



Transgenic alfalfa : development and characterization  
by Nancy KayBlake

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in  
Agronomy  
Montana State University  
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**Abstract:**

Alfalfa (*Medicago sativa* L.) is the world's premier forage crop because of its nutritional quality, performance and broad adaptation. Several problems exist in the production of alfalfa, including disease and insect damage, herbicide sensitivity and limited nutritional quality.

Plant transformation (the introduction of foreign genes into a species) has been proposed as a potential solution to each of these problems. One consideration in alfalfa transformation research which has not been adequately addressed is the inheritance and expression stability of foreign genes in transgenic alfalfa. This study is the initial phase of a project to examine this question.

Transgenic alfalfa plants were generated and characterized prior to the initiation of crossing studies.

In particular, the usefulness of polymerase chain reaction sequence amplification (PCR) in characterizing transgenic alfalfa was examined.

Three Montana-adapted alfalfa cultivars were transformed using *Agrobacterium*-mediated transformation.

The transformation vector used contained two linked genes: the NPT II gene conferring kanamycin resistance and the GUS gene, a commonly used expression reporter gene. Contrary to expectations, 22% of the transgenic alfalfa plants failed to integrate the GUS gene into their genomes although they did integrate the NPT II gene. Further, 29% of the transgenic alfalfa plants which contained GUS gene failed to express it. This data was based on Southern blot analysis but similar results were obtained using the more rapid PCR method.

The GUS reporter gene system functions in transgenic alfalfa and provides a rapid way to score progeny. PCR also promises to be useful in the continuing inheritance studies of these transgenic alfalfa.

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A thesis submitted in partial fulfillment  
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Master of Science

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MONTANA STATE UNIVERSITY  
Bozeman, Montana

August 1991

N378  
B5816

APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ACKNOWLEDGMENTS

I wish to thank Drs. Ray Ditterline and Rich Stout for their guidance and encouragement throughout the course of my graduate study. Thanks are extended to Pioneer Hi-Bred International, Inc. for their generous financial support for this research.

Special thanks and acknowledgment go to my husband, Tom, and son, Robin. Without their patience and support many meals would have gone unprepared and the house unvacuumed.

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## ABSTRACT

Alfalfa (*Medicago sativa* L.) is the world's premier forage crop because of its nutritional quality, performance and broad adaptation. Several problems exist in the production of alfalfa, including disease and insect damage, herbicide sensitivity and limited nutritional quality. Plant transformation (the introduction of foreign genes into a species) has been proposed as a potential solution to each of these problems. One consideration in alfalfa transformation research which has not been adequately addressed is the inheritance and expression stability of foreign genes in transgenic alfalfa. This study is the initial phase of a project to examine this question.

Transgenic alfalfa plants were generated and characterized prior to the initiation of crossing studies. In particular, the usefulness of polymerase chain reaction sequence amplification (PCR) in characterizing transgenic alfalfa was examined.

Three Montana-adapted alfalfa cultivars were transformed using *Agrobacterium*-mediated transformation. The transformation vector used contained two linked genes: the NPT II gene conferring kanamycin resistance and the GUS gene, a commonly used expression reporter gene. Contrary to expectations, 22% of the transgenic alfalfa plants failed to integrate the GUS gene into their genomes although they did integrate the NPT II gene. Further, 29% of the transgenic alfalfa plants which contained GUS gene failed to express it. This data was based on Southern blot analysis but similar results were obtained using the more rapid PCR method.

The GUS reporter gene system functions in transgenic alfalfa and provides a rapid way to score progeny. PCR also promises to be useful in the continuing inheritance studies of these transgenic alfalfa.

## INTRODUCTION

Alfalfa, *Medicago sativa* L., is the premier forage crop in the world because of its wide adaptation, high yield and superior nutritional quality. It is grown on approximately 32 million ha in the world, about 12 million ha of which are in North America (Hill 1987). Like any major crop, however, there are some problems associated with alfalfa cultivation and utilization. Among these are disease, insect damage, herbicide sensitivity, and undesirable nutritional factors. Many problems are being addressed by classical breeding methods. However, attention has recently been focused on plant transformation as a means of moving foreign genes into alfalfa, which would otherwise not be possible using traditional methods. These include genes for disease resistance, herbicide resistance, condensed tannin production, and insect resistance.

An essential component of a successful plant transformation project using *Agrobacterium tumefaciens* is the ability to grow the plant in culture and regenerate whole plants. Alfalfa tissue culture, including regeneration, has been possible for more than a decade. Alfalfa genotypes with high regenerability were selected and have been used extensively. However, these genotypes are

poor agronomically and would be undesirable in a breeding project. It is desirable to be able to transform and regenerate alfalfa which is already agronomically adapted.

A second consideration in a long-term transformation project is identifying and characterizing transformed plants as well as determining the inheritance and stability of the introduced genes through several generations. While assays for gene expression are useful for this purpose, it is also necessary to have a method for screening for the specific gene in the plant genome. Southern blots have permitted the accurate identification of plants containing introduced genes for over a decade (Thomashow et al. 1980). Polymerase chain reaction (PCR) sequence amplification (Sakai et al. 1988) provides an alternative technique which is more rapid and possibly more efficient at determining which plants contain accurately integrated target genes. This study was undertaken to answer two questions. First, is it possible to create transgenic plants from alfalfa cultivars agronomically adapted to Montana? Second, can PCR be used as a replacement for Southern blot analysis to evaluate transgenic alfalfa and its progeny?

