sativa* L. (alfalfa), a valuable forage legume, could lead to improved varieties for different field conditions and could lead to greater understanding of the molecular basis of symbiotic nitrogen fixation. So far only transformation/regeneration systems based on *A. rhizogenes* have been successful in *M. sativa*. Simpson, et al. (78) transformed *M. sativa* cv.CuF101 with the *npt* gene and established "hairy-root" cultures using the binary vector system from *A. tumefaciens* in *A. rhizogenes*. *Agrobacterium rhizogenes*, having a similar manner of plant transformation, is considered closely related to *A. tumefaciens*. Sukhapinda, et al. (81) transformed and regenerated alfalfa plants using a binary vector containing nopaline synthetase in *A. rhizogenes*. Sterile stems were inoculated and "hairy-roots" that resulted were assayed for nopaline synthetase. The percentage of doubly transformed "hairy-roots" receiving both the Ri T-DNA and the vector T-DNA harboring the nopaline synthetase gene ranged from 43-60%. Transgenic plants were phenotypically normal except for a shallow root system.

*Agrobacterium tumefaciens* has successfully been used to transform and regenerate a related alfalfa species (*Medicago varia*) using strain bo42 which is *A. tumefaciens* A281 harboring plasmid pGA471 with the *npt* gene (26). Stem cuttings of a *M. varia* genotype (A2) of good regenerating capability were inoculated and after several weeks produced transformed embryos that later went on to become transgenic plants. However, this method has not yet been extended to commercial cultivars of *M. sativa*. 
The goals of this study were to develop an efficient regeneration system for several commercial cultivars of *M. sativa*, to establish a gene transfer system for those alfalfa cultivars based on the *A. tumefaciens* pGA472 vector system, and to obtain regenerated transformed alfalfa plants.
MATERIALS and METHODS

Bacterial strains, plasmids, and growth conditions

Agrobacterium tumefaciens strains and plasmids used in this study are listed in Table 1. The plasmids pGA472, pToK9, pTVK178, pTVK291 and pTVK25 were a gift from Dr. E. Nester (57). Plasmid pGA472 contains 1) the 25 basepair (bp) border sequences of pTiT37 T-DNA, 2) a chimaeric gene made up of the TiT37 nos promoter, the coding sequence of the npt gene, and the TiT37 nos termination sequence, 3) the Col E1 replicon, 4) the lambda cos site and 5) a wide host range replicon (5)(Figure 3). Plasmids pToK9, pTVK178, pTVK291 are pVK102 derivatives (56)(Figure 4) containing pTiBo542 virulence region clones (57). They are not capable of self-transmission and can be mobilized by pRK2013 (76) (see Figure 2 for pTiBo542 virulence region map). Plasmid pTVK25 is also a pVK102 derivative with a 26.8 kilobase pair (kb) fragment of the pTiBo542 T-DNA cloned into the Sal I site (see Figure 5 for T-DNA map). All plasmids were maintained in Escherichia coli strain HB101 for use in A. tumefaciens strains A348 (pTiA6NC), A281 (pTiBo542) and PC2760 (pAL4404ΔT-DNA). Bacterial cultures were grown either in Luria broth (5 g yeast extract, 10 g NaCl, 10 g Bacto-tryptone, pH 7.8) or on Luria agar. Agrobacterium and E. coli strains containing plasmids having antibiotic resistance were maintained with 100 µg/ml kanamycin sulfate (Sigma Chemical Co. St. Louis, Mo.) or 20 µg/ml tetracycline hydrochloride (Calbiochem-Behring, La Jolla, Ca.).
Table 1. A. tumefaciens strains and plasmids.

<table>
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<th>b alfalfa</th>
<th>tobacco</th>
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<td><strong>Strains with Tn3 insertions within the virG loci from pTiBo542:</strong></td>
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<td><strong>Strains with disarmed T-DNA vector in trans (pGA472):</strong></td>
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<td>OCT</td>
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<td>+</td>
<td>npt gene</td>
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<td>pGA472</td>
<td>Tc</td>
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<td>npt gene</td>
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<tr>
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<td>Km, Tc</td>
<td>OCT</td>
<td>+</td>
<td>+</td>
<td>npt gene</td>
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<td>Km, Tc</td>
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</tbody>
</table>

*aKm, Kanamycin sulfate; Am, ampicillin; Tc, tetracycline HCl
bOCT, octapine; L, L SAP, L, L succinamopine; LOP, leucinopine; AGR, agropine
*These strains were a gift from Eugene Nester, Dept. of Microbiology and Immunology, Univ. of Washington
Figure 3. Plasmid pGA472. The grey boxed in areas correspond to the nopaline synthetase promoter (Pnos) and terminator (Tnos) sequences. BR indicates the location of the right border repeat and BL indicates the location of the left border repeat.
Figure 4. Plasmid pVK102. The virulence region inserts pToK9, pTVK178, and pTVK291 were cloned into the Sal I site of pVK102, a broad host range plasmid able to replicate in both *E. coli* and *A. tumefaciens*. 
Figure 5. Bam HI restriction map of the T region of pTiBo542. Part of an arbitrary map designating 5 kb increments of the Ti plasmid is shown below the restriction map. BL, left border repeat; BR, right border repeat.

*Sal I sites of a 26.8 kb fragment cloned into plasmid pVK102 to construct pTVK25.
**Media**

**Luria bacterial medium**
10 g Baco-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, volumed to 1000 ml with H2O.

**Minimal medium for A. tumefaciens**
100 mg NaCl, 200 mg MgSO4.7H2O, 500 mg NH4Cl, 230 mg KH2PO4, 230 mg Na2HPO4, trace minerals: 50 mg CaCl2.2H2O, 2.39 mg FeEDDHA, 1.45 mg H3BO3, 1.25 mg FeSO4.7H2O, 700 μg CoCl2.6H2O, 50 μg CuSO4.5H2O, 43 μg MnCl2.4 H2O, 1.08 mg ZnSO4.7H2O, Na2MoO4.2H2O, vitamins: 100 μg of riboflavin, PABA, nicotinic acid, biotin, thiamine-HCl, pyridoxine HCl, Ca pantothenate, Inositol, 10 g mannitol, and 15 g agar, volumed to 1000 ml with H2O.

