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# ENGINEERING HERBICIDE RESISTANCE IN CREEPING BENTGRASS (*Agrostis palustris* Huds.) AND ITS POTENTIAL APPLICATION ON THE PREVENTION OF FUNGAL DISEASES

By

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# ABSTRACT

# ENGINEERING HERBICIDE RESISTANCE IN CREEPING BENTGRASS (*Agrostis palustris* Huds.) AND ITS POTENTIAL APPLICATION ON THE PREVENTION OF FUNGAL DISEASES

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An efficient transformation system to generate transgenic creeping bentgrasses (*Agrostis palustris* Huds.) by microprojectile bombardment is described. Embryogenic callus bombarded with the *bar* gene was cultured on selection medium supplemented with either five mg/l of bialaphos or fifteen mg/l of phosphinothricin (PPT) for twelve weeks. 38 independent resistant callus lines were generated and found able to regenerate plants. Except for the plants regenerated from one of the PPT-resistant callus lines, plants from the rest of the resistant callus lines were shown to be resistant to the application of 1.2% Ignite®. Resistant callus lines and plants regenerated from them expressed functional phosphinothricin acetyltransferase, the product of *bar*. Integration was confirmed by Southern hybridization analysis and transcripts corresponding to the *bar* gene were present in transgenic plants that showed herbicide resistance.

Bialaphos showed a higher level of *in vitro* antifungal activity against the pathogens of brown patch (*Rhizoctonia solani*), dollar spot (*Sclerotinia homoeocarpa*) and *Pythium* blight (*Pythium aphanidermatum*), than phosphinothricin (PPT). While PPT suppressed the mycelial growth of *R. solani* and *S. homoeocarpa*, it had no inhibitory effect on *P.*

*aphanidermatum* up to the highest concentration (600 mg/l) in our testing regimes. Whereas bialaphos was significantly effective in the inhibition of growth of *R. solani* and *S. homoeocarpa*, it was less so in that of *P. aphanidermatum*. Various concentrations of bialaphos solutions were applied to transgenic creeping bentgrasses either three hours before or two days after the fungal inoculation. Bialaphos application was able to significantly reduce the symptomatic infection by *R. solani* and *S. homoeocarpa*. The inhibitory effect of bialaphos spraying on the suppression of *P. aphanidermatum* was not as effective as that of the other two pathogens; however, disease development was still reduced to a significant extent. Better control of the disease development of dollar spot and *Pythium* blight also occurred when bialaphos was applied before the inoculation. These results indicated that bialaphos may be used simultaneously and efficaciously as a herbicide for weed control and as a fungicide for the protection of turf areas with bialaphos-resistant creeping bentgrasses against fungal diseases.

*To God, my lovely wife and parents*

*for their love, support and encouragement*

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# Literature Review

## Introduction

Bentgrass (*Agrostis* spp.) is the cool-season turfgrass most commonly used on golfing greens in cool and transitional climatic regions and in the cooler portions of warm climatic regions, especially the arid zone (Beard, 1982). The genus *Agrostis* L. includes a number of species suitable for many turfs, especially in temperate climates with cool, moist summers. The common name bentgrass is applied to all turfgrass species within the genus *Agrostis,* with the exception of redtop. Bentgrass acquired its name from the characteristic bent of the basal part of each stem when plants are growing in thin stands. These stems, instead of growing upright, tend to follow the ground for a short distance and then bend upward. Its growth habit varies from a bunch type with limited stoloniferous growth to an extensive stolon system (Philipson, 1937). Due to the prostrate growth habit, bentgrass is the cool season turfgrass most tolerant of continuous and close mowing. Some species are annuals, but most are perennials and have excellent low temperature hardiness, including all those utilized for turfgrass purposes. They form an extremely fine-textured, dense, uniform, high quality turf when closely mown. Bentgrass is generally tolerant of acid soils and can thrive at lower levels of soil fertility relative to that needed for good performance of Kentucky bluegrass or perennial ryegrass. Future breeding work could significantly expand the areas of adaptation and usefulness of these grasses (Funk *et al*. 1994).

There are three widely used turf-type bentgrass species : (a) *A. palustris*---creeping bentgrass, (b) *A. tenuis*---colonial bentgrass, and (c) *A. canina*---velvet bentgrass, which is not used as extensively as the other two. A wide range of divergent types and intermediate forms with chromosome numbers ranging from 14 to 42 exist among creeping, colonial, and velvet bentgrass (Stuckey and Banfield, 1946; Vaatnou, 1967).

The creeping bentgrass (*Agrostis palustris* Huds.) is native to Eurasia but has been distributed throughout the world for use on golf putting greens, tees, and closely mowed fairways. It is the most widely used *Agrostis* species in North America. The common name creeping bentgrass is derived from the vigorous creeping stolons that develop at the surface of the ground and initiate new roots and shoots from the nodes. Considerable variation exists in the literature concerning the scientific name of the creeping bentgrass, with some authors referring to it as *Agrostis stolonifera* var. *palustris* (Farwell) (Piper, 1918; Armstrong, 1937; Philipson, 1937; Bradshaw, 1958). It is a cross-pollinated tetraploid (alloploid) with 28 chromosomes ($2n=4x=28$; Church 1936).

Creeping bentgrass forms its best quality turf at a cutting height of 1.5 cm or less. It is a soft, fine-textured grass with a vigorous growth habit and is capable of producing a very attractive, dense, and compact turf tolerant of very close mowing. The poor wear resistance of creeping bentgrass is compensated by its excellent recuperative potential. Its quality surpasses that of any other northern turfgrasses. However, such turfs normally receive very high maintenance inputs, including frequent irrigation and mowing, aerification and topdressing for thatch reduction, and pesticides for disease and insect control. The finer textured cultivars tend to be compact and low growing, while the coarser textured are usually more upright and open (Holt and Payne, 1952). The turfgrass color varies among the cultivars from greenish-yellow to dark green to blue green.

The creeping bentgrass can be subdivided into two types based on propagation method. Many of the early cultivars of creeping bentgrass for fine putting greens were selected from old turf areas (Beard, 1973) and propagated by chopping the creeping stems (stolons) into short sections with one or more nodes. There are a number of vegetatively propagated cultivars available, and they are preferred where turfgrass uniformity is to be maintained for an extended period of time. However, there is an abundance of commercial seed cultivars available for this species, such as Penncross, Emerald, Seaside, and Penneagle. Penncross, released in 1954 (Hein, 1958), is a first generation synthetic cultivar produced by the random crossing of three vegetatively propagated strains and is the most widely used cultivar throughout North America, except for the hot and humid southeast (Beard, 1982). The seeded creeping bentgrass cultivars possess a certain degree of heterogeneity and, over a period of time, tend to segregate into distinct patches under turfgrass conditions (Holt and Payne, 1952). Segregation into various strains complicates cultivation practices. In spite of this disadvantage, the seeded cultivars have been widely utilized, primarily because they are more economical to establish.

## Disease Problems of Creeping Bentgrass

Turfgrass disease is a greater problem for golf courses than it is for most other types of turf use, due to close mowing, heavy fertilization, intense irrigation, and constant bruising from traffic and divoting. Disease problems are most severe on putting greens, especially those composed of bentgrass in contrast to bermudagrass. Tees are also subject to high disease activity if the cultural system utilized involves a cutting height and nutritional level similar to those employed on greens. Fungicide programs are commonly used on these types of turf during periods when the potential for disease development is high. The severity of disease problems is typically greater on cool-season grasses than on warm-season grasses, especially in the more humid climatic regions.

The creeping bentgrasses are susceptible to a wide range of diseases, including dollar spot (*Sclerotinia homoeocarpa*), brown patch and yellow patch (*Rhizoctonia solani*, *R. cerealis*, and *R. oryzae*), *Helminthosporium* diseases., *Fusarium* patch (*Gerlachia nivalis*), *Pythium* blight, red thread and pink patch (*Laetisaria fuciformis*), stripe smut (*Ustilago* and *Urocystis* spp.), takeall patch (*Gaeumannomyces graminis*, var.

*avenae*), copper spot (*Gloeocercospora sorghi*), fairy ring (*Marasmius oreades*), downy mildew (*Sclerophthora macrospora*), and *Typhula* blight (*Typhula incarnata*) (Beard, 1973; Sprague, 1982; Smiley, 1983).

## Insect Problems of Creeping Bentgrass

There are over two million different kinds of insects, but relatively few pose a significant problem to turfgrasses. The severity of insect attacks on golf course turfs is not as highly correlated to the intensity of culture as is the disease-causing pathogens. The severity and frequency of insect attacks tend to be greater on warm-season than on cool-season turfgrasses. The occurrence of insect problems on bentgrasses increased noticeably during the mid-1970s with the passage of laws that eliminated the use of persistent insecticides, such as the chlorinated hydrocarbons. Appropriate insecticides are now applied when potentially serious insect injury symptoms first appear. White grub (black turfgrass ataenius, *Ataenius spretulus*), chinch bug (*Blissus leucopterus*), cutworm (*Noctuidae* family), and frit fly (*Oscinella frits*) are of particular concern.

## Weed Problems of Creeping Bentgrass

Five major components of turfgrass quality are uniformity, density, smoothness, texture, and color. Plants that disrupt the uniformity of turfs in terms of either texture or density are considered undesirable in high-quality turf and, thus, are referred to as weeds. An high number of weeds in the turf results from poor establishment and management practices. A permanent, properly managed turfgrass species makes a tight sod that prevents most weeds from becoming established. However, perfect turf maintenance is difficult to achieve, particularly on general turf areas receiving less than intensive care. Herbicides are usually employed to supplement a good weed-control management program to make a weed-free turf.

The characteristics of turfgrass weed species vary tremendously, but all have the ability to persist even though the grass is cut regularly. Generally, they survive mowing because of a low growth habit. Weed species are classified as grassy type (monocotyledon) or broadleaf type (dicotyledon) and are subdivided into annuals, biennials, and perennials. Selecting the proper herbicide and application time for controlling a weed species is determined by the life cycle of the weed and whether it is a broadleaf or grassy-type weed. Generally, weeds fall into three control groups---annual grasses, perennial grasses, and broadleaf weeds.

The broadleaf weeds that infest turfs are much more numerous than grass weeds. Fortunately, they are dicots and can be controlled very effectively with selective and systematic herbicides, such as 2, 4-D, mecoprop, and dicamba, with little or no injury to the turf if applied properly. A combination of 2,4-D and dicamba or mecoprop is most effective since some broadleaf weeds are resistant to one of the herbicides and a mixture ensures broad-spectrum weed control.

Annual grass weeds can usually be controlled with the application of pre-emergence herbicides. Most applications occur in the early spring because pre-emergence herbicides should be sprayed one to two weeks before seed germination. Post-emergence herbicides are also used to control annual grasses in the early stage after emergence. However, the post-emergence method of weed control is often unsatisfactory aesthetically because slowly dying grasses in a turf are unsightly. Another concern is that post-emergence herbicides can be phytotoxic to desirable turfgrasses. Cool season grasses are especially susceptible to this type of injury. Creeping bentgrass is more prone to herbicide injury than Kentucky bluegrass (Albrecht, 1947; Skogley and Jagschitz, 1964; Callahan, 1966). Root and leaf injuries frequently result from the applications of 2,4-D and 2,4,5-TP (Callahan and Engel, 1965).

Perennial weed grasses cause serious weed problems because selective herbicides are not available. Perennial grassy weeds are so similar to desirable turfgrasses from a physiological and anatomical standpoint that herbicides used for killing perennial grass in turf also destroy the perennial turfgrass. A nonselective herbicide such as glyphosate or dalapon must be used with great care. Therefore, the development of a herbicide-resistant creeping bentgrass variety has become imperative so as to achieve a perfect turf management program in a convenient and inexpensive way.

Herbicides have become an indispensable tool of modern agriculture in that they allow economically superior weed control and are more labor- and energy-efficient than manual or mechanical cultivation methods. In recent years, the demand for environmental safety has made it necessary to develop less toxic compounds, so that corporate competition in this field has resulted in the development of several new, better, and safer herbicides, including a number of selective compounds. However, only a very few of these fulfill the ideal requirements of a truly selective herbicide in controlling all plants except the cultivated crop (Schulz *et al.*, 1990).

The information available elucidating the mode of action of several herbicides has rapidly increased in the past few years (Schulz *et al.*, 1990). This fact, coupled with the progress achieved in the genetic manipulation of plants and the ability to transfer foreign DNA from a variety of sources to plants, has opened up an exciting area of research. These observations are further enhanced in that many possible herbicide resistant gene determinants are single dominant traits, making them amenable to gene transfer techniques. Hopefully, the ensuing result will be the expanded use of environmentally safer herbicides which favor a broader weed control spectrum in response to increasing concerns over contamination of the environment, toxicity to animals, and persistence of residues in soil and water. The cultivation of herbicide-resistant turfgrasses also brings economic advantages to the management of golf courses by reducing the quantity needed and simplifying the use of herbicides.

## Traditional Breeding and Plant Biotechnology

Since the beginning of this century, Mendel's law of genetics has provided the scientific foundation for plant breeding. Traditional plant breeding methods have resulted in substantial crop improvement and will continue to provide us with useful crop varieties and enhance our quality of life.

In conventional plant breeding, plant breeders are generally faced with the following three challenges. First of all, they must attempt to locate and utilize the maximum possible range of genetic variability that is available. Then, they need to be able to transfer the trait(s) of interest into the other breeding lines and to select the recombinants that show the desired characteristics. Finally, they have to generate a large number of progeny with predictable properties. By crossing parent plants each holding some of the useful traits and selecting the progeny of those individuals showing the proper combination of traits, tremendous improvements have been achieved in crop performance and will continue to be made.

However, the traditional plant breeding method of crossing has its limitations. It is evident that only traits present within the species or its related species can be combined, since crosses between distantly related or unrelated species usually yield sterile plants or, most often, no progeny at all. Besides the problem of compatibility, the availability of desirable genes and the conservation of genetic diversity are also limitations to plant breeding research due to the erosion of the natural environment.

During the past twenty years, in addition to the development of plant tissue culture techniques, much progress has been made in the fields of cell biology and molecular biology, which, in turn, has had its influence on modern plant breeding research and has offered alternative routes to resolving some of the limitations of traditional plant breeding and production. This relatively recent development in plant biotechnology, the technology to manipulate and regenerate plants *in vitro* and to isolate and construct specific DNA fragments, more or less at will, has led to advances in the ability to selectively alter the genetic make-up of certain plant species.

In 1984, stable foreign gene transfer into plant species became possible with the application of recombinant DNA technology and with the availability of *Agrobacterium tumefaciens* transformation methodology (De Block *et al.*, 1984; Horsch *et al.*, 1984). The ability to transfer genes among organisms without sexual crossing provides plant breeders with new opportunity to improve the efficiency of production and to increase the utility of agricultural crops. Plant genetic engineering has become a useful tool to plant breeding research in that it helps to overcome problems associated with the incompatibility between different species and to broaden the gene pool plant breeders can utilize. Plants with new traits, such as herbicide resistance and insect resistance, have been genetically engineered using genes from unrelated organisms. Scientists are also trying to improve the quality of our agricultural products by altering the nutritional values of proteins and oils. However, it should be emphasized that plant biotechnology is not a substitute or replacement for the conventional plant breeding methods. Rather, it can supplement the traditional plant breeding approaches to improve crop species. The major differences between these two methodologies lie neither in goals nor processes, but rather, in speed, precision, reliability, and scope.

## Methods of Plant Genetic Transformation

The revolutionary progress in recombinant DNA technology makes it necessary to continue to improve the techniques needed for the genetic engineering of plants. Improvement of these techniques requires better tissue culture methodologies and gene transfer techniques. Before describing gene transfer and genetic transformation, the following definitions are adopted to eliminate the ambiguity often found in the literature.

### Gene Transfer

Gene transfer (or DNA uptake) refers to a process which moves a specific fragment of DNA (usually a foreign gene ligated to a bacterial plasmid) into protoplasts or cells. If the gene transfer is efficient and the foreign gene is introduced into a sufficient number of cells, transient gene expression can be quantitatively measured (approximately 12 to 48 hours after the gene transfer). Usually, the level of the protein encoded by the foreign gene is measured to show the success of gene transfer, because it is simpler and easier to measure than the level of mRNA. For studying transient gene expressions, stable integration of the introduced gene is not necessary, but a reproducible and efficient gene transfer method is needed.

### Genetic Transformation

Plant genetic transformation refers to the stable integration of foreign genes isolated from plants, bacteria, or animals into the genome of a plant regenerated from DNA-treated protoplasts or intact cells. The plants carrying the stably integrated foreign genes are defined as transgenic plants. The process of genetic transformation involves several distinct stages, namely, insertion, integration, expression, and inheritance of the newly transferred DNA. Integration can be demonstrated by Southern blot analysis. The analysis should include the use of several restriction enzymes to provide proof of integration of the foreign gene.

For example, it is useful to include (1) an enzyme that cuts twice within the plasmid to show the presence of the foreign gene and to show that there is no rearrangement of the foreign gene on the plasmid in the transgenic plants; (2) an enzyme that cuts once within the plasmid to yield restriction fragments which vary in size depending on the nature of the flanking DNA at the site of insertion of the plasmid in the transformed plants; and (3) an enzyme that does not cut within the plasmid to distinguish between the free plasmid (in monomeric or oligomeric form) and the integrated plasmid.

## Techniques for Gene Transfer

Although straightforward in principle, in practice plant genetic transformation can be quite complex. Once a gene of interest is isolated, it is introduced into a plant transformation vector. The vector DNA facilitates the manipulation of the gene in *Escherichia coli* prior to plant transformation, as well as the transfer of the gene into the host plant. An idealized vector would contain a multiple cloning site, an antibiotic resistance gene allowing for selection in both *E. coli* and *A.. tumefaciens* (e.g., a gene encoding ampicillin resistance), a broad-host bacterial origin of replication, and an antibiotic or a herbicide resistant gene for selection of the foreign DNA in transformed plants. If the *Agrobacterium* system is used for transformation, the vector may contain the Ti plasmid virulence gene and T-DNA borders as well. There are many approaches to introducing foreign DNA into plants, of which the most commonly used are the *A. tumefaciens* "agro-infection" system, PEG- and electroporation-mediated direct gene transfer, and microprojectile bombardment.