## LITERATURE REVIEW

Alfalfa Genetics

Cultivated alfalfa is an autotetraploid,  $2n = 4x = 32$ , with tetrasomic inheritance (Stanford 1951). The alfalfa  $1n = 1x$  haploid genome is  $1.0 \times 10^9$  base pairs (bp), with 22% highly repetitive, 42% mid repetitive, and 36% single copy sequences (Winicov et al. 1988). Compared to other plant species, e.g., *Arabidopsis thaliana* L. ( $7 \times 10^7$  bp) and *Vicia faba* L. ( $4.4 \times 10^{10}$  bp), this is an average genome size (Blake and Kleinhofs 1988).

Alfalfa is a naturally cross-pollinating crop. Alfalfa plants exhibit self-incompatibility to a greater or lesser degree, making production of selfed seed difficult. Alfalfa can usually be clonally propagated by stem cuttings, allowing the production of identical plants for genetic studies.

Alfalfa cultivars are heterogeneous populations, because of alfalfa's cross-pollinating nature. As with other cross pollinated crops, alfalfa exhibits inbreeding depression, resulting in yield and vigor loss. Heterosis, the opposite of inbreeding depression, is the aim in the creation of new alfalfa cultivars. Heterosis is the

postulated consequence of maximum heterozygosity in a polyploid species (Bingham 1980). To approximate the state of maximum heterozygosity, the breeder must create the population from unrelated parents. The greater the number of unrelated parents within the initial population, the greater the heterozygosity (Hill 1987). Another pertinent characteristic of alfalfa genetics is its tetrasomic inheritance. Consequences of tetrasomic inheritance include a slower change in gene frequency under selection, the masking of recessive alleles and difficulty in obtaining populations uniform for dominant alleles.

Heterosis and tetrasomic inheritance pose a problem for the successful introduction of a novel gene into a new alfalfa cultivar. It is not possible to modify a single plant and create a cultivar from it alone as in self-pollinated species. In alfalfa it is most desirable to start with several unrelated parents each with the novel gene as the initial population in creating a new cultivar.

#### Alfalfa Tissue Culture

Alfalfa tissue culture work began in the 1970's with Saunders and Bingham (1972) developing the first system to take alfalfa from callus through regeneration into a whole plant. Through selection, Bingham et al. (1975) developed an alfalfa germplasm, Regen S, which had enhanced capability for regeneration. Other researchers, (Walker et al. 1978,

1979, Walker and Sato 1981, Stuart and Strickland 1984ab) have refined the media and conditions for regeneration and expanded the number of alfalfa germplasms which have been successfully regenerated. Alfalfa has been regenerated from callus derived from immature anthers, immature ovaries, hypocotyl, internodes, stem, petiole, sepal, petal and leaf (Bingham et al. 1988). Alfalfa protoplasts have also been regenerated into whole plants (Kao and Michayluk 1980, Johnson et al. 1981).

Based on this accumulated research, a consensus protocol for alfalfa culture and regeneration has developed. Callus formation is promoted by auxin-cytokinin media. Somatic embryogenesis induction is triggered by a short (3-4 day) pulse of high concentration 2,4-D (2,4-dichlorophenoxyacetic acid), low concentration kinetin exposure, followed by growth on media with high reduced nitrogen, proline and no hormones (McCoy and Walker 1984, Stuart and Strickland 1984ab). After shoot formation, growth on basal media promotes root formation. Regeneration is by somatic embryogenesis not organogenesis (Stuart et al. 1985). Variations on this protocol abound and usually reflect the use of different alfalfa cultivars.

Studies on the inheritance of regeneration capability have found it to be controlled by two dominant genes in diploid alfalfa (Reisch and Bingham 1980). Wan et al. (1988) also found two dominant genes in tetraploid alfalfa.

Allelism tests have not been done to determine if the genes controlling regeneration are the same in diploid and tetraploid alfalfa.

Variation for regenerability in alfalfa has been observed and falls into three categories. The first is variation among genotypes within a cultivar. In a review article, Bingham et al. (1988) stated that approximately 10% of the genotypes within a cultivar have regeneration capability. The second type is variation among cultivars. Brown and Atassnov (1985) tested 76 alfalfa cultivars, representing the nine alfalfa germplasm sources, for embryogenesis and regenerability. They found significant differences among cultivars and that the best regenerating cultivars had a genetic background which included 'Ladak' and *Medicago falcata* L., a close diploid relative of *M. sativa*. The third category of variation is the interaction between genotype and culture media. Seitz Kris and Bingham (1988) reported statistically significant interaction among genotypes of two unrelated alfalfa cultivars and culture media in regeneration ability. Chen et al. (1987) tested three alfalfa cultivars with six media regimens. They found genotype x medium interaction both for somatic embryogenesis and regeneration.

#### Agrobacterium tumefaciens

*Agrobacterium tumefaciens* is a gram negative bacteria,

which is pathogenic on many dicotyledonous plant species. It causes crown gall disease characterized by the formation of a tumorous mass at the infection site. Infection is through wounds in plant tissue, generally the stem.

The molecular mechanism of *Agrobacterium tumefaciens* infection of plants has been extensively studied. *Agrobacterium tumefaciens* contains a 200 kilobases (kb) plasmid called the Ti plasmid. This plasmid has a region of DNA termed the T-DNA. T-DNA is delineated by 25 base pair (bp) direct repeat sequences on the left and right borders which are required for T-DNA transfer. T-DNA is transferred from the bacteria to the plant, where it is incorporated into the plant cell's nuclear genome. Once incorporated, genes in the T-DNA are expressed by the plant. These genes are involved in the synthesis of phytohormones, the auxin, indoleacetic acid and the cytokinin, isopentyl adenosine (Klee et al. 1987), which lead to tumor formation in infected plant tissue.

Transfer of the T-DNA to the plant cell is controlled by six virulence (*vir*) genes encompassing 35 kb of the Ti plasmid but not within the T-DNA borders. The *vir* genes are inducible by bacterial exposure to plant cell exudates, hence the requirement for wounded plant tissue. The induction process takes 8-16 hours (Stachel et al. 1985). The *vir* gene proteins act in *trans* to catalyze T-DNA movement. Protein products of *virD* nick the T-DNA borders.