**MS medium**
1.9 g KNO3, 1.65g NH4NO3, 170 mg KH2PO4, 440 mg CaCl2.2H2O, 370 mg MgSO4.7 H2O, 37.25 mg Na2 EDTA, 27.85 mg FeSO4.7H2O (as FeEDTA), 6.2 mg H3BO3, 16 mg MnSO4.H2O, 8.6mg ZnSO4.7 H2O, 830 μg KI, 250 μg NaMoO4.2H2O, 25μg CuSO4.5H2O, 25 μg CoCl2.6H2O, 100 mg myo-inositol, 0.40 mg thiamine, 0.2 mg kinetin, 2.0 mg IAA (indole acetic acid), volumed to 1000ml with H2O.

**BA medium**
1.9 g KNO3, 1.65g NH4NO3, 170 mg KH2PO4, 440 mg CaCl2.2H2O, 370 mg MgSO4.7H2O, 37.25 mg Na2 EDTA, 27.85 mg FeSO4.7H2O (as FeEDTA), 6.2 mg H3BO3, 16 mg MnSO4.H2O, 8.6mg ZnSO4.7 H2O, 830 μg KI, 250 μg NaMoO4.2H2O, 25μg CuSO4.5H2O, 25 μg CoCl2.6H2O, 1.0 mg nicotinic acid, 100 mg myo-inositol, 1.0 mg pyridoxine, 10 mg thiamine, 1.0 mg BA (benzylaminopurine), 0.1 mg IAA, volumed to 1000ml with H2O.

**SH and B24 media base**
300 mg NH4H2PO4, 2.5 g KNO3, 195.9 mg MgSO4.7H2O, 492 mg CaCl2.2H2O, 10 mg MnSO4.H2O, 5 mg H3BO3, 1 mg ZnSO4.7H2O, 1 mg KI, 200 μg CuSO4.5H2O, 100 μg NaMoO4.2H2O, 100 μg CoCl2.6H2O, 20 mg Na2EDTA, 15 mg FeSO4.7H2O (as FeEDTA), vitamins: 5 mg thiamine HCl, 5 mg nicotinic acid, 500 μg pyridoxine HCl, 1 g inositol, 30 g sucrose, 15 g agar, volumed to 1000mls with H2O.

For SH medium 11.05mg 2,4-D, 1.08 mg Kinetin were added to the above medium base.
For B24 medium: 1.0mg 2,4-D, 0.2mg Kinetin were added to the above medium base.
**BII medium**

500 mg KNO₃, 1.0 g NH₄NO₃, 300 mg KH₂PO₄, 221 mg KCl, 34.2 mg MgSO₄·7H₂O, 32 mg Na₂EDTA, 24.5 mg FeSO₄·7H₂O (as FeEDTA), 4.4 mg MnSO₄·H₂O, 1.6 mg H₃BO₃, 1.5 mg ZnSO₄·7H₂O, 800 μg KI, vitamins: 20 mg glycine, 500 μg nicotinic acid, 100 μg pyridoxine HCl, 103 μg myo-inositol, 100 μg thiamine, 2.0 g yeast extract, 30 g sucrose, 15 g agar, volumed to 1000mls with H₂O.

**Strain construction using bacterial matings**

Bacterial matings were used to transfer pGA472 and the pTiBo542 virulence region plasmids pToK9, pTVK178, and pTVK291 maintained in HB101 into A. tumefaciens (Figure 6). The recipient strain (A. tumefaciens), the donor strains (HB101 pGA472 and HB101 pToK9, pTVK178, or pTVK291) and the helper strain (HB101 pRK2013) were grown on fresh Luria agar containing the appropriate antibiotics. An isolated colony taken from each strain was placed in the center of a Luria agar plate minus antibiotics, mixed together and incubated for 4 hours at 37°C. The cells were then retrieved with a sterile bacterial loop, spread out on minimal medium agar containing 100 μg/ml kanamycin and 20 μg/ml tetracycline and incubated at 28°C for 3-5 days. Only the A. tumefaciens transconjugates are able to grow on the minimal medium since E. coli HB101 is a proline auxotroph. The strains were screened for oncogenicity on Nicotiana tobacum.

**Plasmid preparations and gel electrophoresis**

Plasmids from E. coli were isolated using alkaline lysis as described
Figure 6. Construction of A. tumefaciens strains using bacterial matings. Plasmids pGA472, pTVK178, pTVK291, pToK9 and pRK2013 were each maintained in E.coli proline auxotrophic HB101 strains. Plasmid pRK2013 is able to mobilize itself as well as pTVK178, pTVK291, pToK9 and pGA472. In the first step of the mating pRK2013 is mobilized into the donor strains HB101 harboring pGA472 and into HB101 harboring a virulence region clone (pToK9 in this case). The two donor strains now harboring pRK2013 mobilize their respective plasmids into the recipient A. tumefaciens strain A348. The helper plasmid pRK2013 is not stable in A. tumefaciens. Minimal media minus proline plus tetracycline and kanamycin selects for strain A348 pGA472, pToK9 and against any HB101 strain.
by Maniatis et al. (62). Restriction endonucleases were purchased either from New England Biolabs, Inc. (Beverly, Mass.) or from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). DNA was electrophoresed on 0.7% agarose gels in E Buffer (40mM tris-acetate, pH7.9, 2 mM Na-EDTA), 30 mA for 3-4 hours.

Plant material

The seeds of *M. sativa* cultivars were obtained from North American Plant Breeders (Anchor), Northrup King (Gladiator), or North Central Regional Introduction Plant Station (Saranac). Seeds were surface sterilized by a 95% EtOH wash followed by a 10 minute exposure to 20% Chlorox bleach plus 2 drops Tween 20 per 200 mls solution, rinsed with 6-7 changes of sterile dH2O and sprouted on 1.5% agar. The sprouts were grown aseptically in 50 ml test tubes containing BII medium (13). Leaves and petioles for regeneration and transformation experiments were harvested using aseptic techniques.