## *Agrobacterium*-mediated Genetic Transformation

*Agrobacterium* is a soil-dwelling bacterium that infects wound sites on a wide range of plant species and induces the development of crown gall tumors or hairy roots. These growth responses result from a natural genetic engineering event in which a specific region of DNA from a Ti (tumor-inducing) plasmid or Ri (root-inducing) plasmid is transferred from *Agrobacterium* to a plant cell. This T-DNA (transferred DNA) is integrated and expressed in the nuclear genome of the plant cells. The expression of genes located within the T-DNA results in the development of tumors or hairy roots. The importance of *Agrobacterium* for plant genetic engineering is its natural ability to transfer a segment of DNA into plant cells. Over the past ten years, researchers have capitalized on molecular biology technology to manipulate the T-DNA of *Agrobacterium* for the development of gene vectors to produce transgenic plants.

The molecular mechanisms of plant transformation by *A. tumefaciens* have recently been reviewed (Zambryski, 1988; Kado, 1991; Hooykaas and Schilperoort, 1992). The wild type form of *A. tumefaciens* induces crown gall tumors on many dicotyledonous plants when viable bacteria infect wounded plant tissue (Ream and Gordon, 1982). The crown gall is produced following the transfer of the tumor inducing T-DNA (transferred DNA) region from the Ti plasmid (140 to 235 kilobases) into the genome of an infected plant. The T-DNA fragment, which is integrated into plant nuclear genome and retained in tumor cells, encodes genes for auxin and cytokinin biosynthesis, and it is these hormones in high concentration that promote growth of undifferentiated cells in the crown gall. Transfer of the T-DNA to the plant genome requires the Ti plasmid-encoded virulence genes as well as the T-DNA borders, that is, a set of direct DNA repeats that delineate the region, to be transferred. The tumor inducing genes can be removed from Ti plasmid vectors, disarming the pathogenic nature of the system, without affecting the transfer of DNA fragments between the T-DNA borders. Therefore, the tumor inducing genes are generally replaced with a gene encoding resistant to antibiotics such as kanamycin or to herbicides to allow for the selection of transformants, and with a gene of interest that would confer the desired trait to the recipient plant.

The *Agrobacterium* containing the engineered plasmid is co-cultivated with cultured plant cells or wounded tissue. The de-differentiated cells are then cultured and propagated on selection medium, and transgenic plants can subsequently be regenerated from the resistant cells by altering the levels of auxin and cytokinin in the growth medium. One of the disadvantages of this technique is that most monocots and some dicots are not natural hosts for the bacterium and, for the most part, do not have the proper wound responses to be agro-infected. They are, therefore, generally excluded from *A. tumefaciens*-mediated genetic transformation (Potrykus, 1990, 1991).

## Transformation of Protoplasts

Every single cell of a plant is potentially capable of developing into a whole plant. This phenomenon is known as cell totipotency. Significant advances have been made by researchers during the past decade in plant regeneration from protoplasts (isolated single plant cells without cell walls) in both dicots and monocots. This has not only confirmed the hypothesis of cell totipotency, but has also made it possible to transfer foreign genes into plants using protoplasts as the starting material. There are two major reasons for using protoplasts instead of cells or tissues for genetic manipulation. One is that protoplasts are separated single cells which have been stripped of their cell wall, facilitating the transfer of foreign genes through the plasma membrane. The other is that all cells of transgenic plants regenerated from a protoplast will contain the foreign genes of interest; thus, the transgenic plant will have a uniform genetic makeup, avoiding the problem of chimerism that sometimes complicates the other transformation methods.

Protoplasts capable of gene integration and plant regeneration may be referred to as competent. Protoplasts may be induced to become competent by the alteration of specific experimental conditions. Several methods have been used to stimulate protoplast competence, such as heat shock (Abdullah *et al.*, 1986), low-dose irradiation (Kohler *et al.*, 1989), and media ultrafiltration (Davies *et al.*, 1989). These treatments, in addition to the enzymatic and mechanical treatments used in the protoplast isolation procedure, can increase gene transformation frequency and protoplast division. When competent protoplasts with high viability are available, several methods such as polyethylene glycol (PEG) treatment, electroporation, *Agrobacterium* infection, and sonication can be used for gene transfer.

## PEG- and Electroporation-mediated Direct Gene Transfer

For many years, genetic manipulation in some plants has been accomplished using direct uptake of foreign DNA by plant protoplasts rather than *A. tumefaciens*-mediated gene transfer. The first experiments demonstrating direct gene transfer included the delivery of plasmid DNA to protoplasts of petunia and tobacco in the presence of poly-L-ornithine or polyethylene glycol (PEG) (Davey *et al.*, 1980; Draper *et al.*, 1982; Krens *et al.*, 1982; Paszkowski *et al.*, 1984). During the following years, protoplast transformation mediated by PEG (Negrutiu *et al.*, 1987) or electroporation (Shillito *et al.*, 1985) was substantially simplified, and its efficiency in model systems was increased by several orders of magnitude (reviewed by Paszkowski *et al.*, 1989).

Various chemical treatments have been used to stimulate DNA uptake by protoplasts. At present, PEG is the most common chemical treatment used to stimulate DNA transfer into protoplasts of both dicots and monocots (Negrutiu *et al.*, 1987).

PEG acts to increase the permeability of cell membranes, and has been used as an efficient protoplast fusion agent in somatic cell hybridization of various plant and animal species. It has also been effective in delivering DNA into plant protoplast and in obtaining transformed cells (Wang *et al.*, 1992). Opinion is divided about the mechanism by which DNA crosses the plasmalemma and then enters the nucleus. However, under the influence of the elevated osmotic pressures created by the use of high concentrations of PEG during the transformation of protoplasts, DNA may be incorporated into membrane-bound vesicles in the cytoplasm in a manner similar to the formation of cytoplasmic vesicles on the plasmolysis of cells. These may then fuse with the nuclear membrane systems continuous with the nuclear membrane and effectively transfer DNA to the nucleus. Evidence for this hypothesis comes from work on cation-mediated endocytosis and exocytosis in oat protoplasts. These studies suggest that swelling and shrinking of protoplasts during osmotic adjustment occur by the removal and addition of membrane from the plasmalemma (Glaser and Donath, 1989).

Various parameters important for gene transfer with PEG have been identified. First of all, the concentration of PEG can affect the viability of protoplasts and the efficiency of gene transfer. The optimal concentration of PEG is between 15% and 25%. If the concentration is too high, cell viability will decrease; if it is too low, the efficiency of gene transfer will decrease (Hayashimoto *et al.*, 1990). Secondly, the components of the buffer will also influence gene transfer efficiency (Zhang and Wu, 1988). Finally, culture media for protoplasts will directly affect protoplast division and plant regeneration (Jenes and Pauk, 1989). Currently, to transfer the foreign gene, two major methods, solid culture and nurse culture, are commonly used for efficient protoplast culture after protoplasts have been treated with PEG (Cao *et al.*, 1991).

The electroporation method involves the application of high-voltage electrical pulses to a solution containing protoplasts and foreign DNA. DNA transfer by electroporation is thought to occur as a result of transient changes in the lipid bilayer structure of the plasmalemma induced by the electric field. The introduced DNA enters the cells through reversible pores created in the protoplast membranes by the action of the short electrical pulse treatments. It is known that gene transfer efficiency and protoplast viability are influenced by the amplitude and duration of the electric pulse and by the composition of the electroporation buffer. Fromm *et al.* (1985) first reported gene transfer into maize protoplasts and Langridge *et al.* (1985) first reported the stable transformation of carrot protoplasts with DNA by electroporation. Application of high voltage either directly (both electrodes immersed in the sample solution) or indirectly (without anode contact) to a solution containing plasmid DNA and protoplasts of rice, wheat, or sorghum resulted in different degrees of protoplast viability and gene transfer efficiency (Ou-Lee *et al.*, 1986). Protoplast size and competent state differ from one species to another, and, therefore, the optimal electroporation conditions may vary among species. For example, Shimamoto *et al.* (1989) used a long electrical pulse (10 msec) generated by a large capacitor and a moderate voltage (300 V/cm) for genetic transformation of embryogenic protoplasts of rice (10-20 *u*m in diameter). Rhodes *et al.* (1988) applied a shorter pulse and higher voltage to maize protoplasts (25-40 *u*m in diameter). As in the case of PEG method, the buffer also greatly influences the gene transfer efficiency as well as the protoplast survival rate. Tada *et al.* (1990) demonstrated that replacing the chloride ions in the buffer with an organic acid, aspartic acid monopotassium salt, increased the transformation frequency at least 10-fold and increased the plating efficiency as well.

The production of transgenic plants via direct gene transfer to protoplasts depends on protoplast-to-plant regeneration and on efficient selection systems for transgenic cell clones. Early gene transfer experiments focused on protoplasts of *Solanaceae* species that are easily regenerable, and on the use of the bacterial gene for neomycin phosphotransferase (*npt* II), conferring kanamycin resistance to transformed cell lines. During the past few years, protoplast-to-plant regeneration was achieved for several economically important crops such as Japonica and Indica rice varieties (Shimamoto *et al.*, 1989; Datta *et al.*, 1990; Li *et al.*, 1992a, 1992b), maize (Omirulleh *et al.*, 1993), and forage grasses (Wang *et al.*, 1992). However, the natural resistance of many monocotyledonous species to the antibiotic kanamycin (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989) made the development of other selection systems necessary. In addition to the *npt* II gene, the gene for hygromycin-transferase (*hpt*; Gritz and Davies, 1983) and phosphinothricin-acetyltransferase (*pat*; Thompson *et al.*, 1987) have proven useful for the selection of stably transformed colonies in mono- and dicotyledonous species (Horn *et al.*, 1988; Masson *et al.*, 1989; Shimamoto *et al.*, 1989; Datta *et al.*, 1990; Wang *et al.*, 1992; Omirulleh *et al.*, 1993).

PEG- and electroporation-mediated direct gene transfers are simple and efficient: dozens of protoplast samples can be treated in a single experiment, and thousands of individual transgenic plants can be obtained in model systems with tobacco. Since any DNA fragment can be delivered into the cell, these techniques have the advantage of allowing the assimilation of a gene without having to clone the gene of interest into an *A. tumefaciens* vector. One disadvantage of these techniques is that during incorporation into the host nuclear genome, DNA rearrangement sometimes occurs (Paszkowski *et al.*, 1989). Furthermore, only plants from which protoplasts can be isolated may be transformed by these techniques, and not all protoplast systems can be used to regenerate fertile plants. However, even if a plant cannot be regenerated, these methods still provide an extremely useful means for studying transient gene expression.

### Gene Transfer into Protoplasts Using *Agrobacterium*

Protoplasts of many dicotyledonous and monocotyledonous (nongraminaceous) species can be transformed using *A. tumefaciens* strains harboring foreign gene(s) of interest. Protoplasts must be prepared using optimal conditions for regeneration, co-cultivated with the bacteria, and plated on appropriate media for protoplast culture and regeneration, if desired. Growth of *A. tumefaciens* in the protoplast culture after co-cultivation is retarded by the use of antibiotics in the culture media. In general, *A. tumefaciens*-mediated transformation of plant tissue requires less effort and expertise in tissue culture than transformation of protoplasts. However, transformation of protoplasts using *A. tumefaciens* is an appropriate method in species where *A. tumefaciens*-mediated transformation of tissue explants is difficult or has not been achieved, or in experiments where expression in regenerated transgenic plants is not the primary goal (Thomzik and Hain, 1990).

### Gene Transfer into Protoplasts by Sonication

An alternate transformation method has been reported by Joersbo and Brunstedt (1990) and by Zhang *et al.* (1992). Joersbo and Brunstedt introduced DNA into sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana tabacum* L.) protoplasts by applying a brief exposure of 20 kHz ultrasound in the presence of a plasmid containing the chloramphenicol acetyl transferase (CAT) gene fused to the 35S promoter. A maximal level of CAT activity was achieved in the transformed cells by sonication for 500 to 900 msec at 30 to 70 w electric power. Up to 12% (sugarbeet) and 81% (tobacco) of maximum transient expression could be achieved with no significant loss of viability. The concentrations of plasmid DNA (80 to 110 *u*g/ml) and of sucrose (21 to 28%) in the sonication medium were also determined for the optimal transient expression. The protoplasts surviving the exposure to ultrasound were found to have long-term viability and ability to regenerate to microcalli similar to the untreated protoplasts.

Zhang *et al.* (1992) succeeded in the regeneration of transgenic tobacco plants from protoplasts transformed by sonication. They used dimethyl sulfoxide (DMSO) to raise the transient expression frequency of ß-glucuronidase (GUS) gene to 86% and the shoot differentiation frequency to 60%. The activity of GUS gene in the leaves of regenerated kanamycin-resistant plantlets was found at a frequency of 22 percent. The analysis of $R_1$ regenerated seedlings showed that both kanamycin and GUS genes behaved as typical linked dominant genes. They also found that salmon sperm DNA as carrier DNA was essential for obtaining stable transformants.

One possible advantage of this method is that the system may be simpler than electroporation. The efficiency of transformation was comparable to other transformation methods of protoplasts. It remains to be seen whether this method can be extended to a greater number of plant species.

### Liposome-mediated Transformation of Protoplasts

A variety of approaches have been investigated in order to optimize the delivery of nucleic acids into eukaryotic cells. In mammalian cells, for example, liposomes were successfully used to transfer and express DNA and RNA molecules (Fraley *et al.*, 1982).

Liposomes spontaneously interacted with the plasma membrane of protoplasts and had the advantage that they protected the encapsulated nucleic acids from nucleolytic degradation and, therefore, guaranteed a very efficient delivery of intact nucleic acids into recipient cells. Stable-transformed, fertile, and transgenic tobacco plants expressing kanamycin resistance were obtained through the liposome-mediated transformation method (Caboche, 1990). However, among the various procedures for introducing foreign DNA into higher plant protoplasts, liposome-mediated direct gene transfer was one of the most difficult (Caboche, 1990). The frequency of liposome-mediated transformation was five to ten times less efficient than electroporation in achieving transformation. The problems lay in the preparation of liposomes and the reliable encapsulation of plasmids. Caboche (1990) also suggested that due to the rather low transformation frequency of this technique and to the restriction of the encapsulation procedure to DNA fragments smaller than ten kilobases (above this size the step of sonication involved in the procedure was deleterious to the integrity of the encapsulated material), liposome-mediated transformation of protoplasts was not a recommended technique.

However, since lipofectin, a stable preparation of cationic liposomes, was commercially available, the successful application of lipofectin-mediated transformation has been achieved in animals (Felgner *et al.*, 1987; Felgner and Ringold, 1989) and in plants (Antonelli and Stadler, 1990; Spörlein and Koop, 1991). Complex formation of liposomes and DNA and their introduction into protoplasts occurred spontaneously. Since the DNA did not have to be encapsulated into liposomes, this procedure was significantly simpler than the conventional liposome-mediated transformation method. The efficiency of this method was also enhanced by its combination with other techniques such as PEG treatment or electroporation (Spörlein and Koop, 1991).

### Microinjection into Plant Protoplasts

Microinjection is one of the most precise and direct techniques for delivering macromolecules into specific intracellular compartments of living cells (Reich *et al.*, 1986). Studies with animal cells have shown the utility of intracellular microinjection for efficient transformation (Capecchi, 1980; Brinster *et al.*, 1985). Early attempts with plant cells that were hampered by the cell wall or by the fragility of protoplasts. Methods were, therefore, developed to immobilize protoplasts in agarose or on glass with polylysine or by holding the protoplasts under suction. Successful transformation of wild-type Ti plasmid into alfalfa (Reich *et al.*, 1986) and tobacco protoplasts (Crossway *et al.*, 1986) was achieved. Stable transformation frequencies of 14-66% of injected protoplasts have been reported, but the absolute number of transformants is always low, due to the low number of protoplasts which can be microjected.

Basically, microcapillaries and microscopic devices (micromanipulators) are used to deliver DNA into the protoplasts; if competent, the injected cells can survive and divide. No other transformation method could compete with microinjection if competent cells could be visually identified in plant tissue (Potrykus, 1990). However, it is not yet possible to identify competent cells visually. The disadvantage of this method is that it is very time consuming and is not amenable when large numbers of transformants are desired. Moreover, it requires expensive instruments and considerable skill to carry out.

### Transformation of Intact Cells by Electroporation

Electroporation has been used for more than ten years for transient and stable transformation of protoplasts (Fromm *et al.*, 1985; Shillito *et al.*, 1985). Only recently, however, have electroporation conditions been found that deliver DNA molecules into intact plant cells which are still surrounded by cell walls (Morikawa *et al.*, 1986; Lindsey and Jones, 1987; Dekeyser *et al.*, 1990; D'Halluin *et al.*, 1992b; Klöti *et al.*, 1993; Songstad *et al.*, 1993; Laursen *et al.*, 1994; Arencibia *et al.*, 1995). Optimization of the system mainly involved eliminating explant-released nucleases, prolonging the DNA-explant incubation time and expanding the electrical pulse time. In most reported cases, transformability of intact plant cells or plant tissues depended on pretreatment of the cells or tissues with hypertonic or enzyme-containing solutions. However, certain cells are competent for DNA-uptake by electroporation without any pretreatment, for example, immature embryos of maize and wheat (Klöti *et al.*, 1993; Songstad *et al.*, 1993). The reasons for cell competence for DNA-uptake by electroporation are still unknown (Potrykus, 1990). Compared to particle bombardment, the range of tissues that can be transformed by electroporation, which includes meristem tissue (Dekeyser *et al.*, 1990), immature zygotic embryos, embryogenic calli (D'Haullin *et al.*, 1992b; Arencibia *et al.*, 1995), and suspension culture cells (Laursen *et al.*, 1994), seems to be narrower. However, for tissues that are susceptible to DNA-uptake by electroporation, this is a simple, fast, and inexpensive method for transient and stable transformation in plant tissues.