The nicked bottom strand unwinds, giving rise to a linear single strand T-strand. Repair and polymerase enzymes replace the bottom T-DNA strand. The T-strand is postulated to be the intermediate transfer molecule, with transfer into the plant cell being similar to bacterial conjugation (Zambryski et al. 1988).

Once the T-DNA is transferred into the plant cell, integration into the nuclear genome must occur. Very little is known about this process. T-DNA integration into the genome appears to be at random, except the sites are enriched for adenine and thymine. Multiple T-DNA copies are postulated to occur after T-DNA transfer, as there is no evidence of multiple transferable T-DNA's in *Agrobacterium* (Zambryski et al. 1988). Multiple integration can occur either in tandem array or unlinked at different chromosomal locations. The tandem arrays can be direct or indirect repeats but are usually expressed as a single, dominant Mendelian trait (Jorgenson et al. 1987). Multiple insertions into different chromosomal locations can lead to distinct genes which segregate in the  $F_1$  generation. There is no apparent correlation between the copy number and expression level. Rather, variation in expression is affected by chromosomal location, termed position effect (Jones et al. 1987).

Integration of T-DNA into the plant genome is an efficient process when compared to other DNA insertion

methods into eukaryotic genomes (Gheysen et al. 1987). Despite the efficiency of T-DNA integration, deletions and rearrangements may occur, particularly near the border sequences (Jones et al. 1987, Spielmann and Simpson 1986). One detailed study (Deroles and Gardner 1988b) showed the loss of one or the other chimeric gene, where two genes were present in the original T-DNA. Foreign genes can undergo rearrangements, including truncation, deletions, or tandemization, leading to loss of function (Weising et al. 1988). These rearrangements appear to occur during the integration process since after integration, foreign gene stability is generally maintained through meiosis (Deroles and Gardner 1988a).

#### Agrobacterium-mediated Plant Transformation

*Agrobacterium tumefaciens'* ability to transfer DNA into plant cells and the subsequent incorporation of that DNA into the plant genome makes it a near ideal candidate as a plant transformation vector. Researchers began to experiment with *Agrobacterium* strains, in 1980, to incorporate foreign genes into plant cells (Hernalsteens et al. 1980). The initial problem with *Agrobacterium* vectors was that phytohormone synthesis genes, which cause tumor formation, were incorporated along with the desired foreign DNA. This resulted in plant tissue which grew as callus but failed to regenerate into whole plants. The genes necessary

to transfer the T-DNA were located on the Ti plasmid but not within the T-DNA borders. Bevan et al. (1983) demonstrated that any DNA within the T-DNA borders can be transferred and that the phytohormone synthesis genes are not necessary. This led to the creation of 'disarmed' *Agrobacterium* strains. The hormone genes of the Ti plasmid are removed by homologous recombination leaving an *Agrobacterium* incapable of causing tumor formation. Often the entire T-DNA is removed from *Agrobacterium* strains for use with binary vectors (Ooms et al. 1981).

In 1983, Hoekema et al. proposed the use of a binary vector system and Bevan published the first report of a binary *Agrobacterium* vector in 1984. This vector separates the virulence region and the T-DNA 25 bp borders onto two plasmids. The *vir* region, because of its size, is left on the Ti plasmid (of a disarmed strain) and the T-DNA borders are placed on a smaller, autonomously replicating plasmid, which is easier to manipulate. This vector plasmid contains the origin of replication of a broad host-range plasmid allowing autonomous replication in *Agrobacterium*. In addition to the 25 bp border repeats necessary for T-DNA transfer, there is an enhancer element, 'overdrive', which stimulates transfer (Peralta et al. 1986). Many binary vectors now incorporate the overdrive enhancer as well as the T-DNA borders, though the older vectors function adequately without it.

Within the binary vector's T-DNA borders there is a selectable marker gene as well as the other genes being transferred into the plant. The selectable marker gene (e.g., antibiotic resistance gene) is used in plant transformation to select potentially transformed calli from untransformed calli. Plant material without resistance is killed or growth is arrested in the presence of the antibiotic. Selectable genes used in plant transformation studies include: neomycin phosphotransferase type II (NPT II) conferring kanamycin and G418 resistance; another bacterial phosphotransferase conferring hygromycin resistance, the mutant dihydrofolate reductase enzyme conferring methotrexate resistance; and herbicide resistance genes for chlorsulfuron, biapholos, phosphinothricin and glyphosate (Klee et al. 1987, Weising et al. 1988).

These genes are placed into the T-DNA by triparental mating (Ditta et al. 1980). The genes to be transferred are placed on a *Escherichia coli* plasmid and maintained in an *E. coli* strain. It can then be moved into the disarmed *Agrobacterium* strain with the help of a second *E. coli* strain containing a RK2 plasmid which facilitates wide matings among bacteria. Using appropriate antibiotic selection media, the *Agrobacterium* cells which contain the new plasmid can be preferentially selected. This new strain can be used as the vector in plant transformation studies.

Cocultivation of *Agrobacterium* with regenerating protoplasts was the original method of *Agrobacterium* transformation (Marton et al. 1979). However, many plant species are not easily regenerable from protoplasts. For these species, the leaf disc or explant method is preferred (Horsch et al. 1985). Sterile explants are cocultured for 2-3 days with *Agrobacterium*. Usually a nurse cell culture is included to increase induction of the virulence genes. *Agrobacterium tumefaciens* has been used to transform many plant species, including tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill), *Arapidopsis thaliana*, and *Brassica napus* L. (Klee et al. 1987). *Agrobacterium* tumorigenesis in *Medicago sativa* was first performed by Mariotti et al. (1984), demonstrating that T-DNA could be transferred and expressed in alfalfa. *Agrobacterium*-mediated transformation of alfalfa and its close relatives has previously been reported (Schreier et al. 1985, Deak et al. 1986, Shanin et al. 1986, Chabaud et al. 1988, Halk et al. 1989, D'Halluin et al. 1990, McCoy pers. comm.) with the introduction of kanamycin resistance, alfalfa mosaic virus resistance, hygromycin resistance, herbicide resistance, and proteinase inhibitor genes.