*Nicotiana tobacum* plants were grown from seed and regenerated from leaf disks using MS and BA media (66). Leaf tissue was surface sterilized as described above, disks were cut out using a sterile #5 cork borer (10 mm diameter) and placed on MS medium. After sufficient callus formed around the leaf disks it was transferred to BA medium for shoot induction. Shoots were aseptically transferred to sterile flasks containing BII medium then transferred to soil when plants were 5-6 cm tall.
Transformation of tobacco using *A. tumefaciens*

The constructed *A. tumefaciens* strains containing pGA472 and the virulence region containing pVK102 plasmids were tested on *Nicotiana tobacum*. Leaf disks were immersed in overnight cultures of *A. tumefaciens* and placed on MS agar for 2-3 days. They were subsequently transferred to MS medium containing 500 µg/ml carbenicillin for elimination of *A. tumefaciens* and 200 µg/ml kanamycin for selection of transformed plant cells. Callus tissue that formed was transferred to BA agar plus 200 µg/ml kanamycin for shoot induction. Shoots were placed in sterile flasks containing BII agar plus 200 µg/ml kanamycin until they reached 5-6 cm in height and then planted in soil. Seeds of *npt* transformed plants were surface sterilized and placed on BII medium containing 200 µg/ml kanamycin for sprouting.

Tobacco tissue harboring tumor inducing (wild-type) T-DNA was obtained by cocultivating leaf disks with an overnight culture of strain A281, placing on BII medium for 2-3 days then transferring to BII medium plus 500 µg/ml carbenicillin until tumorous tissue formed around leaf disk edges. Tumor tissue was maintained on BII medium.

**Plant DNA extraction**

Plant DNA was prepared from control (wild-type) alfalfa and tobacco tissue, alfalfa and tobacco tissue transformed with the *npt* gene, and alfalfa and tobacco tissue transformed with tumor inducing (wild-type) T-DNA. A
mortar and pestle were used to grind 2 g of liquid nitrogen frozen tissue. Ten ml of lysis buffer (30 mM EDTA, 50 mM Tris HCl pH 8, 2% sarkosyl, 0.2% β-mercaptoethanol) was added to the powdered plant material and incubated at 4°C for 15 minutes. Cesium chloride was dissolved in the slurry at a concentration of 0.9 g/ml. The plant extract CsCl solution was centrifuged twice at 11,000 rpms in a SS34 fixed angle rotor and the middle layer containing the DNA was filtered through three layers of cheesecloth after each run. Ethidium bromide was added to the solution at a concentration of 400 µg/ml and CsCl concentrations were adjusted to give a final density of 1.57 g/ml. Plant DNA was banded by ultracentrifugation at 45,000 rpms for 15-17 hrs in a VTi80 rotor. The DNA band was collected by puncturing the side of the centrifuge tube with an 18 gauge needle. Ethidium bromide was extracted from the plant DNA solution using multiple changes of water saturated butanol. The DNA solution was dialyzed against several changes of TE (10 mM Tris pH 7.8, 1 mM Na₂EDTA) to remove the CsCl. Plant DNA concentrations were measured using an Aminco-Bowman spectrophotofluorometer. DNA was added to a fluorescent stock solution (2X= 0.1 M NaPO₄ pH 7.4, 4 M NaCl, 0.2 mg H33258 dye) and fluorescence was measured using an excitation wavelength of 356 nm and an emission wavelength of 458 nm. The fluorescence of plant DNA of unknown concentration was compared to a standard curve of fluorescence obtained from known DNA concentrations ranging from 10 ng to 500 ng.
Probe isolation and nick-translation

A 4.2 kb Sac II-Sal I endonuclease digestion fragment of pGA472 contains the coding sequence of the npt gene. The Sac II-Sal I fragment was obtained by digestion of pGA472 first with Sac II endonuclease in a low (50 mM NaCl) salt buffer for one hour at 37°C, then adjusting the NaCl concentration to 150 mM for a one hour Sal I digestion at 37°C. These fragments were separated by electrophoreses for band isolation and nick-translation. A probe containing 5.3 kb of the pTiBo542 T-DNA coding sequence was obtained by digestion of pTVK25 with the endonuclease Bam HI and the resulting fragments were separated by electrophoreses. Digests were electrophoresed for DNA fragment separation in a 1% low melting point agarose gel at 30 mA for 3-4 hours. The 4.2 kb Sac II-Sal I pGA472 band and the 5.3 kb Bam HI pTVK25 band were cut out from the rest of the gel, melted at 65°C, and extracted sequentially in phenol, phenol:chloroform:IAA (25:24:1) and chloroform:IAA (24:1). The DNA was precipitated using two volumes 95% EtOH and one-half volume ammonium acetate at -20°C. Both the pGA472 and the pTVK25 derived fragments were nick-translated using [α-32P]dATP as described by Maniatis, et al. (62). Counts per minute (CPM) were between 1.5 X 10⁴ and 4.8 X 10⁵ CPM/µl probe.