## Microprojectile Bombardment

Biolistic gene transfer is a relatively new and promising approach to plant genetic transformation with the distinct advantages of applicability to any intact plant tissue or region of the plant and with no host range limitation. The term "biolistic" (biological ballistics) was coined to describe the nature of the delivery of foreign DNA into living cells or tissues through "bombardment" with a biolistic device (a particle gun). The concept and process of microprojectile bombardment was first described in detail by Sanford *et al.* (1987). These high velocity microprojectiles are capable of penetrating cell walls. They are coated with a DNA solution, so that when they enter the cells, the DNA is carried as well. Since thousands of tungsten particles are accelerated simultaneously, foreign DNA is also delivered into numerous cells. The biolistic process has resulted in successful biolistic transformation of a wide range of tissues in a wide range of plant species.

Klein *et al.* (1987) first reported a method in which high velocity tungsten microprojectiles accelerated by a device known as a particle gun were employed to deliver nucleic acids into living plant cells. In their experiments, the transient expression of exogenous RNA or DNA was observed in epidermal cells of onions (*Allium cepa*). Evidence that the biolistic process will have broad use has been growing. Following the original observation by Klein *et al.* (1987), the biolistic delivery of foreign DNA into corn cells was also achieved (Klein *et al.* 1988a; 1988b). The technique of particle bombardment has been shown to be the most versatile and effective way for the creation of many transgenic organisms. It has been demonstrated that the process could be used to deliver biologically active DNA into plant cells that results in the recovery of stable transformants (Christou *et al.*, 1988; Wang *et al.*, 1988; Cao *et al.*, 1989; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). Biolistic transformation of higher animals has also been demonstrated *in vitro* (Zelenin *et al.*, 1989), and *in vivo* (Yang *et al.*, 1990; Williams *et al.*, 1991).

Microprojectile bombardment has proven to be effective even in very small cell types, and has, therefore, been useful in transforming diverse microbial species. These include eukaryotes such as yeast, filamentous fungi (Armeleo *et al.*, 1990), and algae (Day *et al.*, 1990); and prokaryotes such as *Bacillus megaterium* (Shark *et al.*, 1991), *Pseudomonas syringae*, *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Erwinia stewartii*, and *Escherichia coli* (Smith *et al.*, 1992).

The biolistic process also made the transformation of organelle genomes possible. Chloroplasts of *Chlamydomonas reinhardtii* can now be routinely transformed (Boynton *et al.*, 1988), along with mitochondria of yeast and *Chlamydomonas* (Johnston *et al.*, 1988; Fox *et al.*, 1988). Higher plant chloroplasts can be either transiently or stably transformed using the biolistic process (Daniell *et al.*, 1990; Ye *et al.*, 1990; Svab *et al.*, 1990).

The ability of microprojectile bombardment to deliver foreign DNA into regenerable plant cells, tissues, or organs appears to provide a reliable and dependable method for achieving truly genotype-independent transformation in many important agronomic crops, bypassing the limitations of *Agrobacterium* host-specificity and the tissue culture-related regeneration difficulties. Transformed plant tissues include cell suspension, calli, immature embryos, mature embryo parts, meristems, leaf pieces, and pollens. Due to the physical nature of this technique, there is no biological limitation and barrier to the actual DNA delivery process; thus, genotype is not a limiting factor. It appears that we have a reliable system in place for plant transformation by combining the relative ease of foreign DNA introduction into plant cells, tissues, or organs with an efficient regeneration protocol that avoids protoplast or suspension culture.

Important advances and refinements in the process of plant transformation, which will be described subsequently, have been made using soybean (McCabe *et al.* 1988; Christou *et al.* 1990) and rice (Christou *et al.* 1991) as model systems for dicotyledonous and monocotyledonous species, respectively. These systems have demonstrated the power and versatility of the microprojectile bombardment method in achieving genotype-independent transformation. Christou (1994) listed four major advantages that make microprojectile bombardment the method of choice for genetically engineering various crops: (1) transformation of organized tissue, (2) universal delivery system, (3) transformation of recalcitrant species, and (4) study of basic plant development processes.

### Instruments

A number of different instruments are currently in use based on various mechanisms to accelerate microscopic particles to supersonic speeds. Of these various acceleration methods, the only method that has been proven to be of general value thus far is the acceleration of microprojectiles on the face of a macroscopic carrier, or " macroprojectile". In all cases, the macroprojectile is driven by a gas shock, which can be achieved by using a gunpowder (chemical explosion) device (Sanford *et al.*, 1987), an apparatus based on electric discharge (an electric explosion of a water droplet) (Christou *et al.*, 1990), a microtargeting apparatus (Sautter *et al.*, 1991), a pneumatic instrument (Iida *et al.*, 1990), an instrument based on flowing helium (Takeuchi *et al.*, 1992; Finer *et al.*, 1992), or an improved version of the original gunpowder device utilizing compressed helium (Sanford *et al.*, 1991). Hand-held devices for both the original Biolistics® device and the Accell® device are also in use. The most popular and widely-used instrument is the one currently marketed by Bio-Rad, Inc. (Biolistics®). The macroprojectile may be any lightweight object that has a front surface that can carry microprojectiles, a back surface that can receive the energy of the gas shock, and sufficient cohesive integrity to withstand the gas shock, sudden acceleration, and violent deceleration.

## Critical Parameters in Optimizing the Biolistic Process for Different Applications

In order to optimize the frequency of genetic transformation, a number of parameters have been identified which need to be considered carefully when using particle bombardment. The most convenient measure of efficiency for DNA delivery into plant cells by microprojectile bombardment is the number of cells transiently expressing a GUS (ß-glucuronidase) gene. The GUS reporter system of Jefferson *et al.*, (1987) has been the most useful method in determining the number of transiently expressing cells per bombardment. One to two days after bombardment with the GUS reporter gene, target cells can be incubated with GUS histochemical substrate (5-bromo-4-chloro-3-indoyl-ß-D-glucuronic acid). Those cells which express GUS stain blue, and the number and distribution of the cells receiving and expressing the GUS gene are easily visualized. However, investigators should not over-emphasize the significance of transient expression data. Because optimization or maximization of transient activity does not necessarily result in optimal stable transformation, transient expression studies should only be used as a guide to develop systems for the stable transformation of a given species.

At least five key factors interact to affect the frequency of stable transformation in the bombarded cells (Birch and Franks, 1991):

1. size and composition of the microprojectiles,
2. DNA attachment to the microprojectiles prior to bombardment,
3. impact velocity of the microprojectile/DNA complex,
4. type of tissue and degree of cell damage suffered on bombardment, and
5. genetic construct.

### *Microprojectile Size and Composition*

Several features need to be considered when choosing materials to be used for microprojectiles in gene transfer work.

1. Particles should be of high density in order to possess adequate momentum to penetrate into the appropriate tissue.
2. Microprojectiles should be available in a range of defined sizes.
3. Metals should be relatively inert to reduce the likelihood of explosive oxidation and to prevent adverse reactions with cell components.
4. There should be no reactivity between particles and DNA and other components of the precipitating mixes.
5. They should have desirable agglomeration and dispersion properties.

Tungsten and gold microprojectiles meet most of these requirements and have been widely used in biolistic bombardment experiments. Tungsten particles are extremely irregular in shape and heterogeneous in size. Although different mean sizes range from 0.4 to 1.7 μm, their distributions overlap extensively. The advantages of tungsten are that it is very inexpensive, it is available in numerous sizes, each size represents a broad spectrum of particle diameters, and it is easily coated with DNA. The disadvantages are that it is potentially toxic to certain cell types; it is subject to surface oxidation that can alter DNA binding; over time, it catalytically degrades the DNA bound to it; and it is highly heterogeneous in shape and size, which prevents optimization of size for a particular cell type.

Gold particles which are available in a very limited range of sizes, are much rounder and more uniform in size than tungsten. A principal advantage of gold particles is their uniformity, which allows for optimization of size relative to a given cell type, assuming one of the few available sizes happens to be optimal. An even more important advantage of gold is that it is biologically inert. Unlike tungsten, gold does not catalytically attack the DNA bound to it. A major disadvantage of gold is that it is relatively expensive. Another concern with gold particles is that it is not stable in sterile aqueous suspensions and over a period of time agglomerates irreversibly (Sanford, 1993).

The optimal size and impact velocity of the microprojectiles depend on properties of the target cells such as size, penetrability, and resilience. Those working with plant cells generally utilize microprojectiles 1.0-2.0 μm in diameter. For example, Klein *et al.* (1988b) found higher transient expression levels when maize suspension culture cells were bombarded with DNA-coated tungsten particles with an average diameter of 1.2 μm than with average diameters of 0.6 μm and 2.4 μm. However, Casas *et al.* (1993) bombarded immature zygotic embryos of sorghum with tungsten microprojectiles 1.7 μm in diameter or with gold microprojectiles ranging from 1.5-3.0 μm in diameter and detected little difference based on the types of microprojectiles used.

### *DNA Attachment*

Any effective procedure for attaching DNA to microprojectiles is probably applicable to all cell types regardless of the other optimized bombardment conditions. However, the microprojectile coating is one of the most important sources of variation affecting biolistic efficiency. Apparently, each time DNA is precipitated, its pattern of precipitation and aggregation is unique and nonreproducible. The precipitation occurs so rapidly that it is nearly impossible to obtain a uniform reaction mixture---especially because gold or tungsten particles are difficult to keep in suspension.

DNA is usually bound to tungsten particles by variations of the $CaCl_2$/spermidine co-precipitation method developed by Klein *et al.* (1988a, 1988b). The particles coated with DNA are washed with 70% ethanol. A second wash with 100% ethanol is recommended. The DNA-coated particles are gently pelleted, brought up to final volume with 100% ethanol, and then pipetted onto the surface center of macroprojectiles. It is usually recommended to wait for the particles to dry thoroughly before using them, although DNA-coated particles may dry as they are propelled through the vacuum chamber towards the target. In contrast, DNA-coated gold particles was dried under a stream of nitrogen before resuspened in 100% ethanol. The gold particle suspension was then pipetted and dried onto the carrier surface before acceleration (McCabe *et al.*, 1988). The nature, form, and concentration of the DNA need also be considered. Once particles have been coated with DNA they should be used as soon as possible. This is particularly true when tungsten particles are used, because the tungsten can degrade the DNA. Exposure to humidity during or after drying dramatically reduces transformation frequency, apparently due to hygroscopic clumping and agglomeration (Smith *et al.*, 1992).

### *Microprojectile Velocity*

Even for a single plant species, it may be necessary to alter microprojectile velocity for optimal transformation frequencies with different tissue types, depending on cell wall thickness and the need to penetrate several cell layers. Although there may be substantial variation between consecutive shots, velocity can generally be controlled to some degree by altering the accelerating force, the vacuum in the chamber, or the distance traveled by the microprojectiles.

### *Tissue Type and Tissue Damage*

It is very important to target cells that are competent for both transformation and regeneration. It is apparent that different tissues have different requirements; extensive histology needs to be performed in order to ascertain the origin of regenerating tissue in a particular transformation study. Depth of penetration thus becomes one of the most important variables, and the ability to tune a system to achieve particle delivery to specific cell layers may make the difference between success and failure in recovering transgenic plants from a given tissue. In addition, experiments performed with synchronized cultured cells indicated that transformation frequencies might also be influenced by cell cycle stage (Iida *et al.*, 1991).

When a microprojectile bombardment device is employed to transform plant cells, the velocities of the particles which are sufficient for gene delivery frequently damage target tissues, especially those near the center of the target. The injury is attributed to physical trauma to the cells from the gas blast and acoustic shock generated by the device. The use of baffles or mesh screens reduce cell death and increase transformation frequency significantly (Gordon-Kamm *et al.*, 1990; Russell *et al.*, 1992).

The addition of an osmoticum (i.e., a supplemental agent increasing osmolarity) to the bombardment medium can increase the frequencies of transformation. Armaleo *et al.* (1990) observed that yeast cells which received bombarding particles usually suffered cytoplasmic extrusions. Higher yeast transformation frequencies were achieved by using a medium of high osmolarity, possibly due to protection against osmotic disruption following cell wall damage and improvement of particle penetration. In tobacco cell suspension cultures, 2- to 10-fold higher rates of both transient and stable transformations were obtained when at least 300-900 mOsm/kg $H_2O$ osmoticum was included in the bombardment medium (Belefant and Fong, 1989). Osmotic pretreatment of target tissues have also been shown to be important in other cases (Russell *et al.*, 1992; Vain *et al.*, 1993).

### *Genetic Construct*

When optimizing a microprojectile bombardment system, it is important to choose a genetic construct that will be expressed at reasonably high frequencies in the target tissue. The GUS histochemical assay is sometimes insensitive, and cells expressing the GUS gene at a relatively low level may not be revealed. It was found that a change from CaMV 35S to a stronger monocot promoter with sugarcane suspension cultures resulted in a 100-fold increase in the apparent frequency of transiently expressing cells based on GUS histochemical staining (Franks and Birch, 1991). Bruce *et al.* (1989) also demonstrated the effects of the gene regulatory sequences, the genetic background of the target tissue, and the environment stimuli on transient expression levels in bombarded tissues.

As for other direct gene transfer techniques, genes for transfer on high-velocity microprojectiles need not be arranged between any *Agrobacterium* T-DNA or viral sequences, and are generally in a small, high copy number *E. coli* cloning vector with appropriate regulatory sequences. Genes can be biolistically delivered as RNA or DNA (Klein *et al.*, 1987), in circular or linear form (Blower *et al.*, 1989), and as single-stranded (Blower *et al.*, 1989) or double-stranded DNA. However, there are some indications that large plasmids (>10 kbp) may be more subject to fragmentation during particle bombardment, resulting in lower expression frequencies and cotransformation frequencies (Mendel *et al.*, 1989; Fitch *et al.*, 1990).

### Remaining problems

The powerful biolistic transformation method, however, is still subject to a number of limiting factors (Hunold *et. al.*,. 1994). Apart from technical considerations, including the type of gun and experimental set-up, some tissues may be resistant to particle penetration due to a strong cuticle, lignified cell walls or a hairy surface. Even in the best experimental systems, only a small fraction of the total cell population will be penetrated by microprojectiles and, of these, not all will express the introduced gene. Transient expression in bombarded suspension culture cells may be observed in 0.1-0.3% of all cells (Iida *et. al.*, 1990; Yamashita *et. al.*, 1991), but the level of stable transformants is much lower (< 0.05%) (Klein *et. al.*, 1989; Russell *et. al.*, 1993). This low efficiency requires either a very high number of experiments or an extremely efficient selection and regeneration protocol. The unfavorable ratio between total cell population and cells capable of expressing the foreign gene, and the even lower proportion of stable transformation events are probably the most serious limitations to the particle bombardment technique.

## Microinjection of DNA into Plant Cells

Microinjection of DNA into cells using capillary micropipettes is one of the most direct methods of delivering foreign DNA into specific cell compartments. The most successful example is the microinjection of plasmid DNA carrying the *nptII* gene into embryoids derived from microspores (12-cell stage) of oilseed rape (Neuhaus, 1987). Among the 80% of embryoids regenerating to plants, half were stably transformed. Although many of these plants proved to be chimeric, they segregated *in vitro* through embryogenesis to give stable transformants. This clearly demonstrates that cells in meristematic clumps capable of plant regeneration can be transformed by microinjection of DNA. Microinjection may also be of some value in transient assays where gene transfer to particular cell types can give information about cell-specific expression and cell lineage. A visual marker such as *gus* can be detected in a single cell. The major limitations to this method are that it is technically demanding, requires the availability of small clusters of embryogenic cells and often results in chimeric plants.

## Macroinjection

Zhou *et al.* (1983) injected total DNA from one cotton variety into the embryos of another cotton variety and obtained a large number of plants. Several of the recipient plant progeny ($R_1$ through $R_4$) showed morphological characteristics that resembled those of the donor plant. de la Peña *et al.* (1987) reported the recovery of transgenic rye plants containing a foreign gene that conferred resistance to the antibiotic kanamycin. In their method, plasmid DNA containing a selectable antibiotic resistance gene was suspended in a buffer medium and injected with a syringe needle directly into the lumen of the developing inflorescence about 14 days before meiosis. The injected plants were allowed to produced seeds, which were screened for resistance to kanamycin. Seven seedlings survived the kanamycin selection and two of these showed neomycin phosphotransferase activity. Stable integration of the resistance gene was demonstrated in resistant seedlings by Southern analysis. However, the subsequent work with barley was disappointing and the frequency of transformation was very low (Mendel *et al.*, 1990). The NPTII activity corresponding to about one percent of that of stably transformed tobacco plants was detected, but the genes were not stably inherited.

## Pollen Tube Pathway

This method was reported by Lou and Wu (1988) using a plasmid-encoded gene, neomycin phosphotransferase, as a reporter. Plasmid DNA carrying the selectable antibiotic resistance gene was simply applied to the cut surface of the stigma for some time after pollination (5-20 minutes to 2-3 hours, depending upon the rate of pollen tube growth). They presented preliminary molecular evidence of gene transfer by both genomic blot hybridization and NPTII enzyme assays in transgenic plants. The transformation frequency was about 2% based on the number of rice florets treated with plasmid DNA solution. In similar work with barley, transformation frequencies of $10^{-3}$ to $10^{-4}$ in $F_1$ seedlings were obtained, but only a low level of expression of the introduced *nptII* gene was detected and this was lost in mature plants and in the next generation (Mendel *et al.*, 1990).

## Direct DNA Uptake into Imbibing Dry Zygotic Embryos

The uptake of DNA by dry plant tissue through membranes, whose physiochemical characteristics change while natural desiccation occurs, was proposed as an alternative and simple way of gene transfer (Töpfer *et al.*, 1989; Senaratna *et al.*, 1991). Töpfer's group isolated embryos of seven cereals and three legumes by blowing off the bran of the cereals and subsequently separating the endosperms from the embryos using cyclohexane and carbon tetrachloride (Töpfer *et al.*, 1989). The intact embryos were selected and imbibed in a solution of plasmid DNA carrying a chimeric NPTII gene. Molecular evidence was presented for the transient expression of the foreign gene. The authors suggested that this method was suitable for transient gene expression studies.