#### $\beta$ -Glucuronidase Reporter Gene System

A reporter gene, also termed a scorable gene, allows identification of those plants which have incorporated the

foreign gene into their genome and are expressing the gene. This should be distinguished from a selectable marker gene, although it is possible for the selectable gene to also function as the reporter gene.

Reporter genes used in plant transformation have included octopine synthase and nopaline synthase, encoded on the T-DNA, chloramphenicol acetyltransferase, neomycin phosphotransferase type II, and both bacterial and firefly luciferase (Weising et al. 1988). One of the more recent reporter genes is the  $\beta$ -glucuronidase (*uidA*) gene of *E. coli*. Jefferson et al. (1986) cloned *uidA* from *E. coli* and fused it to plant promoter sequences which allow for its expression in plants.

$\beta$ -glucuronidase is an acid hydrolase enzyme which cleaves  $\beta$ -glucuronides at the  $\beta$ -linkage between glucuronic acid and the R group. When a glucuronide substrate with an indoxyl group is cleaved, the indoxyl dimerizes to indigo which is insoluble in aqueous solution. Tissue expressing glucuronidase activity is marked by blue staining in the cells. Jefferson et al. (1987) assayed tissues of several plant species: wheat (*Triticum aestivum* L.), tobacco, tomato, potato (*Solanum tuberosum* L.), *Brassica napus*, and *Arabidopsis thaliana*. They found no detectable endogenous  $\beta$ -glucuronidase activity in any of these species.

$\beta$ -glucuronidase (GUS) has been transformed into several plant species including tobacco, tomato, potato, and rice

(*Oryza sativa* L.), with concomitant expression (Jefferson et al. 1987, Plegt and Bino 1989, Terada and Shimamoto 1990). It was originally thought that, fused to the CaMV35S promoter,  $\beta$ -glucuronidase would be constitutively expressed. However further studies have shown the CaMV35S is not a constitutive promoter in plants and GUS expression is found in specific locations, such as the phloem tissue around the vascular ring, parenchyma cells in the cortex and trichomes on the epidermis (Jefferson et al. 1987, Williamson et al. 1989). Plegt and Bino (1989) found endogenous GUS activity in the mature pollen of untransformed plants with binucleate pollen. Conversely, plants with trinucleate pollen exhibit no endogenous GUS activity. This renders the GUS assay of pollen from transformed plants useless for species with binucleate pollen, such as alfalfa.

The GUS assays are easy and versatile. Several substrates may be used, allowing histochemical, spectrometric and fluorometric assays. The histochemical assay is useful both as a qualitative assay and to localize expression. It works well for screening many plants or calli, requiring only a small tissue sample and leaving most of the plant or callus intact. Fluorometric assays are very sensitive and allow for expression quantification (Jefferson et al. 1987).

### Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplifies distinct DNA sequences. As originally described (Saiki et al. 1985, Mullis and Faloona 1987), it used the *E. coli* DNA polymerase I - Klenow fragment. This enzyme denatures at the high temperatures necessary for the reaction. Fresh enzyme had to be added throughout the 4-5 hour reaction and limited the utility of PCR. Using the DNA polymerase from *Thermus aquaticus*, *Taq* polymerase, (Saiki et al. 1988) has made PCR a practical and extremely useful technique for molecular biology. The temperature optimum for DNA synthesis using *Taq* polymerase is 75-80°C, and the enzyme can repeatedly withstand temperatures of 94-95°C without denaturing (Gelfand 1989). These enzyme properties allow repeated cycles of DNA denaturation, annealing and replication, leading to the amplification of specific DNA sequences. Starting with nanogram amounts of genomic DNA, a single copy gene can be amplified sufficiently to be visualized on an agarose gel.

A pair of oligonucleotide primers complementary to the right and left border are needed to target a specific DNA sequence. Genomic sequences of up to 3 kb have been successfully amplified (Saiki 1990). DNA is first denatured at 94-95°C, then cooled to 40-60°C to allow the primers to anneal to the target sequence, and finally heated to 70-75°C for the *Taq* polymerase to synthesize copies of the target.

sequence. This process is repeated 30-40 times, leading to a geometric increase in the copies of the target sequence.

A variation on the standard PCR protocol, simultaneous or multiplex amplification, has been used in a variety of applications. Primer sets specific for two or more fragments of distinguishable sizes are used in one reaction mixture. Up to nine primer sets, amplifying nine distinct fragments have been used in a single PCR (Chamberlain et al. 1988). Lassner et al. (1989) proposed simultaneous PCR as a means of rapid screening of transgenic plants.

## EXPERIMENTAL PROCEDURES

### Plant Material

Alfalfa cultivars 'Spredor II', 'Ladak 65' and 'Rangelander' and a Pale leafed germplasm developed from Ladak 65 are well adapted to Montana and were used for transformation studies. Transformation experiments were performed on identified, individual plants within each cultivar. Parents in each cultivar were normal, unregenerated plants with the exception of two untransformed regenerated Pale plants.