Hybridization assay

A nitrocellulose filter was prepared with 1 µg of each of the prepared plant DNA's listed in "Plant DNA extraction" using a HYBRI-SLOT™ Manifold
apparatus (Bethesda Research Lab.). Plant DNA dissolved in 50 μl boiling buffer (1 mM EDTA, 20 mM Tris pH7.6) was denatured by heating in a boiling water bath for five minutes, incubated in 0.5 M NaOH for 20 minutes, placed on ice, neutralized with cold 400 μl buffer (1.4 M NaCl, 0.15 M Na-citrate, 0.25 M Tris HCl pH 8, 0.25 M HCl), and applied to the nitrocellulose filter under gentle suction. The filter was baked at 80°C for two hours under vacuum. Filters were exposed to pre-hybridization mix (7.5 ml formamide deionized using an anion mixed bed resin, 3.35 ml 20X SSC, 3.75 ml 20X Denhardt’s solution, 4 mg denatured salmon sperm DNA) for 1-4 hours at 42°C (Denhardt’s solution: 0.4% Ficoll 400, 0.4% BSA, 0.4% PVP-300; 20X SSC: 175 g NaCl, 88.2 g Na-citrate, 5 drops HCl per liter solution). The pre-hybridization mix was replaced with hybridization mix (7.5 ml deionized formamide, 3.75 ml 20X SSC, 0.75 ml 20X Denhardt’s solution, 3 ml 50% Dextran sulfate, 4 mg denatured salmon sperm DNA) plus 100 μl of the nick-translated probe. Hybridization was allowed to take place overnight at 42°C (16-19 hours). Hybridized filters were washed three times with 2X SSC-0.1% SDS at 42°C, two times with 0.1X SSC-0.1% SDS at 56°C and allowed to air dry. Kodak XAR-5 film was used for the autoradiogram. One ng of plasmid pGA472 DNA for the npt gene probe and 1 ng of pTVK25 DNA for the T-DNA probe was used as a positive control for hybridization. Plant DNA was applied in duplicate to the nitrocellulose filter to be probed separately with the labeled 4.2 kb npt gene and the labeled 5.3 kb fragment of pTiBo542 T-DNA.
RESULTS

System for efficient regeneration of alfalfa

Petioles excised from alfalfa plants, maintained aseptically in tubes of BII medium, were used in regeneration experiments. To find the optimal ratio of auxin to cytokinin for regeneration of the cultivars used in this study, a matrix of kinetin vs 2,4-D was made (Table 2). SH nutrients (73) were used for the matrix medium base. Callus tissue was induced from petioles of the cultivar Saranac using MS medium then transferred to plates labeled B1-B25 containing the outlined ratios of kinetin and 2,4-D (1-100 μM). B24 was the only medium in the matrix that contained the proper ratio of hormones for regeneration of alfalfa. Callus on plates B2-B5, B7-B10, B13-B15, B20 and B25 became hardened and crystalline and tended to die when transferred to BII. Callus on the rest of the matrix plates either did not form embryos or formed abnormal embryos.

Schemes using different combinations of B24, SH and MS media to determine the most efficient method for regenerating alfalfa were used. It was shown that calli transferred to SH medium after induction on B24 formed greater numbers of normal embryos when transferred to BII than calli transferred directly from B24 to BII medium. Placing alfalfa petioles on B24 medium for 14 days, transfer of the induced callus tissue to SH medium for an additional 14 days, then transfer of callus tissue to BII medium proved most efficient and was used in this study to regenerate alfalfa. Normal alfalfa plants were obtained after embryos were separated from the callus tissue, formed roots and were transferred to tubes containing BII medium (Figure 7).
Table 2. Matrix for determination of optimal hormonal ratios for regeneration of alfalfa (*Medicago sativa*).

<table>
<thead>
<tr>
<th>2,4-D (µM)</th>
<th>100</th>
<th>50</th>
<th>10</th>
<th>5</th>
<th>1</th>
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<td>B2</td>
<td>B3</td>
<td>B4</td>
<td>B5</td>
</tr>
<tr>
<td>Kinetin</td>
<td>50</td>
<td>B6</td>
<td>B7</td>
<td>B8</td>
<td>B9</td>
</tr>
<tr>
<td>(µM)</td>
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<td>B11</td>
<td>B12</td>
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<td>5</td>
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<td>B17</td>
<td>B18</td>
<td>B19</td>
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<td>1</td>
<td>B21</td>
<td>B22</td>
<td>B23</td>
<td>B24</td>
<td>B25</td>
</tr>
</tbody>
</table>

Calli transferred to media denoted in bold italics became hardened and crystalline and tended to die when placed on BII media. As 2,4-D levels were increased callus became softer and whiter. Only calli on B24 went on to develop normal embryos and whole plants.
Figure 7. Regeneration of *Medicago sativa* using biphasic hormone induction. Top left: alfalfa petioles forming callus tissue on B24 media. Top right: callus tissue on SH media. This shows the characteristic appearance of alfalfa callus tissue that tends to regenerate well. Bottom left: embryo buds developing leaves on B11 media. Bottom right: a regenerated alfalfa plant.
Biphasic hormonal induction appeared to be most efficient for the regeneration of the commercial alfalfa cultivars tested: a lower ratio of 2,4-D to kinetin (5:1) initially followed by a higher ratio of 2,4-D to kinetin (10:1) secondly. Stocks were maintained indefinitely through continuous regeneration.

**Regeneration of commercial varieties of alfalfa**

Three cultivars of *Medicago sativa* L. were chosen for regeneration experiments; Anchor, Gladiator, and Saranac. It had been shown that the genotype of a specific alfalfa plant determined its ability to regenerate (12). Clonal selection for plants having the desired genotype was done by placing petioles from many individual plants through the outlined regeneration scheme. Plants showing good regeneration capability were chosen from each of the three cultivars. Plants that regenerated easily retained that ability for successive regenerations. Sterile explants placed on B24 medium produced soft, pale colored callus tissue after 6-9 days (Table 3) and up to four times the original explant tissue volume within 14 days. After transfer to SH medium calli became smooth with a rounded surface, sometimes developing pale green areas towards the end of the 14 day period. Embryos tended to form after the tissue was transferred to BII medium, between 30-45 days after callus induction on B24 medium. Whole plants 1-2 cm in height were obtained after 45-60 days. Stock cultures of Anchor, Gladiator, and
Table 3. Regeneration of control and transformed alfalfa explants.