Senaratna *et al.* (1991) also employed this simple strategy for gene transfer which took advantage of two phenomena occurring in dry tissues : the huge water potential gradient existing between the cell and a water solution (Hegarty, 1978) and the high permeability of disorganized cell membranes at low water contents (Simon, 1974; Hoekstra *et al.*, 1989). Dry somatic embryos of alfalfa (*Medicago sativa* L.) produced *in vitro* from cell cultures were induced to become desiccation tolerant, dried to approximately 10 to 15% moisture, and then used as a target for direct DNA uptake.

Water uptake into these dry somatic embryos was much more rapid than in a true seed because they lacked a seed coat and endosperm (McKersie *et al.*, 1989). To determine whether it was also permeable to large plasmid DNA molecules, dry somatic embryos were imbibed in a solution containing a plasmid carrying the *ß*-glucuronidase (GUS) reporter gene. Transient expression of the GUS gene was observed visually in germinating embryos and seedlings. Though PCR analysis of plant DNA from epidermal cells of somatic embryos was presented, further studies are required to test the stability and hereditability of these transformants. Since this system is suited for transient expression assays as a simple and quick method of testing the effectiveness of vector constructs (Lee *et al.*, 1989), it may be necessary to redesign these direct DNA uptake vectors to allow stable integration of the introduced DNA into the plant genome.

## Laser-mediated Gene Transfer

Laser microbeam irradiation has been used successfully for more than twenty years to manipulate subcellular structure, induce cell fusion, microdissect chromosomes and perform other subcellular microsurgery (Berns and Rounds, 1970; Berns *et al.*, 1991). Recently, this application has been expanded to introduce foreign DNA into mammalian cells (Tsukakoshi *et al.*, 1984; Tao *et al.*, 1987). Working with plant cells, Weber *et al.* (1989, 1990) reported that they could use a laser to puncture holes in the cell walls and the membranes of *Brassica napus* when their chloroplasts and cells were treated with a hypertonic buffer. Therefore, it was theoretically possible to introduce foreign DNA into plant cells and cell organelles.

Guo *et al.* (1995) reported an effective system for introducing exogenous DNA into cells of embryogenic calli of Japonica cultivar ZY1 of *Oryza sativa* L.. Plant cells were pretreated in the hypertonic buffer to draw some of the water from the cells, put into a medium of less negative osmotic potential containing exogenous DNA, and treated immediately with a laser microbeam (wavelength = 355 nm) to puncture holes in the cell wall and membrane. The pretreatment of the cells generated a gradient of osmotic pressure between the inside and outside of the cells, which facilitated the uptake of material into the cells through the laser perforations. *B*-glucuronidase (GUS) genes were successfully introduced into rice cells, as indicated by gene expression both in post-treated cells and in plantlets regenerated from kanamycin-resistant calli that had been treated by this method.

### Silicon Carbide Whisker-mediated Transformation

In contrast to some of the technically demanding methods described above, there have also been reports on the use of silicon carbide whiskers to develop a simple and inexpensive system for the regeneration of fertile and transgenic plants. Transformation of cell lines of maize (Kaeppler *et al.*, 1990, 1992), tobacco (Kaeppler *et al.*, 1990), *Agrostis alba* (Asano *et al.*, 1991), and, most recently, *Chlamydomonas reinhardtii* (Dunahay, 1993) have been demonstrated using such whiskers. The method involves the mixing (e.g., by vortex treatment) of cells in a liquid medium with whiskers and plasmid DNA. The resulting collisions between cells and whiskers appear to lead to cell penetration and DNA delivery (Kaeppler *et al.*, 1990).

Although stable transformation has been achieved, fertile transgenic plants were never obtained in any of the cases mentioned above. Frame *et al.* (1994), however, reported the production of fertile transgenic maize plants using the silicon carbide whisker transformation method. Cells from embryogenic maize suspension cultures were transformed using silicon carbide whiskers to deliver plasmid DNA carrying the bacterial *bar* and *uid*A (GUS) genes, and stable transformants were selected on a medium containing bialaphos (one mg/l). Integration of the *bar* gene and activity of the enzyme phosphinothricin acetyl transferase (PAT) were confirmed in all bialaphos-resistant callus lines analyzed. Fertile transgenic maize plants were regenerated from those cell lines.

Herbicide spraying of progeny plants revealed that the *bar* gene was transmitted in a Mendelian fashion.

## Range of Transformed Species

In the late 1980s, it became clear that some plant species were amenable to genetic engineering (Goldberg, 1988; Gasser and Fraley, 1989). This is because foreign genes can be stably introduced into plant chromosomal DNA by a variety of techniques (Potrykus, 1991). If a given foreign gene contains the appropriate regulatory sequence, the gene product will be synthesized by the transgenic plant (Benfey and Chua, 1989). Since most plant cells are totipotent, this allows regeneration of a fertile 'transgenic' plant from a single transgenic cell. However, in practice, not all plant species have proven to be amenable to regeneration from dedifferentiated tissue (Potrykus, 1991).

In general, dicot plants are easier to transform and regenerate from protoplasts or explant sections than most monocot species (Potrykus, 1991). It has been difficult to generate transgenic monocot plants, which include the economically important crops such as rice, corn, and wheat. One reason is that this group of plants is not usually susceptible to infection by *A. tumefaciens*. While it is not a problem to deliver exogenous DNA to protoplasts derived from monocot crop species, it is difficult to regenerate viable or fertile plants from the transformed cells (Potrykus, 1990; 1991). However, there has been progress in the area of regeneration, especially in the cases of rye, corn, and rice (Gasser and Fraley, 1989; Gordon-Kamm *et. al.*, 1990; Lynch *et al.*, 1991). Also, with the successful development of biolistic transformation methods for plant genetic engineering, one can obtain transgenic monocot plants if the foreign DNA can be stably introduced prior to or during embryogenesis and the embryos are still alive after being bombarded with microprojectiles. For instance, corn and rice embryos bombarded with

**Table 1**. Examples of Transgenic Plants and Transformation Methods Used.

| Plants | Transformation method | Tissue source | Selective agent | Referrence |
|---|---|---|---|---|
| **Dicots** | | | | |
| *Actinidia deliciosa* (kiwi) | *Agrobacterium* | leaf | kanamycin | Rugini *et al*., (1991) |
| *Apium graveolens* (celery) | *Agrobacterium* | petiole | kanamycin | Catlin *et al*., (1988) |
| *Arabidopsis thaliana* (arabidopsis) | *Agrobacterium* | leaf | kanamycin | Lloyd *et al*., (1986) |
| | *Agrobacterium* | seed | kanamycin, G418 | Feldmann and Marks, (1987) |
| | *Agrobacterium* | root | kanamycin | Valvekens *et al*., (1988) |
| | *Agrobacterium* (inoculation *in planta*) | apical shoot | none | Chang *et al*., (1994) |
| | *Agrobacterium* | hypocotyl | phosphinothricin | Akama *et al*., (1995) |
| | Microprojectile | root | kanamycin | Seki *et al*., (1991) |
| *Arachis hypogaea* (peanut) | Microprojectile | meristem | phosphinothricin | Brar *et al*., (1994) |
| *Armoracia lapathifolia* (horseradish) | *Agrobacterium* | leaf | none | Noda *et al*., (1987) |
| *Atropa belladona* (belladona) | *Agrobacterium* | leaf | kanamycin | Mathews *et al*., (1990a) |

**Table 1** (Cont'd).

| Plants | Transformation method | Tissue source | Selective agent | Referrence |
|---|---|---|---|---|
| *Beta vulgaris* (sugarbeet) | *Agrobacterium* | shoot | kanamycin | Lindsey and Gallois, (1990) |
| | *Agrobacterium* | embryogenic callus | phosphinothricin<br>chlorsulfuron | D'Halluin *et al*., (1992a) |
| *Brassica jaucea* (mustard) | *Agrobacterium* | cotyledonary callus | kanamycin | Mathews *et al*., (1990b) |
| *Brassica napus* (oilseed rape) | *Agrobacterium* | stem | kanamycin | Fry *et al*., (1987) |
| | *Agrobacterium* | stem | methotrexate | Pua *et al*., (1987) |
| | *Agrobacterium* | suspension cell | kanamycin<br>chloramphenicol | Charest *et al*., (1988) |
| | *Agrobacterium* | hypocotyl | kanamycin | Radke *et al*., (1988) |
| | *Agrobacterium* | hypocotyl | kanamycin<br>paromomycin | De Block *et al*., (1989) |
| | *Agrobacterium* | petiole | kanamycin | Moloney *et al*., (1989) |
| | *Agrobacterium* | microspore<br>proembryo | kanamycin<br>hygromycin | Pechan, (1989) |
| | *Agrobacterium* | cotyledon | kanamycin<br>hygromycin | Boulter *et al*., (1990a) |
| | *Agrobacterium* | inflorescence | kanamycin | Boulter *et al*., (1990a) |

**Table 1** (Cont'd).

| Plants | Transformation method | Tissue source | Selective agent | Referrence |
|---|---|---|---|---|
| *Brassica napus* (oilseed rape) | *Agrobacterium* | protoplast | kanamycin | Thomzik and Hain, (1990) |
| | Electroporation | protoplast | kanamycin | Guerche *et al*., (1987) |
| | Microinjection | microspore embryoid | kanamycin | Neuhaus *et al*., (1987) |
| (canola) | *Agrobacterium* | stem | kanamycin | Miki *et al*., (1990) |
| *Brassica oleracea* (cauliflower) | *Agrobacterium* | leaf | kanamycin | Srivastava *et al*., (1988) |
| | *Agrobacterium* | hypocotyl | kanamycin<br>phosphinothricin | De Block *et al*., (1989) |
| *Carica papaya* (papaya) | Microprojectile | somatic embryo hypocotyl | kanamycin | Fitch *et al*., (1990) |
| | Microprojectile | zygotic embryo | kanamycin | Fitch *et al*., (1992) |
| *Citrus jambhiri* (lemon) | PEG | protoplast | paromomycin | Vardi *et al*., (1990) |
| *Convolvulus arvensis* (morning glory) | *Agrobacterium* | stem | kanamycin | Slightom *et al*., (1985) |
| *Craterostigma plantagineum* | *Agrobacterium* | leaf | kanamycin | Furini *et al*., (1994) |
| *Cucumis melo* (muskmelon) | *Agrobacterium* | cotyledon | kanamycin | Fang and Grumet, (1990) |

**Table 1** (Cont'd).

| Plants | Transformation method | Tissue source | Selective agent | Referrence |
|---|---|---|---|---|
| *Cucumis sativus* (cucumber) | *Agrobacterium* | hypocotyl | kanamycin | Trulson *et al*., (1986) |
| | *Agrobacterium* | cotyledon | kanamycin | Chee, (1990) |
| | Microprojectile | embryogenic callus | kanamycin | Chee and Slightom, (1992) |
| *Datura innoxia* | *Agrobacterium* | pollen embryo | kanamycin | Sangwan *et al*., (1993) |
| | *Agrobacterium* | zygotic embryo | kanamycin | Ducrocq *et al*., (1994) |
| *Daucus carota* (carrot) | *Agrobacterium* | suspension cell | kanamycin | Scott and Draper, (1987) |
| | *Agrobacterium* | hypocotyl | kanamycin | Thomas *et al*., (1989) |
| | *Agrobacterium* | callus | kanamycin | Wurtele and Bulka, (1989) |
| *Dendranthema grandiflora* (chrysanthemum) | *Agrobacterium* | leaf | kanamycin | Courtney-Gutterson *et al*., (1992) |
| | *Agrobacterium* | node | hygromycin | Renou *et al*., (1993) |
| | *Agrobacterium* | leaf | kanamycin | Pavingerová *et al*., (1994) |
| *Dendrobium orchid* (dendrobium) | Microprojectile | protocorm | kanamycin | Kuehnle *et al*., (1992) |
| *Festuca arundinacea* (tall fescue) | Electroporation | protoplast | hygromycin | Ha *et al*., (1992) |
| | PEG | protoplast | hygromycin<br>phosphinothricin | Wang *et al*., (1992) |
| *Fragaria* x *ananassa* (strawberry) | *Agrobacterium* | leaf, petiole | kanamycin | James *et al*., (1990) |
| | *Agrobacterium* | leaf | kanamycin | Nehra *et al*., (1990) |

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| *Glycine canescens* | *Agrobacterium* | hypocotyl | kanamycin | Rech *et al.*, (1989) |
| *Glycine max* (soybean) | *Agrobacterium* | cotyledon | kanamycin | Hinchee *et al.*, (1988) |
| | *Agrobacterium* | germinating seed | none | Chee *et al.*, (1989) |
| | *Agrobacterium* | immature embryo | kanamycin | Parrott *et al.*, (1989) |
| | Microprojectile | zygotic embryo | kanamycin | Christou *et al.*, (1988) |
| | Microprojectile | meristem | none | McCabe *et al.*, (1988) |
| | Microprojectile | meristem | glyphosate<br>kanamycin<br>phosphinothricin | Christou *et al.*, (1990) |
| | Electroporation | protoplast | kanamycin | Dhir *et al.*, (1992) |
| *Gossypium hirsutum* (cotton) | *Agrobacterium* | cotyledon | kanamycin | Firoozabady *et al.*, (1987) |
| | *Agrobacterium* | hypocotyl | kanamycin | Umbeck *et al.*, (1987) |
| | *Agrobacterium* | hypocotyl | 2,4-D | Bayley *et al.*, (1992) |
| | Microprojectile | suspension cell | kanamycin | Finer and McMullen, (1990) |
| | Microprojectile | meristem | none | McCabe and Martinell, (1993) |
| ***Helianthus annuus*** (sunflower) | *Agrobacterium* | hypocotyl | kanamycin | Everett *et al.*, (1987) |
| | *Agrobacterium* | meristem | kanamycin | Schrammeijer *et al.*, (1990) |

*Table 1 (Cont'd).*

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| (sunflower) | *Agrobacterium*, Microprojectile (for wounding) | meristem | kanamycin | Bidney *et al.*, (1992) |
|  | *Agrobacterium*, Glass bead (for wounding) | seedling | kanamycin | Grayburn and Vick, (1992) |
| *Ipomoea batatas* (sweet potato) | *Agrobacterium* | leaf | kanamycin | Otani *et al.*, (1993) |
|  | *Agrobacterium* | storage root | kanamycin | Newell *et al.*, (1995) |
| *Kalanchöe laciniata* | *Agrobacterium* | leaf | kanamycin | Jia *et al.*, (1989) |
| *Juglans regia* (walnut) | *Agrobacterium* | somatic embryo | kanamycin | McGranahan *et al.*, (1988; 1990) |
| *Lactuca sativa* (lettuce) | *Agrobacterium* | cotyledon | kanamycin | Michelmore *et al.*, (1987) |
|  | Electroporation | protoplast | kanamycin<br>paromomycin | Chupeau *et al.*, (1989) |
| *Linum usitatissimum* (flax) | *Agrobacterium* | hypocotyl | kanamycin | Basiran *et al.*, (1987) |
|  | *Agrobacterium* | hypocotyl | kanamycin | Jordan and McHughen, (1988) |
|  | *Agrobacterium* | hypocotyl | kanamycin, G418 | Dong and McHughen, (1993) |
| *Liriodendron tulipifera* (yellow poplar) | Microprojectile | suspension cell | kanamycin | Wilde *et al.*, (1992) |

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| *Lotus corniculatus* (trefoil) | *Agrobacterium* | seedling | hygromycin | Stougaard-Jensen *et al.*, (1986) |
| *Lycopersicon esculentum* (tomato) | *Agrobacterium* | leaf | kanamycin | Horsch *et al.*, (1985) |
| | *Agrobacterium* | leaf | kanamycin | McCormick *et al.*, (1986) |
| | *Agrobacterium* | cotyledon | kanamycin | Shahin *et al.*, (1986b) |
| | *Agrobacterium* | cotyledon | kanamycin<br>glyphosate | Fillatti *et al.*, (1987a) |
| | *Agrobacterium* | leaf | chloramphenicol | Benfey and Chua, (1990) |
| | *Agrobacterium* | leaf | kanamycin | Perlak *et al.*, (1991) |
| | Microprojectile | suspension cell | kanamycin | Van Eck *et al.*, (1995) |
| | PEG | protoplast | kanamycin | Koornneef *et al.*, (1986) |
| *Lycopersicon pennelli* | Microprojectile | suspension cell | kanamycin | Van Eck *et al.*, (1995) |
| *Lycopersicon peruvianum* | Electroporation | protoplast | chlorsulfuron<br>paromomycin | Bellini *et al.*, (1989) |
| *Malus pumila* (apple) | *Agrobacterium* | leaf | kanamycin | James *et al.*, (1989) |
| *Malus* X *domestica* (commerical apple) | *Agrobacterium* | leaf | kanamycin | Yao *et al.*, (1995) |
| *Medicago sativa* | *Agrobacterium* | stem | kanamycin | Shahin *et al.*, (1986a) |

*Table 1 (Cont'd).*

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| (alfalfa) | *Agrobacterium* | stem | none | Sukhapinda *et al.*, (1987) |
| | *Agrobacterium* | stem, petiole | hygromycin<br>kanamycin<br>phosphinothricin | D'Halluin *et al.*, (1990) |
| | *Agrobacterium* | leaf, petiole | kanamycin | Thomas *et al.*, (1994) |
| | Micorprojectile | embryogenic callus | kanamycin | Pereira and Erickson, (1995) |
| *Medicago varia* (*M. sativa* x *M. falcata*) | *Agrobacterium* | stem | kanamycin | Deak *et al.*, (1986) |
| | *Agrobacterium* | leaflet | kanamycin | Chabaud *et al.*, (1988) |
| *Nicotiana alata* | *Agrobacterium* | hypocotyl | kanamycin | Ebert and Clarke, (1990) |
| *Nicotiana edwardsonii* | *Agrobacterium* | leaf | kanamycin, G418 | Kiernan *et al.*, (1989) |
| *Nicotiana plumbaginifolia* | *Agrobacterium* | protoplast | chloramphenicol<br>kanamycin<br>methotrexate | De Block *et al.*, (1984) |
| | *Agrobacterium* | protoplast | kanamycin | Horsch *et al.*, (1984) |
| | *Agrobacterium* | protoplast | kanamycin | Jones *et al.*, (1987) |
| | *Agrobacterium* | epidermal peel | kanamycin | Trinh *et al.*, (1987) |
| | *Agrobacterium* | leaf | kanamycin | Ellis *et al.*, (1988) |
| | PEG | protoplast | kanamycin | Negrutiu *et al.*, (1987) |

Table 1 (Cont'd).