### Plant Transformation

The *Agrobacterium* binary vector, pBI121, containing the CaMV35S-GUS fusion gene linked to the Nos-NPT II gene (Jefferson et al. 1987; obtained from Clontech, Inc.) was the transformation vector. It was mobilized into the disarmed *Agrobacterium tumefaciens* strain LBA4404 (Ooms et al. 1981) by a triparental mating between LBA4404, the *E. coli* strain HB101 with pBI121 and HB101 with the helper plasmid RK2013 (Bevan 1984, Ditta et al. 1980). Alfalfa transformation followed the explant method of Horsch et al. (1985), using young stem sections as explants. Explants

were sterilized in 10% bleach (5.25% sodium hypochlorite), 0.1% SDS (sodium dodecylsulfate) for 15 minutes with stirring, rinsed three times with sterile water for 30 seconds with stirring, rinsed once with 95% ethanol for 10 seconds and then rinsed twice with sterile water. Stem sections were then cut into 2-3 mm lengths and inoculated for five minutes with LBA4404/pBI121 grown in TY media (White and Greenwood 1987) supplemented with 25 mg/L streptomycin and 50 mg/L kanamycin and diluted to  $5 \times 10^7$  cells/ml. Explants were cocultivated for two days on MS media (Sigma M 5524 MS Basal Salt Mixture and N 0390 Nitsch and Nitsch vitamin mixture) supplemented with  $9 \mu\text{M}$  2,4-D and  $1.1 \mu\text{M}$  benzyl-amino purine (BAP) with the surface of the media covered with sterile filter paper. Selection of transformed callus was on the same media supplemented with 50 mg/L kanamycin and 300 mg/L carbenicillin. The kanamycin selection killed all untransformed callus within eight weeks.

Whole plant regeneration from selected calli followed the protocol of Redenbaugh et al. (1986). Callus was placed on SH media (Sigma S 6765 Basal Salt Mixture and N 0390 Nitsch and Nitsch vitamin mixture) supplemented with  $50 \mu\text{M}$  2,4-D and  $5 \mu\text{M}$  kinetin for a 3-4 day induction period. It was transferred to hormone-free SH media supplemented with 25 mM ammonium nitrate and 50 mM proline for embryogenesis. Embryos usually developed in 4-6 weeks. Embryos were

converted to whole plants on half-strength SH media supplemented with 25  $\mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ) and 0.25  $\mu\text{M}$  NAA ( $\alpha$ -naphthalene-acetic acid). Kanamycin was not included in the regeneration media as it retarded regeneration. Whole plants were regenerated 4-6 months after the initial inoculation with *Agrobacterium*.

#### DNA Extraction

Tissue was collected from mature plants which had been cut back and allowed to regrow at least once prior to collection. DNA was extracted from plants as described by Lassner et al. (1989). Generally a minimum of 50 mg leaf tissue was ground with extraction buffer heated to 65°C and 2 g sand in a mortar and pestle. For PCR analysis of F1 progeny, DNA was extracted from a single trifoliolate leaf. The sample was ground in a microfuge tube, using a glass rod, with heated extraction buffer and 100 mg sand and processed the same as for larger extractions.

#### Polymerase Chain Reaction

Amplification by PCR was performed according to manufacturer's instructions using Perkin Elmer/Cetus (Norwalk, CT) AmpliTaq Recombinant *Taq* DNA polymerase. The NPT II primers GAACAAGATGGATTGCACGC and GAAGAACTCGTCAAGAAGGC border a 785 bp DNA fragment from the NPT II gene (nucleotides 157 to 942 according to Beck et al. 1982). The

GUS primers ACGTCCTGTAGAAACCCCAA and CCCGCTTCGAAACCAATGCC border a 1097 bp DNA fragment from the GUS gene (nucleotides 23 to 1120 according to Jefferson et al. 1986). Reactions were performed using 10-20 ng plant DNA or 0.01 ng pBI121 DNA in 25  $\mu$ l volumes. DNA concentration was determined by ethidium bromide fluorescence in an agarose gel. Samples were heated to 94°C 10 min, followed by 30 cycles of 94°C 1 min, 50°C 1 min, 72°C 3 min, with a final extension step of 72°C 7 min in a Coy tempcycler (Model #50, Coy Laboratory Products, Inc., Ann Arbor, MI). Simultaneous amplification using NPT II and GUS primers was performed as described by Lassner et al. (1989).

#### Southern Blot Analysis

Southern blot analysis was of two types: genomic blots and PCR blots. For genomic blots, five micrograms genomic DNA were digested with restriction enzymes according to manufacturer's directions, electrophoresed on 0.8% agarose gel, and blotted to Zetaprobe nylon membrane (BioRad) using the alkaline transfer method of Reed and Mann (1985). The membranes were hybridized with probes radioactively labeled using BRL Random Primers DNA Labeling System according to manufacturer's directions. Radioactive probes were purified using the BioRad Prep-a-Gene Kit according to manufacturer's directions. Hybridization followed the protocol of Sambrook et al. (1990) at 65°C in a Robbins Scientific

hybridization incubator. DNA double digested with *SstI/SphI*, was probed with pBI121 linearized with *SstI* to verify incorporation of the T-DNA. DNA digested with *EcoRI* or *Hind III* was probed with the 3 kb *SstI/SphI* GUS fragment and the 1.7 kb *SphI* NPT II fragment of pBI121, corresponding to the CaMV35S-GUS gene and a portion of the NPT II gene respectively, for copy number determination and verification of insertion of both genes.

Blots of PCR gels were made from amplified samples electrophoresed on a 1.4% agarose gel and blotted to Zetaprobe. The 3 kb *SstI/SphI* fragment and 1.7 kb *SphI* fragment, described above, were used as probes. Fragments were labeled and purified as described above. Each PCR blot was probed first with the 3 kb *SstI/SphI* fragment and then with the 1.7 kb *SphI* fragment, with the probe being removed from the filter after the first hybridization as described by Sambrook et al. (1989).