<table>
<thead>
<tr>
<th>Alfalfa tissue</th>
<th>Callus tissue</th>
<th>Leaves or embryos</th>
<th>Whole plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed petioles on kanamycin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Untransformed callus on kanamycin</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Untransformed callus (-) kanamycin</td>
<td>6-9 days</td>
<td>30-45 days</td>
<td>45-60 days</td>
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<tr>
<td>Petioles inoculated with strain A348 pToK9, pGA472</td>
<td>*6-9 (90) days</td>
<td>210 days</td>
<td>0</td>
</tr>
</tbody>
</table>

*Petioles were inoculated then allowed to form callus tissue on B24 without kanamycin. Selection started two weeks later on SH media. The transformed calli started growing after 90 days.
Saranac with a good regenerating capability were maintained by continuing regeneration of explants.

**Cloned virulence genes from the Ti plasmid pTiBo542 enhance infectivity of A. tumefaciens**

The *A. tumefaciens* strains A348 and A281 were tested for their ability to form tumors on the *M. sativa* L. cultivars Anchor, Apollo, Gladiator, Saranac, and Vernal. Neither of these wide-host range strains were able to induce tumors on any of the five cultivars. Jin, et al. (52) showed that virulence of strain A348 could be enhanced by a plasmid containing the *virG* locus and the 3' end of the *virB* operon from the supervirulence Ti plasmid pTiBo542 of A281. It was also shown that increased virulence was correlated with increased expression of the virulence genes which was achieved by introducing the *virG* loci, or for maximum virulence *virG* plus the 3' end of *virB*. Three plasmids containing pTiBo542 virulence region clones (pToK9, pTVK178, and pTVK291) were obtained and mated into *A. tumefaciens* strains A348 and A281. These plasmid-containing strains were then tested on commercial cultivars Anchor, Apollo, Gladiator, Saranac, and Vernal. Each of the five cultivars were tested with one or more of strains A348 pToK9, A348 pTVK178, A348 pTVK291, A281 pTVK178, and A281 pTVK291. Two week old alfalfa seedlings grown aseptically in 150 ml test tubes were inoculated with a 26 gauge needle at the first leaf internode. Each of the five cultivars responded to infection by at least one of the three A348 strains with formation of a tumor within 45-70 days. Neither of the A281 strains with the extra *virG* copies were able to induce tumors on these
cultivars (Table 4 and Figure 8). The only virulence gene in common to all three virulence region plasmids was \textit{virG}. Plasmids pTVK79Ω9 and pTVK79Ω16 have Tn3 insertions within the \textit{virG} gene. Cultivars Saranac and Anchor were infected with strains A348 pTVK79Ω9 and A348 pTVK79Ω16 with no resulting tumor formation. It was apparent that extra copies of \textit{virG} were important for increased frequency of infection of these alfalfa cultivars.

**Virulence of constructed \textit{A. tumefaciens} strains on tobacco**

\textit{Nicotiana tobacum} was used as a positive control system to test \textit{A. tumefaciens} strains used in this study. Tobacco is a natural host for \textit{A. tumefaciens} and transforms easily when infected. Strains A281 and A348 induced tumors on tobacco leaf disks 18 days post infection, strains A348 pToK9 and A348 pTVK178 induced tumors began forming at 16-19 and 17 days, respectively (Table 5). The difference in rate of tumor formation was not significant for any of the strains tested on tobacco.

Tobacco leaf disks were successfully transformed with the \textit{npt} gene from strains A348 pGA472, A348 pToK9, pGA472, PC2760 pGA472, and PC2760 pToK9, pGA472. Since strain PC2760 is T-DNA-less, only the disarmed T-DNA on pGA472 was available for transfer into the plant genome.

Transformed tobacco leaf disks were placed directly on MS medium plus 200 \( \mu \text{l/ml kanamycin} \) and still produced callus tissue almost as soon as control leaf disks (16-23 vs 13-22 days). Transformed leaf disks initially
Table 4. Virulence of A. tumefaciens strains on commercial cultivars of alfalfa.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Strain tested</th>
<th>Formation of tumor</th>
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</thead>
<tbody>
<tr>
<td>Anchor</td>
<td>A348</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A281</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC2760</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A348 pToK9</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK178</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK291</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK79Ω9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK79Ω16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A281 pTVK291</td>
<td>-</td>
</tr>
<tr>
<td>Apollo</td>
<td>A348</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A291</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK291</td>
<td>+</td>
</tr>
<tr>
<td>Gladiator</td>
<td>A348</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A291</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK178</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A348 pToK9</td>
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<td></td>
<td>A281</td>
<td>-</td>
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<tr>
<td></td>
<td>PC2760</td>
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<td></td>
<td>A348 pToK9</td>
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<tr>
<td></td>
<td>A348 pTVK178</td>
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<td>A348 pTVK291</td>
<td>+</td>
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<tr>
<td></td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK79Ω16</td>
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</tr>
<tr>
<td></td>
<td>A281 pTVK291</td>
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</tr>
<tr>
<td>Vernal</td>
<td>A348</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A281</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK178</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A348 pTVK291</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 8. Crown gall on commercial cultivars of alfalfa. Two week old plants were inoculated at the first leaf internode with the *A. tumefaciens* strain indicated, using a 26 gauge needle.
Table 5. Tumor induction of tobacco using *A. tumefaciens*

<table>
<thead>
<tr>
<th>Strain used in tobacco</th>
<th>Days post inoculation until</th>
<th>Leaf disk inoculation</th>
<th>Tumor formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A281</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A348</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A348 pToK9</td>
<td>16-19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A348 pTVK178</td>
<td>17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
formed smaller amounts of calli than controls and tended to lag behind controls in leaf and whole plant formation (Table 6). Control leaf disks placed on MS medium containing 100 μg/ml kanamycin turned brown within 30 days without ever producing callus tissue. Control callus tissue transferred to medium containing 200 μg/ml kanamycin turned brown without any further growth after 16 days (Figure 9). Leaf disks prepared from transformed tobacco plants formed callus tissue, shoots, and whole plants on 200 μl/ml kanamycin at the same rate as control leaf disks on medium without kanamycin. Seeds from a tobacco plant transformed with A348 pToK9, pGA472 sprouted on BII medium with or without 200 μg/ml kanamycin after six days. Transformed sprouts developed normally on kanamycin.