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| *Nicotiana plumbaginifolia* | Lipofectin (Electropoation) | protoplast | kanamycin | Spörlein and Koop, (1991) |
| *Nicotiana tabacum* (tobacco) | *Agrobacterium* | protoplast | chloramphenicol<br>kanamycin<br>methotrexate | De Block *et al*., (1984) |
| | *Agrobacterium* | suspension cell | kanamycin | An *et al*., (1985) |
| | *Agrobacterium* | leaf | glyphosate<br>kanamycin | Comai *et al*., (1985) |
| | *Agrobacterium* | leaf | kanamycin | Horsch *et al*., (1985) |
| | *Agrobacterium* | leaf | kanamycin<br>phosphinothricin | De Block *et al*., (1987) |
| | *Agrobacterium* | leaf | kanamycin<br>streptomycin | Jones *et al*., (1987) |
| | *Agrobacterium* | leaf | chlorsulfuron<br>kanamycin | Haughn *et al*., (1988) |
| | *Agrobacterium* | leaf | hygromycin<br>kanamycin | Landsmann *et al*., (1988) |

**Table 1 (Cont'd).**

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| (tobacco) | *Agrobacterium* | protoplast | chlorsulfuron<br>kanamycin | Lee *et al.*, (1988) |
| | *Agrobacterium* | leaf | bromoxynil<br>kanamycin | Stalker *et al.*, (1988) |
| | *Agrobacterium* | leaf | hygromycin<br>kanamycin | Hatamoto *et al.*, (1990) |
| | *Agrobacterium* | leaf | chloramphenicol | Benfey and Chua, (1990) |
| | *Agrobacterium* | leaf | kanamycin | Perlak *et al.*, (1991) |
| | *Agrobacterium* | pollen embryo | kanamycin | Sangwan *et al.*, (1993) |
| | Calcium phosphate | protoplast | kanamycin | Hain *et al.*, (1985) |
| | Electroporation | protoplast | kanamycin | Riggs and Bates (1986) |
| | Electroporation, PEG | protoplast | kanamycin | Shillito *et al.*, (1985) |
| | Electroporation, PEG | protoplast | chloramphenicol<br>kanamycin | Pietrzak *et al.*, (1986) |
| | PEG | protoplast | kanamycin | Paszkowski *et al.*, (1984) |
| | PEG | protoplast | kanamycin | Negrutiu *et al.*, (1987) |
| | Liposome | protoplast | kanamycin | Deshayes *et al.*, (1985) |

*Table 1 (Cont'd).*

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| (tobacco) | Lipofectin (Electropoation) | protoplast | kanamycin | Spörlein and Koop, (1991) |
| | Microprojectile | leaf, suspension cell | kanamycin | Klein *et al.*, (1988c) |
| | Microprojectile | leaf | kanamycin | Tomes *et al.*, (1990) |
| | Microprojectile | embryogenic pollen | none | Stöger *et al.*, (1995) |
| *Pelargonium* spp. (lemon geranium) | *Agrobacterium* | leaf, stem | none | Pellegrineschi *et al.*, (1992) |
| *Petunia hybrida* (petunia) | *Agrobacterium* | leaf | kanamycin | Horsch *et al.*, (1985) |
| | *Agrobacterium* | protoplast | kanamycin | Nagy *et al.*, (1985) |
| | *Agrobacterium* | leaf | kanamycin<br>glyphosate | Horsch *et al.*, (1986) |
| | *Agrobacterium* | leaf | methotrexate | Eichholtz *et al.*, (1987) |
| | Electroporation, PEG | protoplast | kanamycin | Tagu *et al.*, (1988) |
| | *Agrobacterium* | meristem | kanamycin | Ulian *et al.*, (1988) |
| *Phaseolus vulgaris* (common bean) | Microprojectile | meristem | bialaphos | Russel *et al.*, (1993) |
| *Picea abies* (Norway spruce) | Microprojectile | somatic embryo | kanamycin | Robertson *et al.*, (1992) |

**Table 1** (Cont'd).

| Plants | Transformation method | Tissue source | Selective agent | Referrence |
|---|---|---|---|---|
| *Picea glauca* (white spruce) | Microprojectile | embryogenic callus | kanamycin | Ellis *et al.*, (1993) |
| *Pisum sativum* (pea) | *Agrobacterium* | shoot | hygromycin | Puonti-Kaerlas *et al.*, (1992) |
|  | *Agrobacterium* | meristem | kanamycin | Davies *et al.*, (1993) |
|  | *Agrobacterium* | zygotic embryo | phosphinothricin | Schroeder *et al.*, (1993) |
| *Populus alba* x *P. tremula* (aspen) | *Agrobacterium* | stem | kanamycin<br>phosphinothricin | De Block, (1990) |
| *Populus alba* x *P. didentata* (poplar) | *Agrobacterium* | leaf | kanamycin | Fillatti *et al.*, (1987b) |
|  | *Agrobacterium* | stem | kanamycin<br>phosphinothricin | De Block, (1990) |
|  | Microprojectile | nodule | kanamycin | McCown *et al.*, (1991) |
| *Populus tremuloides* (quaking aspen) | *Agrobacterium* | leaf | kanamycin | Tsai *et al.*, (1994) |
| *Prunus domestica* (plum) | *Agrobacterium* | hypocotyl | kanamycin | Scorza *et al.*, (1994) |
| *Prunus subhirtella autumno rosa* (cherry rootstock) | *Agrobacterium* | embryogenic callus | kanamycin | Machado *et al.*, (1995) |

**Table 1 (Cont'd).**

**Table 1** (Cont'd).

| Plants | Transformation method | Tissue source | Selective agent | Referrence |
|---|---|---|---|---|
| *Pseudotsuga menziesii* (Douglas-fir) | *Agrobacterium* | seedling, shoot | kanamycin | Dandekar *et al.*, (1987) |
| *Rosa hybrida* (rose) | *Agrobacterium* | embryogenic callus | kanamycin | Firoozabady *et al.*, (1994) |
| *Rubus ideaus* (red raspberry) | *Agrobacterium* | leaf, petiole | geneticin<br>hygromycin | Mathews *et al.*, (1995) |
| *Solanum melongena* (eggplant) | *Agrobacterium* | leaf | kanamycin | Guri and Sink, (1988) |
|  | *Agrobacterium* | leaf | kanamycin | Filippone and Lurquin, (1989) |
|  | *Agrobacterium* | leaf | kanamycin | Rotino and Gleddie, (1990) |
| *Solanum tuberosum* (potato) | *Agrobacterium* | shoot | kanamycin | Ooms *et al.*, (1987) |
|  | *Agrobacterium* | tuber | kanamycin | Stockhaus *et al.*, (1987) |
|  | *Agrobacterium* | leaf | kanamycin | De Block, (1988) |
|  | *Agrobacterium* | leaf | kanamycin<br>hygromycin | Knapp *et al.*, (1988) |
|  | *Agrobacterium* | tuber | kanamycin | Sheerman and Bevan, (1988) |
|  | *Agrobacterium* | microtuber | kanamycin | Ishida *et al.*, (1989) |
|  | *Agrobacterium* | leaf | kanamycin | Wenzler *et al.*, (1989) |
| *Solanum* spp. | *Agrobacterium* | microtuber | kanamycin | Kumar *et al.*, (1995) |

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| *Stylosanthes guianensis* | *Agrobacterium* | leaf | kanamycin<br>phosphinothricin | Sarria *et al*., (1994) |
| *Stylosanthes humilis* | *Agrobacterium* | leaf | kanamycin | Manners, (1988) |
|  | *Agrobacterium* | leaf, stem | kanamycin | Manners and Way, (1989) |
| *Trifolium repens* (white clover) | *Agrobacterium* | stolon internode | kanamycin | White and Greenwood, (1987) |
| *Trifolium subterraneum* | *Agrobacterium* | hypocotyl | phosphinothricin | Khan *et al*., (1994) |
| *Vaccinium macrocarpon* (cranberry) | Microprojectile | stem | kanamycin | Serres *et al*., (1992) |
| *Vigna aconitifolia* (moth bean) | *Agrobacterium* | protoplast | kanamycin | Eapen *et al*., (1987) |
| *Vitis rupestris* (grape) | *Agrobacterium* | hypocotyl | kanamycin | Mullins *et al*., (1990) |
| **Monocots** |  |  |  |  |
| *Agrostis palustris* (creeping bentgrass) | Microprojectile | embryogenic callus | none | Zhong *et al*., (1993) |
|  | Microprojectile | suspension cell | bialaphos | Hartman *et al*., (1994) |
| *Asparagus officinalis* (asparagus) | *Agrobacterium* | spear | kanamycin | Bytebier *et al*., (1987) |

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| *Avena sativa* (oat) | Microprojectile | embryogenic callus | phosphinothricin | Somers *et al.*, (1992) |
| *Dactylis glomerata* (orchardgrass) | Electroporation, PEG | protoplast | hygromycin | Horn *et al.*, (1988) |
| Hordeum chilense x *Triticum durum* (tritordeum) | Microprojectile | immature inflorescence | G418 | Barcelo *et al.*, (1994) |
| *Hordeum vulgare* (barley) | Microprojectile | zygotic embryo<br>embryogenic callus | bialaphos | Wan and Lemaux, (1994) |
| | Microprojectile | microspore | phosphinothricin | Jähne *et al.*, (1994) |
| | Microprojectile | immature embryo | none | Ritala *et al.*, (1994) |
| | Microprojectile | immature embryo | hygromycin | Hagio *et al.*, (1995) |
| *Musa acuminata* (banana) | *Agrobacterium* | apical meristem, corm | kanamycin | May *et al.*, (1995) |
| | Microprojectile | suspension cell | hygromycin | Sági *et al.*, (1995) |
| *Oryza sativa* (rice) | *Agrobacterium* | immature embryo | G418 | Chan *et al.*, (1993) |
| | *Agrobacterium* | embryogenic callus | hygromycin | Hiei *et al.*, (1994) |
| | Microprojectile | immature embryo | bialaphos<br>hygromycin | Christou *et al.*, (1991) |
| | Microprojectile | suspension cell | phosphinothricin | Cao *et al.*, (1992) |

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| (rice) | Electroporation | protoplast | G418 | Toriyama *et al.*, (1988) |
| | Electroporation | protoplast | bialaphos | Toki *et al.*, (1992) |
| | PEG | protoplast | hygromycin | Datta *et al.*, (1990) |
| | PEG | protoplast | G418 | Peng *et al.*, (1990) |
| | PEG | protoplast | chlorsulfuron<br>hygromycin | Li *et al.*, (1992b) |
| | PEG | protoplast | hygromycin | Li *et al.*, (1992a) |
| | Laser microbeam | embryogenic callus | kanamycin | Guo *et al.*, (1995) |
| *Saccharum officinarum* (sugarcane) | Microprojectile | embryogenic callus | kanamycin | Bower and Birch, (1992) |
| | Electroporation | embryogenic callus | none | Arencibia *et al.*, (1995) |
| *Secale cereale* (rye) | Microprojectile | embryogenic callus | phosphinothricin | Castillo *et al.*, (1994) |
| | Marcoinjection | immature inflorescence | none | De la Peña *et al.*, (1987) |
| *Sorghum bicolor* (sorghum) | Microprojectile | immature embryo | bialaphos | Casas*et al.*, (1993) |
| *Triticum aestivum* (wheat) | Microprojectile | embryogenic callus | phosphinothricin | Vasil *et al.*, (1992) |
| | Microprojectile | embryogenic callus | bialaphos | Weeks *et al.*, (1993) |
| | Microprojectile | immature embryo | phosphinothricin | Becker *et al.*, (1994) |

**Table 1** (Cont'd).

| **Plants** | **Transformation method** | **Tissue source** | **Selective agent** | **Referrence** |
|---|---|---|---|---|
| (wheat) | Microprojectile | somatic embryo | phosphinothricin | Nehra *et al.*, (1994) |
| | Marcoinjection | spikelets | none | Hess *et al.*, (1990) |
| | Electroporation | protoplast | phosphinothricin | He *et al.*, (1994) |
| *Zea mays* (maize) | *Agrobacterium* | shoot apex | kanamycin | Gould *et al.*, (1991) |
| | Microprojectile | embryogenic callus | chlorsulfuron | Fromm *et al.*, (1990) |
| | | suspension cell | phosphinothricin | |
| | Microprojectile | suspension cell | phosphinothricin | Gordon-Fromm *et al.*, (1990) |
| | Microprojectile | immature embryo (osmotic treatment) | phosphinothricin | Koziel *et al.*, (1990) |
| | Microprojectile | embryogenic callus | hygromycin | Walters *et al.*, (1992) |
| | Microprojectile | suspension cell | phosphinothricin | Vain *et al.*, (1993) |
| | Electroporation | suspension cell | phosphinothricin | Laursen *et al.*, (1994) |
| | Electroporation | immature embryo<br>embryogenic callus | kanamycin | D'Halluin *et al.*, (1992) |
| | PEG | protoplast | methotrexate | Golovkin *et al.*, (1993) |
| | Silicon carbide whisker | suspension cel | phosphinothricin | Frame *et al.*, (1994) |
| | mixture of pollen and exogenous DNA | silk | none | Ohta, (1986) |

DNA coated microprojectiles have been transformed by this method (Gordon-Kamm *et. al.* 1990; Christou *et. al.*, 1991).

By applying plant transformation methodology and recombinant DNA technology, many transgenic plants have been genetically engineered with different foreign DNA during the past ten years. Table 1 lists some examples of transgenic plants that have been produced and the transformation methods, tissue sources, and selective agents used to transform them. The number of plant species which has been transformed is increasing rapidly because the technology for stably inserting and expressing foreign genes in plants has been continuously improved, and more and more genes have been identified and linked together with the traits they control.

## Markers for Plant Gene Transfer

Genetic analysis of plant species would not be possible without the application of marker genes to distinguish differences between individual plants. Naturally occurring mutations which can be used as markers range from single base pair differences or restriction fragment length polymorphisms between homologous stretches of DNA to altered genes or chromosomes which affect morphological characters or enzyme-mediated biochemical reactions. In the past, plant genetic research has frequently been limited by the availability of desirable mutations which could be used as convenient markers. Now that an increasing number of plant species are amenable to genetic transformation by foreign DNA, marker genes can be constructed at will by recombining pieces of DNA from various *in vitro* sources.

## Applications of Marker Genes

### *Confirmation of DNA Delivery and Stable Transformation*

Marker genes play a crucial role in the development of transformation procedures because they can be used to ascertain how effectively the transforming DNA is being introduced into plant cells using different treatments (Walden, 1989). Selectable markers are generally used for stable transformation. They either allow living cells, tissue, or whole plants to grow under conditions which would prevent the growth of untransformed plant cells, or confer a phenotype which could allow transformed and untransformed tissue to be differentiated. In contrast, assayable markers direct the expression of a readily detectable gene product (e.g., an enzyme), which is not necessarily distinguishable in nontransformed living cells.

Assayable markers are frequently employed to optimize DNA delivery in the early stages of developing a transformation procedure. This is because a high level of marker gene expression in the first few days following the gene transfer is thought to be essential for stable transformation. However, a high level of transient gene expression may not always be sufficient. Some of the variables which affect transient gene expression (e.g., the number of transformed cells, the number of active templates inside those transformed cells, and the viability of the transformed cells following DNA delivery) are important parameters for ensuring stable transformation.

Other parameters, such as the integration of marker genes into the target cell genome and the cell division following DNA integration, are prerequisites for stable transformation, but are not necessarily related to the efficiency of transient marker gene expression. Similarly, the selection of stable transformants can be affected by the efficiency of marker gene expression at the site of integration, and this may be different from the level of expression observed prior to integration (Kay *et al.*, 1987; Odell *et al.*, 1988).

### *Quantification of Gene Expression*

Assayable markers are frequently used to quantify gene expression for reasons other than optimizing DNA delivery. Such markers are also commonly referred to as reporter genes. When fused to plant regulatory sequences *in vitro*, reporter genes are indispensable for the analysis of gene expression in transient assays or in stable transformants. Recombinant gene fusion can be used to determine when, where, and at what level regulatory sequences direct gene expression *in vivo* and which intra- or extracellular signals are involved (Benfey and Chua, 1989, 1990). Reporter genes can also be fused to peptide coding sequences to determine whether they are involved in targeting proteins either outside of the cell (Denecke *et al.*, 1990) or into membrane-bound organelles such as chloroplasts (Schreier and Schell, 1986; Svab *et al.*, 1990), mitochondria (Boutry *et al.*, 1987), endoplasm reticulum (Iturriaga *et al.*, 1989), peroxisomes (Gould *et al.*, 1990), or nuclei (Restrepo *et al.*, 1990).

### *Visualization of Gene Expression*

Markers which can be directly detected in plant tissues are very important and powerful tools. In transient expression studies, such markers can indicate the number and location of transformed cells, both of which are important parameters for optimizing stable transformation. In stable transformants, it is possible to determine whether an introduced gene is expressed evenly in all cells of a particular tissue or at higher levels in certain selected cells or tissue. This information often provides greater insight into the functional role of a given gene at different stages of plant development and in response to different environmental stimuli.