#### GUS Histological Assay

$\beta$ -glucuronidase expression was qualitatively assayed using the procedure of Jefferson et al. (1987). Hand sectioned tissue was stained with 2 mM X-gluc (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide) in 0.1 M NaPO<sub>4</sub> buffer pH 7, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.01 M NaEDTA overnight at 37°C. Tissue sections were then placed in 95% ethanol to remove plant

pigments which interfere with seeing the stain. Pollen was stained according to Plegt and Bino (1989) with 1 mM X-gluc in 50 mM NaPO<sub>4</sub> buffer pH 7, 10% sucrose, 50 mg/L boric acid overnight at room temperature.

#### NPT II Expression

Expression of the NPT II gene, measured as kanamycin resistance, in regenerated putative transgenic plants and their progeny was determined as described by De Block et al. (1989). Sterile explants (usually 5-10 leaf and stem pieces) from an individual plant were placed on the callusing media described previously and supplemented with 50 mg/L kanamycin. The explants were grown for four weeks under previously described conditions. Callus from each explant was subdivided and transferred to fresh media for an additional 2-3 weeks. Each callus was then scored as either: green, actively growing or brown, non-growing. The green, growing callus indicated the expression of kanamycin resistance.

#### Pollen Germination

Male fertility of transformed plants and progeny was determined by germinating fresh pollen in 10% sucrose, 50 mg/L boric acid for three hours and then staining with acetocarmine. Pollen was scored as sterile (nongerminating) if pollen tube length was less than the diameter of the

pollen grain. Germination percentage was compared to pollen from untransformed plants.

#### Chromosome Counts

Mitotic chromosome counts on transgenic plants were performed as described by Blake and Bingham (1986).

#### Crosses

Transgenic plants were used as female parents in crosses to wild type alfalfa and as male parents in crosses to male sterile alfalfa. Hand crosses were made under greenhouse conditions. Chi-square test was used to test genetic ratios.

## RESULTS AND DISCUSSION

Plant Regeneration

Genotypes within Ladak-65, Spredor II, Rangelandier and Pale were inoculated with the *Agrobacterium* binary vector pBI121. Approximately 20% of the inoculated genotypes were regenerated into putative transgenic plants (Table 1). This surpasses the published average regeneration capability (Bingham et al. 1988). However, creeping rooted cultivars, including Ladak, Spredor II, and Rangelandier, have been identified as having greater than average regeneration capability (Bingham et al. 1988). Ladak and Rangelandier have been regenerated previously, but this is the first report of regeneration of Spredor II.

Table 1. Genotypes regenerated within alfalfa cultivars after *Agrobacterium*-mediated transformation.

Cultivar	# Genotypes Inoculated	# Genotypes Regenerated	% Regeneration
Ladak 65	67	9	13.4
Spredor II	30	6	20
Rangelandier	26	8	30.8

Data from Pale is not shown (Table 1) as it was treated in a different manner. Two Pale genotypes were previously

regenerated from callus tissue culture without *Agrobacterium* inoculation. Since these had proven regeneration capability, they were the only two Pale genotypes used in the transformation experiments.

The regenerated plants were designated as putative transgenic alfalfa. A total of 181 putative transgenic alfalfa were regenerated from all genotypes. Eighty-seven plants survived for further testing. It was assumed, because of the regeneration method employed, that many of these 87 plants were "clones" of each other and not independently derived transformation events. The remainder of the study entailed the verification and characterization of these plants.

#### GUS Expression

The histological GUS assay was performed at a very early age as only a small leaf portion was needed and it did not interfere with further plant development. This initial screening showed 58 of 87 plants expressed GUS, as indicated by blue-staining cells. The remaining 29 plants never exhibited GUS expression even after repeated assays.

The initial GUS assay was performed on leaf tissue. Several GUS positive plants thus identified were studied for tissue specific expression. Stem cross-sections showed blue staining in the phloem tissue and blue staining was seen in a developing ovule in an ovary (pers. comm. R.G. Stout).

Leaves showed GUS expression in the parenchyma cells and the vascular bundles of roots also stained blue. Results with pollen paralleled those published by Plegt and Bino (1989) for binucleate pollen of other species. Pollen from both transgenic and normal alfalfa stain blue, rendering this an unsuitable tissue for assay. Plegt and Bino (1989) suggest that an endogenous  $\beta$ -glucuronidase gene exists which is highly tissue and developmentally specific. However, if such a gene exists in alfalfa, it probably has limited homology to the bacterial gene, as cross-hybridization is not seen with normal, non-transgenic alfalfa. This pattern of expression throughout the plant is similar to other published studies (Jefferson et al. 1987, Terada and Shimamoto 1990).

#### NPT II Expression

Forty-seven putative transgenic plants were tested for kanamycin resistance. Explants from 46 of 47 plants formed callus and grew on 50 mg/L kanamycin. Control, untransformed plants died at this concentration. The one regenerated plant which failed to express kanamycin resistance was later classified as an escape. Fewer plants were tested for kanamycin resistance than for GUS expression. Some plants died and some were prematurely disposed of because they did not exhibit GUS expression.

Southern Blot Analysis

Southern blot analysis is the standard method of demonstrating that plants contain foreign genes in their genomes, as well as estimating gene copy number, and discerning independently derived transgenic plants from sibling transgenic plants. Thirty-five (40%), of the original 87 putative transgenic plants, are derived from independent transformation events (Table 2).

Table 2. Percentage of transgenic alfalfa plants which are derived from independent transformation events.

Cultivar	# Transgenic Alfalfa	# Independent Transgenic Alfalfa	% Independent Transgenic Alfalfa
Ladak 65	21	12	57
Spredor II	33	7	21
Rangelander	24	12	50
Pale	9	4	44
Total	87	35	40

DNA of the first regenerated transgenic plants were probed with the entire pBI121 plasmid. Hybridization occurred with all putative transgenic plants tested and no hybridization occurred with control, untransformed plants.