An interesting result was obtained when strains A348 pGA472 and A348 pToK9, pGA472 were used during the same experiment to transform tobacco leaf disks from the same leaf. Callus began forming around transformed leaf disks of either strain after 16 days. However, calli transformed by A348 pToK9, pGA472 formed leaves and then whole plants 10 days sooner than calli transformed by A348 pGA472. Calli transformed by A348 pGA472 produced 4 kanamycin resistant plants after 60 days whereas calli transformed by A348 pToK9, pGA472 produced 13 kanamycin resistant plants during that period and twice as much callus material. Transformed tissues were maintained on 200 μg/ml kanamycin throughout the experimental period. Control leaf disks callused after 13 days, formed leaves within 29 days, and whole plants after 36 days.

Strains PC2760 pGA472 and PC2760 pTVK178, pGA472 were not used during the same experiment, however both produced transformed calli
<table>
<thead>
<tr>
<th>Tobacco Leaf disks</th>
<th>callus tissue</th>
<th>leaves</th>
<th>whole plants</th>
<th>Number of whole plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls on Km</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls for A348 pGA472 and A348 pKToK9, pGA472 infection</td>
<td>13</td>
<td>29</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>Other controls</td>
<td>13-22</td>
<td>23-50</td>
<td>36-60</td>
<td>not determined</td>
</tr>
</tbody>
</table>

**Inoculated with strain:**

- A348 pToK9, pGA472: 16 40 44 13 after 60 days
- A348 pGA472: 16 50 55 4 after 60 days
- †PC2760 (ΔT-DNA) pToK9, pGA472: 23 66 120 8 after 150 days
- †PC2760 (ΔT-DNA) pGA472: 23 135 0 3 after 220 days

†Strains PC2760 pToK9, pGA472 and PC2760 pGA472 were used in separate experiments from each other and separate from the other two strains listed.

*Allowed control callus to produce only 4 plants.
Figure 9. Kanamycin resistant (Km\textsuperscript{r}) phenotype conferred on tobacco transformed with the \textit{npt} gene from pGA472. Top left: untransformed callus tissue on kanamycin (L) contrasted with untransformed callus tissue from the same source forming shoots off kanamycin (R). Top right: strain A281 inoculated leaf disks not able to form tumorous tissue on kanamycin (L), but able to form tumor tissue off kanamycin (R). Bottom left: untransformed callus tissue on kanamycin (L) contrasted with \textit{npt} transformed callus tissue on kanamycin (R). Bottom right: \textit{npt} transformed tobacco callus forming shoots on kanamycin.
after 23 days. PC2760 pTVK178, pGA472 went on to produce leaves after 66 days, whole plants within 120 days, and after 150 days 8 kanamycin resistant plants. Strain PC2760 pGA472 transformed calli produced leaves by 135 days and three plants by 220 days.

Regeneration of tobacco

Tobacco callus tissue began forming 13-22 days after induction on MS medium. Shoots formed after transfer to BA medium within 23-50 days and tobacco plants (3-4 cm in height) were obtained within 36-60 days after callus induction (Table 6). Shoots could be induced by placing sterile leaf disks directly on BA medium. However, MS medium was used initially because it induced abundant amounts of callus tissue that ultimately could produce much larger numbers of shoots when later placed on BA medium.

Transformation of alfalfa using constructed A. tumefaciens strains

Medicago sativa plants showing good regeneration capability from the cultivars Anchor, Gladiator, and Saranac were used in transformation experiments. Petioles were infected with A. tumefaciens strains containing pGA472 and either pToK9, PTVK178 or pTVK291 for npt T-DNA transfer. Petioles were immersed in overnight cultures of A. tumefaciens and placed on B24 agar for 2-3 days. Some of the inoculated petioles were transferred to
B24 agar containing 500 µg/ml carbenicillin for bacterial elimination and callus induction without selection and some petioles were placed on B24 medium containing 500 µl/ml carbenicillin plus 100 µl/ml kanamycin for elimination of bacteria, callus induction, and immediate selection of transformed plant cells. After 2 weeks any resulting callus tissue was transferred to SH medium containing 100 µg/ml kanamycin. Surviving callus tissue was maintained on BII medium containing 100 µg/ml kanamycin. To obtain alfalfa tumor tissue, petioles were infected with A348 pToK9 for wild-type T-DNA transfer and placed on BII agar (no plant hormones). After 2-3 days petioles were transferred to BII plus 500 µg/ml carbenicillin. Callus tissue formed within 30 days and was assumed to be tumorous since no hormones were added to the medium.

Placement of alfalfa petioles directly on 100 µl/ml kanamycin after inoculation did not result in npt transformed callus. It was only when petioles were allowed to form callus tissue on B24 medium before selection with 100 µl/ml kanamycin that transformation occurred and then only when inoculated with strain A348 pToK9 pGA472.

Transfer into and expression of a foreign gene in the cultivar Gladiator

Once A. tumefaciens virulence was enhanced for alfalfa, then pGA472 could be used to transfer the npt gene into this plant genome. The npt gene was successfully transferred into the genome of the cultivar Gladiator using strain A348 pToK9, pGA472. The other strains containing
virulence region clones and pGA472 have not yet been successfully used to transfer the *npt* gene into alfalfa. During the infection stage of Gladiator there was visible growth of *A. tumefaciens* around the explants. After bacterial elimination, inoculated petioles on B24 minus kanamycin began forming healthy, pale-colored callus by about the seventh day along with controls. Petioles on B24 plus kanamycin turned brown, occasionally forming small amounts of white fuzzy callus material that was not able to grow on SH medium plus kanamycin. Calli induced on B24 minus kanamycin and transferred to SH medium containing 100 µg/ml kanamycin stopped showing growth and areas of the callus tissue began turning brown. Later transfers onto BII medium plus kanamycin furthered the browning reaction of the inoculated callus tissue. No new growth was apparent until 90 days post infection when three isolated spots of calli were detected growing among the browning callus tissue. This new tissue grew readily on BII medium plus kanamycin and had a crystalline texture with diffuse green areas. This was not the usual appearance of callus tissue that is able to regenerate but at day 210 dark green centers began to form. These centers developed groups of small leaves and embryo-like structures (Figure 10). Hormonal adjustments in the medium were not successful in inducing whole plant formation. Hormonal induction did, however, produce calli that were smooth and rounded like that which tends to regenerate. Control Gladiator petioles regenerated within the usual time frames for development. Infection of alfalfa explants with strains A3438 pTVK178, pGA472, A348 pTVK291, pGA472, PC2760 pToK9, pGA472, PC2760 pTVK178, pGA472, and PC2760 pTVK291, pGA472 have not yet resulted in transformed calli.
Slot Blot assay of transformed plant DNA