The ß-D-glucuronidase (GUS) reporter gene system has proven remarkably versatile in many investigations of this type (Jefferson, 1989). One current limitation of this system, however, is that the histochemical procedure for detecting GUS expression usually means sacrificing the tissue. However, markers which can be used to visualize gene expression in living plant tissue have been developed. Some of these have already proven useful as markers for whole plant transformation. In *Arabidopsis*, trichome formation requires the product of the *gl1* gene. This gene has recently been cloned and reintroduced into a *gl1* mutant which completely lacks trichomes (Herman and Marks, 1989). Trichome formation was restored by gene transfer, indicating that the defect was complemented by the introduced gene. Plants with trichomes can be easily distinguished from plants without, so this marker could be very useful for *Arabidopsis* transformation. The pigmentation marker gene isolated from maize is another example of the visible markers which can assist the identification of transgenic plants (Meyer *et al.*, 1987).

## Selectable Markers

Selectable markers confer a dominant phenotype on transformed cells because they result in the addition of a new trait not normally associated with untransformed cells. Traits which can be used to select transformed cells from those that are not transformed generally fall into two categories: those which confer either cell viability or lethality in the presence of a selective agent; and those which have a negligible effect on cell survival, but which confer transformed cells with some distinguishable physical characteristic.

## Criteria for Choosing Selectable Markers

Plant cell transformation frequencies are generally low, so most stable transformation systems use markers which ensure the survival of transformed cells in the presence of a selective agent. The choice of an appropriate selective agent should be made on the basis of several factors. At the very least, the selective agent should be readily available and soluble in plant tissue culture medium. However, the most important factor is whether the frequency at which untransformed cells or plants can survive at a given level of the selective agent is greater than or similar to the expected or observed transformation frequency. If the frequency of "escapes" is high, an alternate selection should be sought. Ideally, the $LD_{50}$ (i.e., the level at which 50% growth inhibition is observed) for untransformed cells should be at least an order of magnitude lower than that for transformed cells (Nutter *et al.*, 1987; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989). In practice, however, this can only be ascertained when transformed cells are available.

The nature of the target tissue and the DNA delivery system should also be considered when developing a selection procedure with a given marker gene. For example, green tissue may be more sensitive to selective agents which affect plastid-localized processes than callus cells. Furthermore, exposure of callus cells to high levels of these agents during the selection procedure can affect the regeneration of green plants or the subsequent development of plastids. With other selective agents, the survival of transformed cells under selection conditions can be affected by the release of toxic compounds from surrounding untransformed cells. With bulky explants, transformed cells on the surface may also be starved of nutrients if secondary thickening is induced by the selective agent in adjacent untransformed cells. In these cases, transformants can be recovered either by subdividing the target tissue or by using suspension cell cultures or protoplasts where feasible. In addition, large explants or clumps of untransformed cells often survive for longer periods under selective conditions. This can lead to a higher frequency of escapes, especially if transformed cells deplete the selective agent in the surrounding medium. In some cases, such escapes can easily be "weeded" out by screening procedures which involve rooting or germination in the presence of the selective agent.

## Selectable Marker Genes for Transformation of Monocots

Since monocotyledonous plant species are generally insensitive to *Agrobacterium*-mediated transformation, alternate procedures are necessary for genetic modification of these plants. Gene transfer by microprojectile bombardment is currently one of the successful methods for the genetic transformation of monocots. In this approach, the efficiency of DNA delivery and especially the efficiency of stable integration of the introduced genes are rather low compared to those obtained by *Agrobacterium*-mediated transformation. For instance, after particle bombardment, only 1.9% of the cells initially expressing the ß-glucuronidase gene became transformed by integration of the gene (Klein *et al.*, 1988b). Although Spencer *et al.* (1991) obtained rather high values of stable transformation that ranged from five to ten percent after the bombardment of maize cells, others obtained very low frequencies of about 0.1% (Gordon-Kamm *et al.*, 1990). Such a low efficiency of stable integration points out the importance of an effective selection procedure to identify the small number of the stably transformed cells from untransformed cells.

The antibiotic kanamycin, which has been most widely used as a selective agent for the transformation of dicotyledonous species, can not easily be applied to monocots (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989). The endogenous resistance of many monocotyledonous species to kanamycin makes the development of other selection systems necessary. Monocots are often insensitive to relatively high levels of kanamycin, which allows the regeneration of untransformed plant cells on kanamycin-containing medium. For example, a concentration at 100 mg/l of kanamycin allowed the growth of 70% of untransformed rice calli (Dekeyser *et al.*, 1989), and protoplasts isolated from suspension cells of *Lolium perenne* were even able to divide in the presence of 800 mg/l of kanamycin (Potrykus *et al.*, 1985).

Therefore, many attempts have been made to replace the selective agent kanamycin by other antibiotics or herbicides. This seems to be a reasonable approach, because the various substances differ in their mode of action and in the way they are absorbed and transported in plant tissue. The expression level of the resistance gene is also important in ensuring a sufficient level of resistance in the transformed cells. In this way, the transformed cells might be allowed to divide and further develop among the majority of untransformed, highly susceptible cells. High expression levels can be obtained by using specific plasmid vectors constructed for monocotyledonous species.

## Herbicide and Antibiotic Resistance Markers

The repertoire of selectable markers which result in resistance to antibiotics, herbicides, or other phytotoxins is now fairly extensive. Table 2 lists those that have been successfully used for plant gene transfer. Resistance to the selective agents listed in Table 2 is conferred by one of the following three principle mechanisms.

1. Overexpression of the protein which is the target for the selective agent. This will cause a plant tolerant to a given level of the selective agent by ensuring a proportion of the total amount of the target protein synthesized remains unaffected, thereby allowing the cell to survive.
2. Alteration of the site of action. In this case, mutation of the target protein renders it less able to bind to the selective agent, but still capable of carrying out its biochemical function.
3. Introduction of genes from bacteria that can render detoxification either by enzymatic modification or sequestration of the selective agent .

In general, plant genetic engineers have adapted the first two strategies whereas third strategy is novel to genetically manipulated plants. In addition to the three mechanisms mentioned above, some selective agents could also be detoxified by the plant itself, for example, by glutathione-*S*-transferase. This enzyme occurs in plants (as well as other organisms) and renders xenobiotic compounds ineffective by conjugating them to reduced glutathione.

**Table 2**. Selectable Marker Genes for Plant Transformation.

| **Gene** | **Gene product** | **Origin** | **Selective agent** | **Reference** |
|---|---|---|---|---|
| *aacC3*,<br>*aacC4* | gentamycin-3-N-acetyltransferase | *Serratia marcescens*;<br>*Klebsiella pneumoniae* | gentamycin | Hayford *et al.*, (1988) |
| AK | aspartate kinase | *Escherichia coli* | high concentrations of lysine and threonine | Perl *et al.*, (1993) |
| *aphIV* | hygromycin phosphotransferase | *E. coli* | hygromycin B | van den Elzen *et al.*, (1985)<br>Waldron *et al.*, (1985) |
| *bar*<br>(PAT) | phosphinothricin acetyltransferase | *Streptomyces hygroscopicus*,<br>*Streptomyces viridochromogenes* | bialaphos,<br>phosphinothricin | De Block *et al.*, (1987)<br>Thompson *et al.*, (1987)<br>Wohlleben *et al.*, (1988) |
| (GS) | glutamine synthetase | *Medicago sativa* | | Tischer *et al.*, (1986)<br>Eckes *et al.*, (1989) |
| Ble | bleomycin resistance determinant | Tn5;<br>*Streptoalloteichus hindustanus* | bleomycin<br>phleomycin | Perez *et al.*, (1989)<br>Hille *et al.*, (1986) |

**Table 2** (Cont'd).

| **Gene** | **Gene product** | **Origin** | **Selective agent** | **Reference** |
|---|---|---|---|---|
| *bxn* | bromoxynil specific nitrilase | *Klebsiella ozaenae* | bromoxynil | Stalker *et al.*, (1988) |
| *cat* | chloramphenicol acetyl transferase | phage p1Cm | chloramphenicol | Herrera-Estrella *et al.*, (1983b) |
| *csr1-1* | acetolactate synthase | *Arabidopsis thaliana* | sulfonylurea herbicides | Haughn *et al.*, (1988) |
| *dhfr* | dihydrofolate reductase | plasmid R67 | methotrexate | Herrera-Estrella *et al.*, (1983a) |
| DHPS | dihydrodipicolinate synthase | *E. coli* | S-aminoethyl L-cysteine | Perl *et al.*, (1993) |
| EPSP | 5-enolpyruvylshikimate-3-phosphate synthase | *Petunia hybrida*,<br>*Escherichia coli*,<br>*Salmonella typhimurium*,<br>*Arabidopsis thaliana* | glyphosate | Shah *et al.*, (1986b)<br>Duncan *et al.*, (1984)<br>Stalker *et al.*, (1985)<br>Klee *et al.*, (1987) |

**Table 2** (Cont'd).

| **Gene** | **Gene product** | **Origin** | **Selective agent** | **Reference** |
|---|---|---|---|---|
| *nptII* | neomycin phosphotransferase | Tn5 | G418, kanamycin, neomycin, paromomycin | Bevan *et al.*, (1983)<br>Fraley *et al.*, (1983)<br>Herrera-Estrella *et al.*, (1983a) |
| *psbA* | $Q_B$ protein | *Amaranthus hybridus* | atrazine | Cheung *et al.*, (1988) |
| SPT | streptomycin phosphotransferase | Tn5 | streptomycin | Jones *et al.*, (1987) |
| *sul* | dihydropteroate synthase | plasmid R46 | sulfonamide | Guerineau *et al.*, (1990) |
| *tdc* | tryptophan decarboxylase | *Catharanthus roseus* | 4-methyl tryptophan | Goddijn *et al.*, (1993) |
| *tfdA* | 2,4-D monooxygenase | *Alcaligenes eutrophus* | 2,4-dichlorophenoxyacetic acid | Lyon *et al.*, (1989) |

Glutathione-*S*- transferase has been extensively studied in maize at the molecular level, but has not yet been exploited in approaches to plant genetic manipulation for herbicide resistance. Besides these intracellular mechanisms of resistance, tolerance to inhibitors can be achieved by reducing their uptake into the cell (Harvey and Harper, 1982).

## Antibiotics and Resistance Genes

Antibiotic resistance genes of bacterial origin, expressed under the direction of plant regulatory elements, have been shown to be effective in plant cells. Expression of such a gene in plant cells resulted in transformed cells showing acquired resistance to the corresponding antibiotic. Representatives of a major class of antibiotics, the aminoglycosides, and the corresponding resistance genes of microbial origin have been widely used for plant transformation research.

The best known member of the aminoglycoside antibiotics is kanamycin, which has been applied as a selective agent in many dicot transformation experiments. Other agents are gentamycin, G418, neomycin, paromomycin, and hygromycin. These antibiotics inhibit the protein synthesis in prokaryotic cells. Kanamycin, gentamycin and its derivative geneticin (G418), neomycin, and paromomycin bind to the 30S ribosomal subunit, thus inhibiting the initiation of translation and, consequently, protein synthesis (Nap *et al.*, 1992). Hygromycin occupies the ribosomal binding site of the elongation factor EF-2, inhibiting peptide chain elongation (Kors, 1991). Ribosomes of mitochondria and chloroplasts of higher plants are related to bacterial ribosomes, and are also susceptible to these antibiotics. The most dramatic visible effect on plants is chlorosis, bleaching of the leaves, caused by the lack of chlorophyll synthesis.

Resistance to kanamycin, G418, neomycin, and paromomycin was obtained by the *aph*A2 gene from Tn5 of *Escherichia coli* (Bevan *et al.*, 1983). This gene encodes for the enzyme APH(3')II (aminoglycoside 3'-phosphotransferase II), also called NPTII (neomycin phosphotransferase II), which phosphorylates a specific hydroxyl group of these antibiotics. Due to this phosphorylation, binding of the antibiotics to the ribosome is inhibited. Kanamycin can also be detoxified by AAC(3') (gentamycin 3-N-acetyltransferase), which acetylates the 3'-amino group of ring II. Gentamycin can also be detoxified by AAC(3'), but, because it does not contain a hydroxyl group at the 3'-position of ring I, it can not be phosphorylated by APH(3'). However, a gentamycin derivative, G418, contains an extra hydroxyl group at the 4'-position of ring I, which enables phosphorylation by NPTII. Genes encoding ACC(3') enzymes have been used for transformation of *Petunia hybrida* and *Arabidopsis thaliana* (Hayford *et al.*, 1988) and tobacco (Carrer *et al.*, 1991). Hygromycin can be inactivated by phosphorylation of a hydroxyl group by the enzyme APH(4'), also called HPT (hygromycin phosphotransferase). The *hpt=aph*IV gene which codes for this enzyme was isolated from *E. coli* and rendered suitable for expression in plant cells (van den Elzen *et al.*, 1985; Waldron *et al.*, 1985).

Generally, kanamycin was added to the growth medium in a concentration previously shown to be inhibitory for the growth and regeneration of untransformed cells. Little has been known about the transport of kanamycin in plant tissues. It did not seem to be mobile in the vascular tissue, but rather to diffuse through plant tissue via intercellular spaces. It was observed that this diffusion only occurred over rather short distances (Weide *et al.*, 1989), which meant that in large explants, not all parts were reached by the antibiotic. From prokaryotic studies, it was known that the strongly positive molecule was bound to the negatively charged cell membrane, then mediated by carriers, and actively transported into the cell. This last step rendered the process pH dependent; if the pH dropped below 6, binding was strongly inhibited. However, the normal tissue culture pH is 5.8 or even lower. Moreover, bivalent ions in the medium ($Ca^{2+}$ and $Mg^{2+}$) competed with the kanamycin for the carrier-binding sites (Nap *et al.*, 1992).

Although the use of kanamycin has proven to be very effective for the development of transformation protocols for dicotyledonous species, research in monocot transformation demonstrated that in these species, kanamycin was less effective as a selective agent. When kanamycin was used for the selection of protoplasts of *Gramineae*, it was shown that, for an optimal effect, the antibiotic had to be applied as soon as possible after protoplast isolation and before restoration of the cell wall (Hauptmann *et al.*, 1988). This suggests that transport over the cell wall is the limiting factor. Additional problems have been reported for rice: regeneration of plants from transformed calli proved to be impossible if kanamycin was used as the selective agent (Peng *et al.*, 1992). Only when G418 was used for selection could calli expressing the *aph*A2 gene be regenerated into plants. It was concluded that kanamycin somehow impaired the regeneration potential of the rice calli. Similar phenomena were reported by Toriyama *et al.* (1988), Zhang *et al.* (1988) and Battraw and Hall (1992).

G418 (geneticin) has been tested as an alternate selective agent in various monocots, *e.g.*, rice, *Lolium*, and other *Gramineae*, which were highly resistant to kanamycin (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989). In all cases, G418 was shown to be more effective, perhaps due to the more effective binding to ribosomes.

Hygromycin was another useful antibiotic for most tissues, including those of monocots, and showed a higher sensitivity compared to kanamycin (Meijer *et al.*, 1991). Hygromycin has been applied in establishing transformation procedures for maize (Walters *et al.*, 1992), rice (Shimamoto *et al.*, 1989; Li *et al.*, 1992a; Datta *et al.*, 1992), and orchardgrass (Horn *et al.*, 1988).

## Herbicides and Resistance Genes

### Phosphinothricin (Bialaphos)

In 1971, a novel tripeptide (bialaphos or bilanaphos) containing the previously unknown amino acid L-2-amino-4-[hydroxyl(methyl)phosphinyl] butyric acid (L-PPT), together with two L-analine groups, was obtained by two different teams working independently of one another---Bayer *et al.* (1972) in Tübingen, from *Streptomyces viridochromogenes*; and microbiologists of the Japanese firm Meiji Seika Kaisha (Kondo *et al.*, 1973), from *Streptomyces hygroscopicus.* The Tübingen authors gave the new amino acid the name phosphinothricin (PPT) (Bayer *et al.*, 1972). Structural proof was obtained by degradation (Bayer *et al.*, 1972; Ogawa *et al.*, 1973a) and synthesis (Bayer *et al.*, 1972; Ogawa *et al.*, 1973b).

DL-Phosphinothricin is the active ingredient of a non-selective, broad-spectrum, postemergent, commercial herbicide formulation and was given the common name glufosinate ammonium (GLA). It is sold worldwide under the following trade names: Basta, Buster, Challenge, Finale, Harvest, and Ignite. The active ingredient is highly stable as a chemical compound, but its degradation is rapid in a microbiologically active environment, such as soil or surface water. The D-isomer is herbicidally inactive. L-Phosphinothricin was initially identified as the active moiety of the herbicidal tripeptide bialaphos (L-Phosphinothricinyl-L-alanyl-L-alanine) produced by *Streptomyces hygroscopicus* (Kondo *et al.*, 1973). PPT is an analog of glutamate and acts as a competitive inhibitor of the enzyme glutamine synthetase (GS; glutamate-ammonia ligase) in both plants and bacteria (Bayer *et al.*, 1972). In plants, GS is critical for the assimilation of ammonia into L-glutamate, forming L-glutamine, and is central to the regulation of nitrogen metabolism (Miflin and Lea, 1977). It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid catabolism, and photo-respiration. Inhibition of this enzyme causes accumulation of ammonia in the cells. The accumulation of ammonia rather than the lack of glutamine caused the death of plant cells (Tachibana *et al.*, 1986).

PPT is synthesized chemically (Basta; Hoechst AG), or by fermentation of *Streptomyces hygroscopicus* (Herbiace; Meiji Seika Kaisha). The latter product, also called Bialaphos, is a tri-peptide compound, which consists of PPT and two alanine residues. In contrast to PPT, bialaphos has little inhibitory activity against glutamine synthetase *in vitro*, but is converted to PPT *in vivo* by intracellular peptidases which remove the alanine residues (De Block *et al.*, 1987).

Resistance to this herbicide is conferred by phosphinothricin-N-acetyltransferase (PAT), which inactivates PPT by acetylation, using acetyl coenzyme A as a cofactor. Two similar genes encode PAT: *bar*, isolated from *S. hygroscopicus* (Murakami *et al.*, 1986; Thompson *et al.*, 1987), and so named because of its resistance to Basta; and *pat*, isolated from *S. viridochromogenes* (Wohlleben *et al.*, 1988).