To address why some transgenic plants failed to express GUS, separate hybridizations were done with the 3 kb *SstI/SphI* GUS fragment and the 1.7 kb *SphI* NPT II fragment as probes. Genomic DNA was digested with *HindIII*, which cuts between the GUS and NPT II genes of pBI121. The Southern blot in Figure 1A was hybridized with the 1.7 kb

*SphI* NPT II fragment and then hybridized with the 3 kb *SstI/SphI* GUS fragment (Figure 1B). Lanes 4, 5, and 6 show hybridization to only the NPT II fragment while lanes 1, 3, 6, 9 show hybridization to both the GUS and NPT II fragments. Lanes 2 and 8 represent control, untransformed plants which show hybridization to neither fragment.

The presence of the NPT II and GUS genes and their expression in regenerated plants was determined on 30 plants including 29 of the 35 independent transgenic plants (Table 3). Seventeen plants (59%) contained and expressed both the NPT II and GUS genes. Five plants (22%) contained and expressed the NPT II gene but were missing the GUS gene. All plants expressed the NPT II gene when present and none contained only the GUS gene. This is not unexpected as selection was based on kanamycin resistance. One regenerated plant did not contain or express either gene. It is probably an escape as described by Klee et al. (1987). The seven plants (29%) which contained both genes but failed to express GUS are more difficult to explain. The often cited "position effect" may not apply in these cases, since the NPT II gene is being expressed and the two genes should be linked. The GUS gene may have been partially deleted or rearranged leading to non-expression. An alternative hypothesis is that the GUS gene has been inactivated by the plant, possibly by methylation (Amasino et al. 1984). This could be determined by sequencing of the genes in these

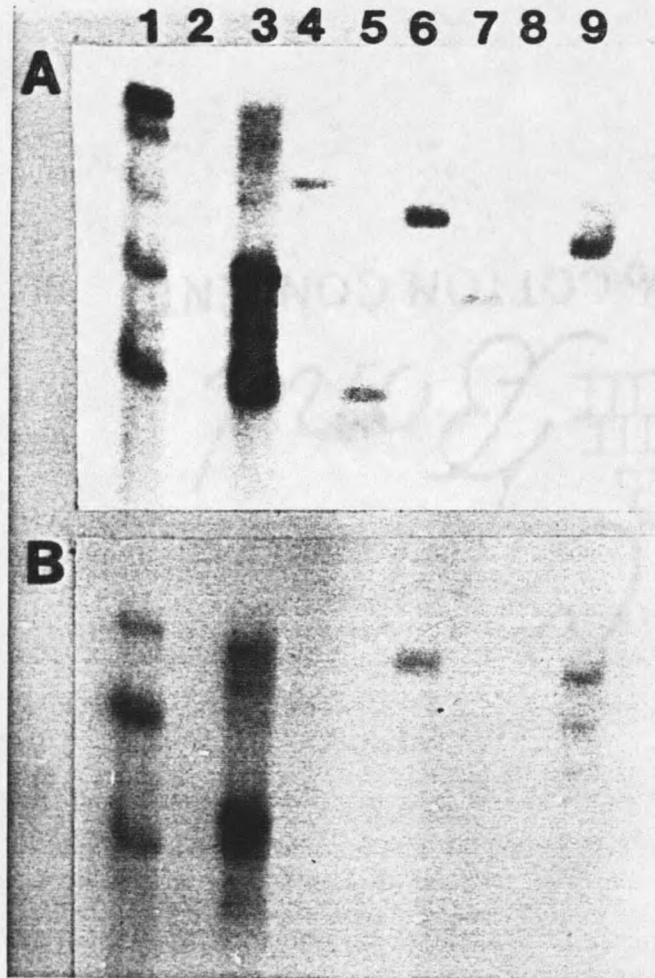


Figure 1. Southern blot of *Hind*III digested genomic DNA of transgenic and control alfalfa plants. A: Blot probed with radioactively labeled 1.7 kb *Sph*I NPT II fragment. B: Same blot, stripped of NPT II probe, and then re-probed with radioactively labeled 3 kb *Sst*I/*Sph*I GUS fragment.

plants and comparing the sequences to the original sequences.

Table 3. The presence and expression of the NPT II and GUS genes in transgenic alfalfa.

NPT II gene	GUS gene	Kanamycin <sup>R</sup>	GUS expression	# Plants
+	+	+	+	17
+	-	+	-	5
+	+	+	-	7
+	-	-	-	0
-	-	-	-	1

These data have implications for those transformation projects where the gene(s) of primary interest is not directly selected for during tissue culture. Based on these results, a significant proportion of the transgenic plants could either not contained the unselected gene or fail to express it.

Copy number was estimated concurrently with testing for the presence of each gene on the independent transgenic plants. Copy number ranged from 1-5 in 33 transgenic plants (Figure 2). In Figure 1 each band for a particular plant theoretically represents one copy of the GUS gene or NPT II gene. The exception is if tandem repeats have occurred, in which case, a single band might represent several tandem copies of a gene. Therefore, the copy number estimation represents the minimum number of copies present in the plant genome. Jorgenson et al. (1987) gave evidence that within a tandem array of inserted genes, there is often only one copy