The \textit{npt} gene was detected in the genomes of \textit{npt} transformed alfalfa and \textit{npt} transformed tobacco using a Slot blot assay. T-DNA was detected in the genomes of alfalfa and tobacco tumors using the same assay (Table 7 and Figure 11). No significant hybridization was seen with the T-DNA probe and DNA from \textit{npt} transformed alfalfa or tobacco. Conversely, the \textit{npt} gene probe did not hybridize with DNA from T-DNA transformed alfalfa or tobacco tumors. Control genomic DNA from alfalfa and from tobacco hybridized with neither the \textit{npt} gene probe nor the T-DNA probe.
Figure 10. Alfalfa tissue transformed with the \textit{npt} gene from pGA472. Top left and right: leaves forming on \textit{npt} transformed callus tissue. Bottom: \textit{npt} transformed plantlet.
Table 7. Slot Blot Hybridization Assay.

<table>
<thead>
<tr>
<th>Plant DNA source</th>
<th>T-DNA probe hybridization</th>
<th>npt gene probe hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control alfalfa*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>npt transformed alfalfa</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T-DNA transformed alfalfa</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control tobacco*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>npt transformed tobacco</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T-DNA transformed tobacco</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Control DNA is from plants that have not been transformed, either by the npt gene nor with T-DNA.
Figure 11. Autoradiogram showing hybridization of plant DNA with [\( \alpha\)-\( ^{32}\)P] labeled probes. DNA was applied to a nitrocellulose filter using a Hybri-slot™ apparatus. DNA in lane A was probed with a 5.3 kb fragment of pTiBo542 T-DNA. DNA in lane B was probed with a 4.2 kb fragment containing the npt gene from pGA472. The concentration of all plant DNA added to the nitrocellulose filter was 1 \( \mu \)g. The control plasmid DNA concentration was 1 ng.
DISCUSSION

The first requirement for the development of Ti plasmid-mediated gene transfer systems for alfalfa requires that the cultivars of choice can be readily transformed. Genetic improvement of certain cultivars of alfalfa could have many commercial applications. Infectivity tests were done on a selection of commercial alfalfa cultivars released as early as 1953 to as late as 1976 using commonly used Agrobacterium strains. Multiple attempts to infect Medicago sativa cultivars Anchor, Apollo, Gladiator, Saranac, and Vernal with either Agrobacterium tumefaciens A348 pTiA6NC or Agrobacterium tumefaciens A281 carrying the supervirulent Ti plasmid pTiBo542 were totally unsuccessful. This was found in spite of expected success based on the findings of Hood et al. (43), which showed that strain A281 was virulent on M. sativa L. cv. RA-3, and the findings of Deak et al. (26), which showed that strain bo542 harboring pTiBo542 was able to transform M. varia, a naturally occurring hybrid of M. sativa and M. falcata. The work of Jin et al. (52) clearly indicated that extra copies of the virG locus of pTiBo542 supplied in trans increased the infectivity and host range of strain A348. A similar approach was pursued in this study in an attempt to extend the host range of some Agrobacterium strains to commercial cultivars of alfalfa. With the construction of strain A348 variants containing overlapping pTiBo542 virulence region clones in trans (plasmids pToK9, pTVK178, pTVK291) we were able to induce tumors on the cultivars Anchor, Apollo, Gladiator, Saranac and Vernal. The smallest virulence region placed in trans that still allowed transformation contained only virG and the 3' end of virB (pToK9). Enhanced virulence of A348 was further localized to the virG gene by
showing that Tn3 insertions in the virG locus abolished enhanced virulence conferred by the pTiBo542 virulence region clones. This suggests that the infection of alfalfa, much like that reported for other plant transformation systems, was dependent upon multiple copies of virG.

Enhanced infection of these cultivars, however, was not only dependent on extra copies of virG, for strain A281 containing the virulence region clones in trans was not able to induce tumors on any of the five cultivars tested. Strain A348’s ability to induce tumors on the cultivars tested and strain A281’s lack of ability may relate to a number of factors. One possibility for the avirulence of strain A281 on the cultivars tested may be that a hypersensitivity response occurs, causing cells at the site of inoculation to be killed due to the infection. Agrobacterium tumefaciens strains containing the wide host range plasmid pTiA6NC are avirulent on some cultivars of Vitis plants. Yanofsky et al. (95) showed that mutations in the virC loci of pTiA6NC prevented a hypersensitive response and allowed formation of tumors on the Vitis cultivars. The pTiA6NC virG gene product in A348 may enhance the expression of virC to a level that is too low for efficient transformation while the pTiBo542 virG gene product in A281 may enhance virC expression to a level that is too high for transformation of the tested cultivars of alfalfa. The copies of the pTiBo542 virG gene in trans in the A348 background may have provided the virG protein at an intermediate level that was conducive for transformation of these alfalfa cultivars. Alternatively, since the difference in virG expression appears to be the primary difference between A348 and A281 (52) the pTiBo542 virG may enhance transcription of the pTiA6NC virulence genes better than its own Ti plasmid virulence genes. It is possible that the pTiBo542 virG protein
interacted with the virA protein of A348 more efficiently than with the virA protein of A281 which could have lead to higher expression of the rest of the A348 virulence genes.