In transformation studies, Basta, PPT, and bialaphos have each been used to select for PPT (bialaphos)-resistant plants by spraying full-grown plants or by adding them to selection medium in earlier stages. In the media, one to three mg/l PPT is generally adequate to select for transformed cells (De Block *et al.*, 1987; Gordon-Kamm *et al.*, 1990). Dekeyser *et al.* (1989) found a concentration of 10 mg/l optimal in discriminating between transformed and untransformed rice calli. They also showed that, for an effective selection, it was important to exclude amino acids from the selection medium, because several amino acids (glutamic acid, proline, arginine) allowed growth of untransformed cells in the presence of PPT. This seems to contradict data indicating that ammonia accumulation leads to cell death (Tachibana *et al.*, 1986). However, it shows that both the composition of the selection medium and the concentration of PPT are important factors in the selection for transformed plant cells.

As selective agents, PPT and Bialaphos have successfully been applied with *Brassica napus* (De Greef *et al.*, 1989) and *Helianthus annus* (Escandón and Hahne, 1991). Transgenic tobacco, potato, and tomato plants, which expressed the *bar* gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987), were tolerant to 4-10 times the field rate of application of the herbicide (De Block *et al.*, 1987). They have also proven to be effective in monocots such as maize (Gordon-Kamm *et al.*, 1990; Spencer *et al.*, 1991; 1992), oats (Somers *et al.*, 1992), and rice (Dekeyser *et al.*, 1989; Datta *et al.*, 1992; Cao *et al.*, 1992), which have a high tolerance for antibiotics such as kanamycin and hygromycin.

### Glyphosate

The herbicide glyphosate (*N*-phosphonomethyl glycine) has a simple molecular structure, as it is an analogue of the amino acid glycine, and is the active component of Roundup™, a herbicide marketed by the Monsanto Company. It is in widespread use as a nonselective post-emergent herbicide and has the desirability of effectiveness with short-lived residual soil activity which seems environmentally sound. Glyphosate is mobile in the phloem and tends to accumulate in the apex of the stem and root, so it affects meristematic and apical cells especially (Comai *et al.*, 1989). Glyphosate kills most herbaceous plants, so it must not come in contact with the crop when applied. This limits its modes of application.

Early investigations into the mode of action of glyphosate indicated that it inhibited aromatic amino acid biosynthesis (Jaworski, 1972). Under the influence of glyphosate, bacteria and plant cell cultures accumulated massive quantities of shikimic acid and shikimic acid 3-phosphate (Amrhein *et al.*, 1980, 1983; Berlin and Witte, 1981), both of which were intermediates of aromatic amino acid biosynthesis. All these facts are accounted for by the finding that, *in vitro*, glyphosate is a potent competitive inhibitor of the enzyme 5-enol-pyruvylshikimic acid 3-phosphate (EPSP) synthase (Steinrücken and Amrhein, 1980).

EPSP synthase has been isolated from a number of plants and microbial organisms and has probably become the most comprehensively investigated enzyme in the shikimic acid pathway (Amrhein, 1985). The *aro*A gene which codes EPSP synthase has been isolated and sequenced from *Escherichia coli* (Duncan *et al.*, 1984), *Salmonella typhimurium* (Stalker *et al..*, 1985) and *Arabidopsis thaliana* (Klee *et al.*, 1987). The amino acid sequence of EPSP synthase has been derived from the nucleotide sequence of these genes.

*S. typhimurium* provided an experimental system based on the availability of defined mutations in the genetic locus (*aro*A) coding for EPSP synthase. If EPSP synthase is the biochemical target for glyphosate, then it should be possible to isolate glyphosate-resistant mutants by mapping at the *aro*A locus which encodes a glyphosate-resistant enzyme. Two types of mutations (an overproduction of EPSP synthase via an up-promoter mutation and a structurally altered EPSP synthase enzyme which is resistant to the inhibition of glyphosate) were obtained from ethylmethanesulphate-mutagenized *S. typhimurium* cultures (Comai *et al.*, 1983). The enzyme activity of the mutant, which was shown to synthesize an EPSP synthase with increased resistance to glyphosate, was 50% inactivated at 1.20 mM glyphosate and that of the wild-type at 0.01 mM. A comparison of the wild-type and mutant gene sequences revealed a cytosine-to-thymine transition mutation that resulted in a proline-to-serine amino acid substitution at position 101 of the 349 amino acid polypeptide (Stalker *et al* ., 1985). Another modified EPSP synthase which was encoded by a mutated EPSP synthase gene was isolated from *Petunia* (Shah *et al.*, 1986b).

This information opens up two possible methods of producing glyphosate-resistant plants by genetic engineering: (1) transfer of a glyphosate-sensitive EPSP synthase under the control of a powerful promoter, causing overproduction of the enzyme and/or (2) transfer of a gene which codes a mutated, glyphosate-resistant EPSP synthase.

Both methods have been used to generate glyphosate-tolerant plants. Using a petunia cell line which overproduces EPSP synthase by gene amplification (Shah *et al*., 1985; Steinrücken *et al*., 1986), a cDNA of the EPSP synthase gene was isolated and, under the control of the 35S cauliflower mosaic virus promoter (CaMV), a chimeric gene was constructed. Leaf discs of petunia incubated with *Agrobacterium* containing the chimeric EPSP gene were able to form callus on medium containing 0.5 mM glyphosate, whereas control leaf discs failed to do so. EPSP synthase activity in transformed callus was up to 40-fold higher than in the control callus, confirming that the engineered EPSP synthase gene could confer glyphosate resistance. From these transformed cells, transgenic plants that show tolerance to glyphosate have been generated (Shah *et al*., 1986b). Four independent transgenic plants selected for resistance to kanamycin (the resistance gene was co-transferred with the chimeric EPSP synthase gene) could tolerate spraying with Roundup herbicide at a dose equivalent to 0.8 LB per acre, which is two to four times the level required to kill wild type plants.

The alternate method, i. e., the insertion and expression of a mutated bacterial aroA gene which codes a glyphosate-resistant EPSP synthase, was also successful (Comai *et al*., 1985; Fillatti *et al*., 1987a; Hinchee *et al.*, 1988; Vasil *et al.*, 1991). The sources of the *aro*A gene were the strains of *Salmonella typhimurium* and *E. coli*. *S. typhimurium* that were able to grow on glyphosate-containing medium and be recovered as spontaneous or chemically induced mutants. A small percentage of these mutations mapped to the *aro*A locus. One of these mutants was shown to synthesize an EPSP synthase with increased resistance to glyphosate (Comai *et al.*, 1983). Enzyme activity was 50% inactivated at 0.01 mM glyphosate and 1.20 mM glyphosate for the wild-type and the resistant EPSP synthases, respectively (Stalker *et al.*, 1985). Comparison of the derived amino acid sequences of the EPSP synthases from mutant and wild-type *S. typhimurium* revealed that the substitution of a proline (Pro 101) for a serine residue accounted for the decreased affinity of the enzyme for glyphosate (Stalker *et al.*, 1985).

A more highly resistant form of EPSP synthase from *E. coli* has also been described (Kishore *et al.*, 1986).

Much attention has been paid to improving the selection system based on glyphosate resistance. This has included isolation of promoters that were effective in apices and meristems, where glyphosate accumulated (Comai *et al.*, 1989). A higher transformation efficiency is also expected to be achieved by targeting the enzyme to the chloroplast, where the shikimate pathway is located. To achieve this, the *aro*A gene from *S. typhimurium* was fused to the region of *RbcS* encoding the transit peptide of the ribulose biphosphate carboxylase small subunit (Comai *et al.*, 1988), but, so far, this chimeric gene has not been used in transformation experiments. However, a fusion between the region of *Petunia Epsps* encoding the transit peptide and the resistance gene *aro*A from *S. typhimurium* improved the selection of transgenic plants (Della-Cioppa *et al.*, 1987; Botterman and Leemans, 1988).

## Atrazine

Atrazine also inhibits photosynthesis by binding to the $Q_B$ protein in the thylakoid membrane, thus blocking electron transport. The resistance gene has been isolated from chloroplasts of an atrazine-resistant *Amaranthus hybridus*. The gene is a mutated *psb*A gene that encodes a modified $Q_B$ protein. It has been fused to a transit peptide sequence for chloroplast targeting. Transgenic tobacco plants appeared to be tolerant to 100 mM atrazine (Cheung *et al.*, 1988), but no other reports concerning the use of atrazine as a selective agent in transformation studies are known. There also exists an atrazine-detoxifying system based on the glutathione-S-transferase *gst* gene (Shah *et al.*, 1986a), but this has not been applied in any transformation research.

## Bromoxynil

Bromoxynil is a herbicide that inhibits photosynthesis in plants by binding to electron-transport components of photosystem II in the thylakoid membrane (Van Rensen, 1982; Sanders and Pallett, 1986). There may also be a low-affinity binding site for bromoxynil in the 32-kd polypeptide of the same complex (Szigeti *et al.*, 1982; Vermaas *et al.*, 1984). The *bxn* gene (bromoxynil nitrilase gene) from *Klebsiella ozaenae* codes for a specific nitrilase that degrades bromoxynil. Transgenic tobacco plants harboring this gene have been obtained (Stalker *et al.*, 1988). The chimeric *bxn* gene was placed in the T-DNA of a binary Ti vector and transferred into tobacco via *Agrobacterium*. After the selection of transformants using a co-transferred kanamycin-resistance marker, the plants were shown to be resistant to bromoxynil up to $10^{-4}$M. Control tissue is normally bleached between $10^{-6}$M and $10^{-5}$M bromoxynil. Resistant plants were shown to have inherited one to three copies of the *bxn* chimeric gene.

## Chlorsulfuron

Chlorsulphuron is a selective herbicide that affects amino acid biosynthesis by inhibiting the enzyme acetolactate synthase (ALS) and can be applied to cereal crops without detriment to their growth (Sweetser *et al.*, 1982; LaRossa and Falco, 1984). ALS is involved in the synthesis of branched-chain amino acids. The gene *crs*1-1, isolated from *Arabidopsis thaliana*, confers resistance to chlorsulfuron by encoding an ALS with a reduced affinity for the herbicide (Haughn *et al.*, 1988).

Chlorsulfuron has been applied as a selective agent in the transformation of tobacco (Haughn *et al.*, 1988), maize (Fromm *et al.*, 1990), and sugarbeets (D'Halluin., 1992a). The ALS gene from chlorsulfuron-resistant *Arabidopsis* was transformed into tobacco as a 5.8-kb genomic fragment inserted into the T-DNA of a disarmed Ti plasmid (Haughn *et al.*, 1988). Unlike all previous examples, this herbicide-resistance gene required no further manipulation and illustrates an advantage of recovering a resistance gene from a plant such as *Arabidopsis* than a bacterium. The ALS coding sequence is flanked by its own transcription regulatory sequences and contains a transit peptide for targeting the pre-ALS into chloroplasts (Mazur *et al.*, 1987; Haughn *et al.*, 1988). Transformed plants were selected on kanamycin-containing medium using an *aph3*'II chimeric gene which was co-resident with the *csr* gene in the T-DNA and was present at two to ten copies per haploid genome. D'Halluin *et al.* (1992a) compared the use of chlorsulfuron, other sulfonylurea compounds, PPT, and kanamycin. They concluded that, after the selection procedure, 90% of the shoots that survived the application of chlorsulfuron were actually transformed, while for PPT and kanamycin, these figures were 30% and 50%, respectively. These results indicated that chlorsulfuron was an efficient selective agent for the transformation of sugarbeets. Transgenic poplars (Brasileiro *et al.*, 1992) and fertile transgenic rice plants (Li *et al.*, 1992b) have also been obtained by using the *crs*1-1 gene in combination with the CaMV 35S promoter.

## 2,4-D

2,4-D (2,4-dichlorophenoxy acetic acid) is an auxin analog that is competitive with indole acetic acid (IAA) in occupying binding sites of auxin receptors. The resistance gene *tfd*A has been cloned from the soil bacterium *Alcaligenes eutrophus* (Lyon *et al.*, 1989). This *tfdA* gene codes for the enzyme 2,4-dichlorophenoxy acetate monooxygenase that is involved in the degradation of 2,4-D. The gene has been introduced into tobacco (Streber and Willmitzer, 1989), resulting in transgenic plants with enhanced tolerance to 2,4-D. However, in this study, kanamycin was used to select for transformed tissues, and selection by 2,4-D alone did not yield any transformed shoots, possibly because of the overgrowth of untransformed tissue. Due to the fact that 2,4-D is very toxic to dicotyledons and monocotyledonous plants are known to be tolerant, the use of 2,4-D as a selective agent has been a question (Streber and Willmitzer, 1989).

### Methotrexate

Methotrexate causes the deficiency of thymidylate by inhibiting the enzyme dihydrofolate reductase. Because thymidylate is a precursor of one of the components of DNA, nucleotide biosynthesis is blocked, and cell death results. Resistance can be conferred by the mouse dihydrofolate reductase gene (*dhfr*) (Eichholtz *et al.*, 1987). This gene encodes an enzyme with a reduced affinity for methotrexate. It has been utilized in the transformation of *Petunia hybrida* (Eichholtz *et al.*, 1987), *Brassica napus* (Pua *et al.*, 1987), and monocots like rice (Meijer *et al.*, 1991). Also for grass species such as *Panicum maximum* , it has shown to be a useful selective agent (Hauptmann *et al.*, 1988). In all cases, transgenic plants or calli have been obtained. However, Meijer *et al.* (1991) questioned the suitability of methotrexate as a selective agent for rice because of an inhibition of the other enzymes involved in the biosynthesis of nucleotides.

## Application of Plant Transformation to Confer Insect Tolerance

If functional insecticides can be genetically engineered into plants, it may be feasible to develop crops that are intrinsically tolerant of insect pests. Such plants would not have to be sprayed with costly and potentially hazardous chemical pesticides. In addition, as in the case with many biologically generated insect pathogens, such insecticides would be required at much lower concentrations than exogenously applied synthetic pesticides. Moreover, since biological insecticides are usually specific, they are generally not hazardous to the intended consumers of the crops.

**Bt Toxin**

Two main strategies have been used to confer resistance against insect pests. The first involves transforming plants with a gene for an insecticidal protoxin produced by one of several subspecies of *Bacillus thuringiensis* (Bt toxin) (Vaeck *et al.*, 1987; Brunke and Meeusen, 1991; Perlak *et al.*, 1990). The crystalline protein with insecticidal properties has received much attention because the protoxin does not persist in the environment nor is it hazardous to mammals. The idea of using such compounds is nothing new, since Bt toxins in various formulations (usually as whole sporulated bacteria) have been employed as insecticidal crop sprays for more than twenty years (Dulmage, 1981), but their application is limited by the high cost of production. Furthermore, the toxic components, i.e., the intracellular crystals which are produced by the bacteria during sporulation, are not stable under field spraying and exposure conditions, and, in particular, under high intensity ultraviolet light.

When ingested by certain insects, the crystals of Bt are partially hydrolyzed under the alkaline conditions of the midgut, thereby releasing proteins of 65,000-160,000 in molecular weight; further natural proteolytic processing of these proteins releases smaller toxic fragments. The mechanism of toxicity appears to depend on the disruption of the membranes of the cells lining the gut, and this action is only carried out by the cleaved fragments (Sacchi *et al.*, 1986). These Bt toxins are very species-specific, and, although commercial toxins effective against more than 50 lepidopteran pest species have been identified, comparatively few with activity against coleopterans have been isolated. Despite this possible limitation, the potential for increasing the utility of Bt toxin by the genetic engineering of plants to express the Bt toxin was recognized, and transgenic tobacco, tomato, potato, and cotton plants have been obtained (Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Gasser and Fraley, 1989; Perlak *et al.*, 1990; Brunke and Meeusen, 1991). These transgenic plants have expressed sufficient quantities of insecticidal crystalline protein to provide protection from damage by feeding larvae of several different insects.

## Protease inhibitor

The presence of inhibitors of mammalian digestive protease in plants, particularly those in legume seeds, has long been known (Read and Haas, 1938). Their incontrovertible demonstration as proteins came in 1945, when the trypsin inhibitor from soybean seeds was isolated and crystallized (Kunitz, 1945). This class of compound has been shown to be very widespread in plants (Richardson, 1977), and those present in the leaves or seeds of *Leguminosae*, *Graminae*, and *Solananceae* have been extensively studied and characterized (Richardson, 1991). The possible role protease inhibitors played in plant defense systems against insect pests was investigated as early as 1947, when it was observed that larvae of certain pests were unable to develop normally on soybean products (Mickel and Standish, 1947). Subsequently, the trypsin inhibitors present in soybean were shown to be toxic to the larvae of the flour beetle, *Tribolium confusum* (Lipke *et al.*, 1954). Following these early studies, there have been many examples of protease inhibitors being active against certain insect species (Birk *et al.*, 1963; Applebaum, 1964; Steffens *et al.*, 1978; Gatehouse *et al.*, 1979; Broadway and Duffy, 1986; Gatehouse and Hilder, 1988).

Additional evidence for the protective role of protease inhibitors has been provided by Ryan and co-workers (Green and Ryan, 1972) when they demonstrated that damage to the leaves of the tomato and potato, either by insect feeding or mechanical wounding, induces the synthesis and accumulation of protease inhibitors both in the damaged leaves (the "local" reaction) and also in undamaged leaves at a distance from the original wound site (the "systematic" reaction). This production of inhibitors was shown to be the result of a wound hormone, protease inhibitor-inducing factor (PIIF), which is released from the damaged leaves and transported to other leaves where it initiates synthesis and accumulation of protease inhibitors (Shumway *et al.*, 1976; Walker-Simmons and Ryan, 1977; Brown *et al.*, 1985). This evidence strongly implicated protease inhibitors in plant protection (Ryan, 1983). Additional evidence indicating the involvement of protease inhibitor in plant defense systems were provided by other research groups.