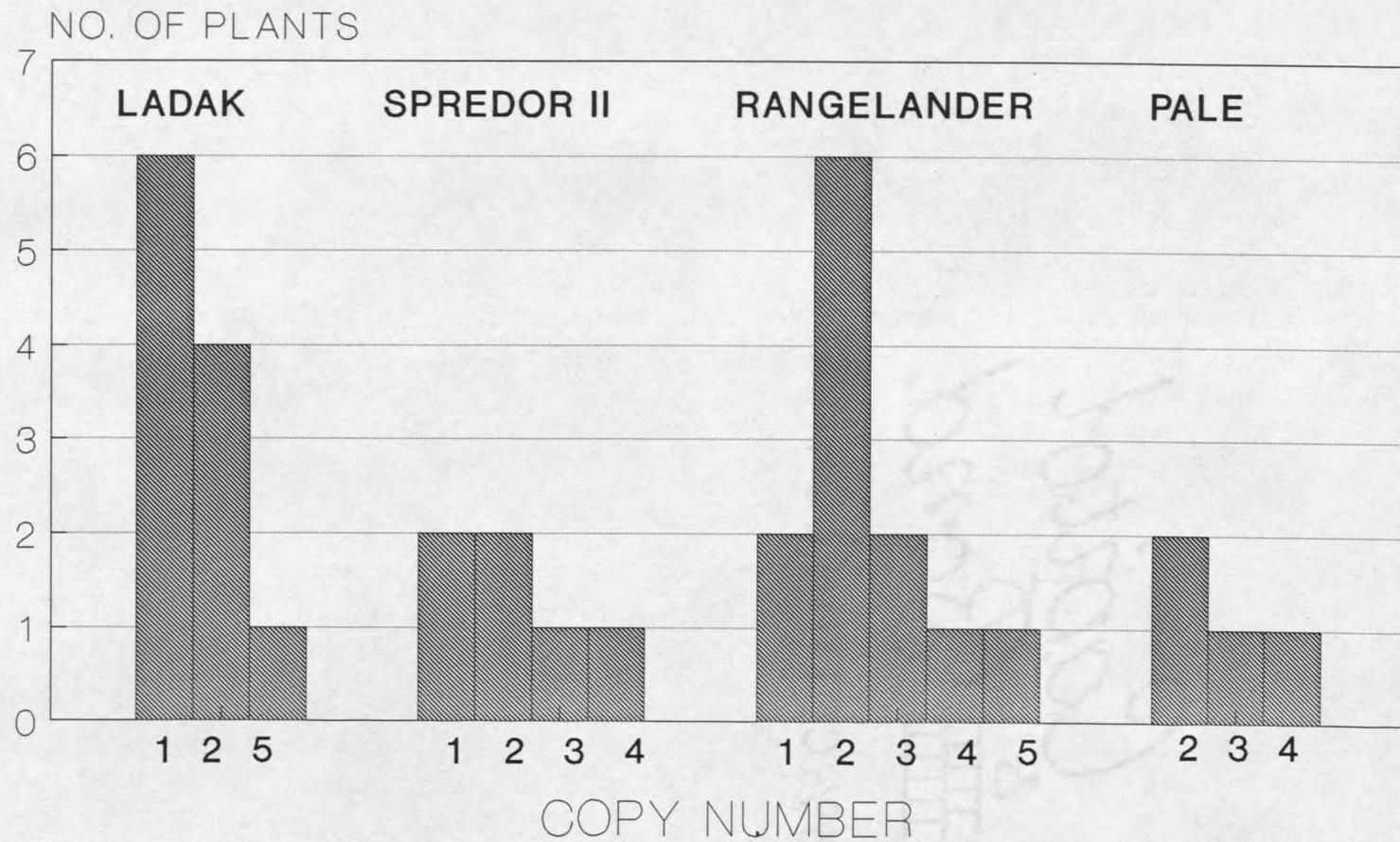


Figure 2. Estimation of copy number of NPT II gene in transgenic alfalfa plants.

of the gene which is expressed. Determining the number of expressed genes is best made by crosses with the transgenic plant and subsequent progeny inheritance analysis.

### Polymerase Chain Reaction

Southern blots have permitted the accurate identification of plants containing introduced genes for over a decade (Thomashow et al. 1980). Polymerase chain reaction sequence amplification provides an alternative technique which is more rapid and possibly more efficient at determining which plants contain accurately integrated foreign genes. This is a particularly important consideration for analyzing hundreds of transgenic progeny.

Establishing a specific protocol for simultaneous amplification of NPT II and GUS genes was undertaken. Determination of optimal annealing temperature for specific primer sets and target sequences is necessary to eliminate artifacts due to nonspecific amplification. An annealing temperature of 50-55° C gave good amplification with minimum nonspecific amplification products using the GUS and NPT II primers and target sequences. A 25  $\mu$ l reaction, rather than the standard 100  $\mu$ l reaction, was adopted for cost savings. This reaction volume reduction required accurate determination of template DNA concentration for each reaction. PCR failures were commonly due to either excess or insufficient template DNA. This problem was solved by

careful DNA concentration determination as described previously. A single leaf yielded enough DNA several PCR reactions.

Simultaneous PCR amplification of the NPT II and GUS fragments was performed on the independent transgenic alfalfa plants. Transgenic plants, known to express GUS, contained both the NPT II and GUS genes based on the presence of PCR amplified fragments from both genes (Figure 3A, lanes 4,7,10,12). Transgenic plants which did not express GUS, apparently contained only the NPT II gene since only this fragment was amplified by PCR (Figure 3A, lanes 3,6,9,11,13). Non-transformed, non-regenerated control plants displayed neither amplified fragment (Figure 3A, lanes 2,5,8,14), although nonspecific amplification is seen in lane 2. DNA from pBI121 was included as a positive control. The control reactions were done using GUS and NPT II primers separately (Figure 3A, lanes 15 and 16, respectively), as well as simultaneously (lane 1), and the expected fragments were amplified in each. Differences in band intensities seen in Figure 3A are probably due to differences in template DNA concentration between reactions and/or differences in copy number of the introduced genes among transformed plants.

To assure that the amplified 785 bp and 1097 bp fragments corresponded to the NPT II and GUS genes, respectively, Southern analysis was performed on the blotted





