In addition to the successful infection of alfalfa cultivars with A. tumefaciens, two other factors had to be addressed; the development of a rapid regeneration system for alfalfa explants and the development of a vector system that incorporated the strain A348 with the additional copies of virG in trans.

A biphasic hormone induction was found to be optimal for regeneration of the alfalfa cultivars used in this work. Regeneration of commercial cultivars of alfalfa to the stage of rooting and multiple leaf formation can be accomplished within 45-60 days with B24 medium (developed for this study) followed by SH medium (73) and BII medium (13). A high ratio of 2,4-D to kinetin promotes optimal shoot formation in alfalfa and a lower ratio of 2,4-D to kinetin promotes root formation (89). The continued presence of 2,4-D may be antagonistic to organogenesis which could be why alfalfa embryos generally do not form until callus is removed from medium containing hormones (BII medium). The precise ratio of induction hormones in B24 medium (1.0 mg/liter to 0.2 mg/liter) may produce the general organization needed for subsequent embryogenesis. SH medium with higher 2,4-D concentration for the second phase of induction (11.05 mg/liter to 1.08 mg/liter) may provide additional hormonal stimulation to enhance embryogenesis after the initial organogenesis has taken place. The sequential treatment of alfalfa petioles on B24, SH, and BII media of the cultivars Anchor, Gladiator, and Saranac proved a reliable and efficient method for regeneration.
The vector systems used in strain A348 were constructed by bacterial matings and their ability to transform needed to be confirmed by transformation of a plant species that was known to be susceptible to Agrobacterium infection. Tobacco was chosen as a positive control for testing the disarmed binary vector strains because of its high susceptibility to infection by A. tumefaciens and ease of regeneration. Several of the constructed A. tumefaciens strains carrying plasmid pGA472 were able to transform tobacco cells with the npt gene showing the system to be effective. During the initial transformation stages, regeneration was slowed slightly during the selection for kanamycin resistant cells. Regeneration of npt transformed tobacco on kanamycin showed that once the transformed cells have been selected, later treatment with kanamycin did not interfere with the rate of regeneration. Seeds of transformed plants showed stable inheritance of the npt gene by their ability to sprout and develop normally on kanamycin.

The disarmed T-DNA vector pGA472 in an A. tumefaciens A348 or PC2760 (ΔT-DNA) background was sufficient to transfer a foreign gene into Nicotiana tobacum. The transferred gene was expressed and stably inherited in the progeny. Addition of plasmid pToK9 to strain A348 pGA472 increased virulence and resulted in formation of larger amounts of callus and higher numbers of transformed plants. The additional copies of virG probably enhanced virulence gene expression. This in turn, would result in the increased frequency of disarmed T-DNA transfer and lead to a greater number of npt transformed tobacco cells.

A tripartite plasmid system in strain A348 that includes pGA472, the virG containing plasmid pToK9 and the Ti plasmid pTiA6NC was sufficient to transform the cultivar Gladiator. However no alfalfa plants were regenerated
in this study. Altering the parameters of bacterial cell induction, plant inoculation, and transformed plant cell selection should lead to increased rates of transformation and to regeneration of transformed alfalfa plants. Any alfalfa plant that can be infected with an *A. tumefaciens* strain is a candidate for transformation using this system.

Hybridization of the *npt* gene to genomic DNA from transformed alfalfa and tobacco confirmed integration of the *npt* gene into the plant chromosome. No hybridization was seen between the DNA of *npt* transformed plants and a fragment of T-DNA. This showed that either the T-DNA from the Ti plasmid was not cointegrated with the pGA472 disarmed T-DNA or too few copies were present in the transformed genome for detection with these methods. Evidence against cointegration was the ability of alfalfa and tobacco *npt* transformed tissues to differentiate. DNA from tumorous tissue of both alfalfa and tobacco hybridized to a fragment of the pTiBo542 T-DNA, the alfalfa DNA showing less hybridization than tobacco DNA. It is possible the alfalfa tumor cells containing T-DNA may have been overgrown by non-T-DNA containing cells during the many subsequent transfers on tissue culture since no selection was made for tumorous cells. The tobacco tumor cultures had been passed on tissue culture for a much shorter period of time relative to the alfalfa cultures. A second alternative could involve the T-DNA fragment used to probe the transformed plant DNAs. Alfalfa was transformed with strain A348 harboring octapine Ti plasmid pTiA6NC while tobacco was transformed with strain 281 harboring L,L succinamopine Ti plasmid pTiBo542. The T-DNA probe was taken from the TC region of pTiBo542 which may not be very homologous with pTiA6NC TC DNA. Also the TC region of pTiA6NC may not have transferred and integrated into the
alfalfa genome as frequently as the pTiBo542 T\(C\) region did in tobacco.

The method for transformation of alfalfa outlined in this study is quick, straight-forward and provides a convenient method for selection of transformed cells. It is feasible that immature transformed alfalfa plants could be obtained as early as 60 days from the time of inoculation with \(A.\) \textit{tumefaciens}. This method has an advantage over the binary vector system used in \(A.\) \textit{rhizogenes} in that the root-inducing step prior to regeneration is avoided and little or no wild-type T-DNA is present in the regenerated transformed plant. This way the plant is as close to its natural state as possible except for the presence of the gene of choice and its T-DNA border repeats.

In this work it is demonstrated that \(A.\) \textit{tumefaciens} with enhanced virulence and the disarmed T-DNA vector pGA472 can transform \textit{Medicago sativa} with a foreign gene. The gene coding for neomycin phosphotransferase shows stable chromosomal integration and is expressed in the plant cell. Strain A348 harboring pGA472 and a plasmid containing a virulence region clone from the supervirulent Ti plasmid pTiBo542 was able to transform the alfalfa cultivar Gladiator. The evidence for the genetic incorporation of the \textit{npt} gene was the expression of neomycin resistance shown by callus growth on kanamycin and the ability of the \textit{npt} gene of pGA472 to hybridize with transformed alfalfa DNA.
REFERENCES