The protection role of protease inhibitor in the "field" was first provided by Gatehouse *et al.* (1979), who demonstrated that elevated levels of these inhibitors in one variety of cowpeas were partially responsible for the observed resistance of the seeds to the major storage pests of this crop, the bruchid *Callosobruchus maculatus*. This particular trait was later exploited by conventional plant breeding, whereby bruchid resistance was transferred to an agronomically improved background (Redden *et al.*, 1983).

The mechanism of antimetabolic action of protease inhibitors is not yet fully understood. Direct inhibition of digestive enzymes is unlikely to be the only effect. Possibly a situation exists that is analogous to the effect of some protease inhibitors on mammals, where the major deleterious effect is loss of nutrients through pancreatic hypertrophy and overproduction of digestive enzymes (Liener, 1980). That they affect the nutritional biochemistry of *C. maculatus* has been clearly demonstrated, since methionine supplementation has been shown to overcome the antinutritional effects of the cowpea trypsin inhibitor (CpTI) (Gatehouse and Boulter, 1983). There is also evidence to suggest that they are only one part of the complex interaction between plant nutritional value and the insects' digestive physiology (Broadway *et al.*, 1986). This may explain why there are some cowpea varieties which bruchids are able to infect even though they contain high levels of CpTI (Xavier-Filho *et al.*, 1989).

Transformation of plants with the protease inhibitor gene has been considered to have the potential to enhance resistance against pathogens and insects (Boulter *et al.*, 1990b; Ryan, 1990) and can also provide information regarding the roles of protease inhibitor in the plant defense mechanism against insects. Direct evidence for the effectiveness of protease inhibitors in reducing insect damage has been obtained by transforming tobacco via *Agrobacterium tumefaciens* with a cowpea protease inhibitor gene encoding a trypsin/trypsin inhibitor (Hilder *et al.*, 1987). Synthesis of the inhibitor in transformed tobacco plants was confirmed immunologically by Western blotting. Transformed tobacco plants which expressed a foreign cowpea trypsin inhibitor gene and produced about one percent of the leaf proteins as the inhibitor were more resistant to feeding by larvae of *Heliothis virescens* than untransformed control plants or transformed plants that did not express the gene.

The cowpea trypsin inhibitor gene is the first gene of plant origin to be successfully transferred to another plant species that resulted in enhanced insect resistance. The protease inhibitor gene was considered to be a particularly ideal candidate for transfer to other plant species via genetic engineering, since it has been demonstrated to be insecticidal towards a wide range of economically important field and storage insects when tested on artificial diets. These include members of the Lepidopterae such as *Heliothis* spp. and *Anthonomus* spp., Coleopterae such as *Diabrotica* spp., *Bruchidae* spp. and *Anthonomus* spp., and Orthopterae such as *Locusta* spp.

Furthermore, and of prime importance, they appear to exhibit low or no mammalian toxicity (Hilder *et al.*, 1991). These protease inhibitors are small polypeptides of about 80 amino acids and belong to the Bowman-Birk type of double-headed serine protease inhibitors, and, while most bind two molecules of trypsin, some of the isoinhibitors are trypsin/chymotrypsin inhibitors (Gatehouse *et al.*, 1980). They are encoded by a moderately repetitive gene family in the cowpea genome (Hilder *et al.*, 1989). One drawback, however, is that relative to the Bt toxin, high concentrations of cowpea trypsin inhibitor are required for effectiveness (Brunke and Meeusen, 1991).

Potato and tomato plants also contain two powerful inhibitors of serine proteases called inhibitor I and inhibitor II (Plunkett *et al.*, 1982). Inhibitor I is an inhibitor of chymotrypsin that only weakly inhibits trypsin at its single reactive site; while inhibitor II contains two reactive sites, one of which inhibits trypsin and the other inhibits chymotrypsin. Both inhibitors are synthesized as precursors and undergo post-translational modification (Cleveland *et al.*, 1987) to form the mature proteins which are sequestered into the vacuole (Shumay *et al.*, 1976). Not only has the gene encoding protease inhibitor isolated from cowpeas been shown to confer resistance when expressed in tobacco (Hilder *et al.*, 1987), but the inhibitor II genes of the tomato and potato, and the inhibitor I gene of the tomato, when expressed in the same model plant, have also been shown to confer insect resistance (Johnson *et al.*, 1989).

High expression of the protease inhibitor II gene severely retarded the growth of *Manduca sexta* larvae feeding on the leaves of transformed tobacco plants. Results showed that the presence of the foreign protease inhibitor II gene in tobacco leaves at levels over 100 $\mu$g/g leaf tissue severely retarded the growth of larvae that fed on them, compared to larvae that fed on untransformed plants or transformed plants that did not express the inhibitor gene. At lower levels (about 50 $\mu$g/g leaf tissue) larvae growth was retarded to a lesser degree than at higher levels, leading the authors to suggest that there is a dose-dependent relationship between the levels of protease inhibitor and larvae growth. In addition to retarding larval growth, the presence of inhibitor II appeared to reduce the amount of leaf tissue consumed. Interestingly, transgenic tobacco plants expressing the tomato protease inhibitor I gene (a strong inhibitor of chymotrypsin) at levels greater than 100 $\mu$g/g leaf tissue supported similar rates of larval growth as those found for control plants. Since the protease inhibitor II is a trypsin, as opposed to a chymotrypsin inhibitor, it would appear that it is the trypsin inhibitor which is responsible for the observed insecticidal activity.

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## Tissue Culture and Genetic Transformation of Creeping Bentgrass

Plantlet regeneration from embryogenic callus in turfgrass was first reported in species which have a dual use as forage and turf. These include ryegrass hybrids (*Lolium multiflorum* x *L. perenne* and reciprocal crosses) (Ahloowalia, 1975), tall fescue (*Festuca arundinacea*, Schreb.) (Lowe and Conger, 1979), and *Agrostis stolonifera* L. (Wu and Antonovics, 1978). Krans *et al.* (1982) first investigated hormonal and environmental requirements of callus induction from mature caryopses, callus maintenance, and plantlet regeneration in creeping bentgrass cv. Penncross (*Agrostis palustris* Huds.). It was found that prolific callus induction occurred on Murashige and Skoog (MS) medium with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) under light incubation and with 1.0 or 10.0 mg/l 2,4-D under dark incubation.

Kinetin supplements were not required for callus induction. However, caryopses incubated in the dark with 0.01 mg/l kinetin had greater callus formation than caryopses incubated without kinetin when combined with 1.0 mg/l 2,4-D. They also indicated that dark incubation provided better callus production from caryopses, and callus cultured on 1.0 mg/l 2,4-D maintained the best plantlet regeneration ability. It was also found that callus induced from caryopses originated from the scutellum, root cortex, and along the coleoptile up to and adjacent to the first node.

Blanche *et al.* (1986) evaluated the effect of suspension culture on callus growth and plantlet formation and reported that callus growth was twofold greater in callus previously cultured in suspension than callus not previously cultured in suspension. However, plantlet formation was inhibited in callus previously cultured in suspension (less than ten plantlets per dish) compared to callus not previously cultured in suspension (250 to 450 plantlets per dish).

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Zhong *et al.* (1991) reported an efficient plant regeneration system via somatic embryogenesis from mature seeds of creeping bentgrass cv. Penncross. They found that in combination with 30 $\mu$M dicamba (3,6-dichloro-o-anisic acid), the presence of 2.25 $\mu$M 6-BA (6-benzyladenine) significantly enhanced the formation of embryogenic callus and provided the best plant regeneration. Over 80% of these somatic embryos germinated and formed normal plantlets on half-strength MS basal medium.

Terakawa *et al.* (1992) produced the first plant regenerates from protoplasts isolated from embryogenic suspension cultures of creeping bentgrass. Protoplasts isolated from the cultures were successfully grown on MS basal medium supplemented with 0.1 mg/l 2,4-D. Although the plating efficiency of protoplasts was rather low (0.36%), 30% of the protocalli formed normal green plants that were established in soil.

The first transgenic plants of creeping bentgrass were achieved by Zhong *et al.* (1993) employing the microprojectile bombardment of embryogenic callus to transfer the ß-glucuronidase (GUS) reporter gene. Transgenic plants were obtained from bombarded embryogenic calli without the application of any selective agent. The efficiencies for the expression of GUS activity in the embryogenic calli one month after bombardment ranged from zero to 28 %. However, of the 500 regenerated plants from their experiments, only 15 were chosen for assay and four had integrated the reporter gene.

Employing a biolistic procedure, Hartman *et al.* (1994) transformed creeping bentgrass with *bar* gene which confers resistance to the herbicide bialaphos. Suspension cells were bombarded with gold particles coated with *bar* gene and transferred to the selection medium containing two or four mg/l of bialaphos. A total of 271 plants out of approximately 900 regenerated plantlets survived the initial spray concentration (1.5 mg/ml) of the commercial herbicide Herbiace™. Of these, 55 survived the higher spray rate (two mg/ml). The authors speculated that the large number of escapes observed could have been due to the interference of amino acids (asparagine and/or glutamine) in the selection medium.

# BIBLIOGRAPHY

Abdullah, R., Cocking, E. C., and Thompson, J. A. 1986. Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technology 4: 1087-1090.

Ahloowalia, B. S. 1975. Regeneration of ryegrass plants in tissue culture. Crop Sci. 15: 449-452.

Akama, K., Puchta, H., and Hohn, B. 1995. Efficient *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the *bar* gene as selectable marker. Plant Cell Rep. 14: 450-454.

Albrecht, H. R. 1947. Strain differences in tolerance to 2,4-D in creeping bent grasses. J. Amer. Soc. Agron. 39 : 163-165.

Amrhein, N. 1985. Specific inhibitors as probes into the biosynthesis and metabolism of aromatic amino acids. Recent Adv. Phytochem. 20: 83-117.

Amrhein, N., Deus, B., Gehrke, R., and Steinrücken, H. C. 1980. The site of inhibition of shikimate pathway by glyphosate. II. Interference by glyphosate with chorismic acid formation *in vivo* and *in vitro*. Plant Physiol. 66: 830-834.

Amrhein, N., Johanning, D., Schab, J., and Schulz, A. 1983. Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture. FEBS Lett. 157 : 191-196.

An, G. 1985. High efficiency transformation of cultured tobacco cells. Plant Physiol. 79: 568-570.

Antonelli, N. M., and Stadler, J. 1990. Genomic DNA can be used with cationic methods for highly efficient transformation of maize protoplasts. Theor. Appl. Genet. 80: 395-401.

Applebaum, W. W., Birk, Y., Harpaz, I., Bondi, A. 1964. Comparative studies on proteolytic enzymes of *Tenebrio molitor* L. Comp. Biochem. Physiol. 11: 85-103.

Arencibia, A., Molina, P. R., de la Riva, G., and Selman-Housein, G. 1995. Production of transgenic sugarcane (*Saccharum officinarum* L.) plants by intact cell electroporation. Plant Cell Rep. 14: 305-309.

Armaleo, D., Ye, G. N., Johnston, S. A., Klein, T. M., Shark, K. B., and Sanford, J. C. 1990. Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. Curr. Genet. 17: 97-103.

Armstrong, J. M. 1937. A cytological study of the genus *Poa* L. Can. J. Res. 15: 281-297.

Asano, Y., Otsuki, Y., and Ugaki, M. 1991. Electroporation-mediated and silicon carbide whisker-mediated DNA delivery in *Agrostis alba* L. (Redtop). Plant Sci. 79: 247-252.

Barcelo, P., Hagel, C., Becker, D., Martin, A., and Lörz, H. 1994. Transgenic cereal (tritordeum) plants obtained at high efficiency by microprojectile bombardment of inflorescence tissue. Plant J. 5 : 583-592.

Barton, K., Whiteley, H. and Yang, N. -S. 1987. *Bacillus thuringiensis* ∂-endotoxin in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol. 85: 1103-1109.

Basiran, N., Armitage, P., Scott, R. J., and Draper, J. 1987. Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase. Plant Cell Rep. 6: 396-399.

Battraw, M., and Hall, T. C. 1992. Expression of a chimeric neomycin phosphotransferase II gene in first and second generation transgenic rice plants. Plant Sci. 86: 191-202.

Bayer, E., Gugel, K. H., Haegele, K., Hagenmaier, H., Jessipow, S., Koenig, W. A., Zaehner, H. 1972. Phosphinothricin und phosphinothricyl-alanyl-alanine. Helv. Chim. Acta 55: 224-239.

Bayley, C., Trolinder, N., Ray, C., Morgan, M., Quisenberry, J. E., and Ow, D., W. 1992. Engineering 2, 4-D resistance into cotton. Theor. Appl. Genet. 83: 645-649.

Beard, J. B. 1973. Turfgrass: Science and Culture. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. pp. 71-78.

Beard, J. B. 1982. Turf management for golf courses. Macmillan Publishing Company, New York. pp. 119-124.

Becker, D., Brettschneider, R., and Lörz, H. 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J. 5 : 299-307.

Belefant, H., and Fong, F. 1989. Abscisic acid ELISA: Organic acid interference. Plant Physiol. 91: 1467-1470.

Bellini, C., Chupeau, M. -C., Guerche, P., Vastra, G., and Chupeau, Y. 1989. Transformation of *Lycopersicon peruvianum* and *Lycopersicon esculentum* mesophyll protoplasts by electroporation. Plant Sci. 65: 63-75.

Benfey, P. N., and Chua, N. -H. 1989. Regulated genes in transgenic plants. Science 244: 174-181.

Benfey, P. N., and Chua, N. -H. 1990. The cauliflower mosaic virus 35S promoter: Combinatorial regulation of transcription in plants. Science 250: 959-966.

Benfey, P. N., Takatsuji, H., Ren, L., Shah, D. M., and Chua, N. -H. 1990. Sequence requirements of the 5-enolpyruvylshikimate-3-phosphate synthase 5'-upstream region for tissue-specific expression in flowers and seedings. Plant Cell 2: 849-856.

Berlin, J., and Witte, L. 1981. Effects on glyphosate on shikimic acid accumulation in tobacco cell cultures with low and high yields of cinnamoyl putrescine. Z. Naturforsch. 36C: 210-221.

Berns, M. W., and Rounds, D. E. 1970. Cell surgery by laser. Sci. Amer. 222: 98-110.

Berns, M. W., Wright, W. H., and Wiegand-Steinbing, R. 1991. Laser microbeams as a tool in cell biology. Rev. Cytol. 129: 1-44.

Bevan, M. W., Flavell, R. B., and Chilton, M. -D. 1983. A chimaeric antibiotic resistance marker gene as a selectable marker for plant cell transformation. Nature 304: 184-187.

Bidney, D., Scelonge, C., Martich, J., Burrus, M., Sims, L., and Huffman, G. 1992. Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Mol. Biol. 18: 301-313.

Birch, R. G. and Franks, T. 1991. Development and optimisation of microprojectile systems for plant genetic transformation. Aust. J. Plant Physiol. 18: 453-469.

Birk, Y., Gertler, A., and Khalef, S. 1963. Separation of a *Tribolium*-protease inhibitor from soybeans on a calcium phosphate column. Biochim. Biophys. Acta 67: 326-328.

Blanche, F. C., Krans, J. V., and Coats, G. E. 1986. Improvement in callus growth and plantlet formation in creeping bentgrass. Crop Sci. 26: 1245-1248.

Blowers, A. D., Bogorad, L., Shark, K. B., and Sanford, J. C. 1989. Studies on *Chlamydomonas* chloroplast transformation: foreign DNA can be stably maintained in the chromosome. Plant Cell 1: 123-132.

Botterman, J., and Leemans, J. 1988. Engineering herbicide resistance in plants. Trends Genet. 4: 219-222.

Boulter, M. E., Croy, E., Simpson, P., Shields, R., Croy, R. R. D., and Shirsat, A. H. 1990a. Transformation of *Brassica napus* L. (oilseed rape) using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*-a comparison. Plant Sci. 70: 91-99.

Boulter, D., Edward, G. A., Gatehouse, A. M. R., Gatehouse, J. A., and Hilder, V. A. 1990b. Additive protective effects of incorporating two different higher plant derived insect resistance genes in transgenic tobacco plants. Crop Prot. 9: 351-354.

Boutry, M., Nagy, F., Poulsen, C., Aoyagi, K., and Chua, N. -H. 1987. Targeting of bacterial chloramphenicol acetyltransferase to mitochondria in transgenic plants. Nature 328: 340-342.

Bower, R., and Birch, R. G. 1992. Transgenic sugarcane plants via particle bombardment. Plant J. 2 (3): 409-416

Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K., and Sanford, J. C. 1988. Chloroplast transformation of *Chlamydomonas* using high velocity microprojectiles. Science 240: 1534-1538.

Bradshaw, A. D. 1958. Studies of variation in bentgrass species, I. Hybridization between *Agrostis tenuis* and *A. stolonifera*. J. Sports Turf Res. Inst. 9 (34): 422-429.

Brar, G. S., Cohen, B. A., Vick, C. L. and Johnson, G. W. 1994. Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from elite cultivars utilizing ACCELL technology. Plant J. 5 : 745-753.

Brasileiro, A. C. M., Tourneur C., C., Leplé, J. C., Combers, V., and Jouanin, L. 1992. Expression of the mutant *Arabidopsis thaliana* acetolactate synthase gene confers chlorsulfuron resistance to transgenic poplar plants. Transgenic Research 1: 133-141.

Brinster, R. L., Chen, H. Y., Trumbauer, M., Yagle, M. K., and Palmiter, R. D. 1985. Factors affecting the efficiency of introducing foreign DNA into mice. Proc. Natl. Acad. Sci. U.S.A. 82: 4438-4442.

Broadway, R. M., and Duffy, S. S. 1986. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J. Insect Physiol. 32: 827-833.

Brown, W. E., Takio, K., Titani, K., and Ryan, C. A. 1985. Wound-induced trypsin inhibitor in alfalfa leaves: identity as a member of the Bowman-Birk inhibitor family. Biochem. 24: 2105-2108.

Bruce, W. B., Christensen, A. H., Klein, T., Fromm, M., and Quail, P. H. 1989. Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. Proc. Natl. Acad. Sci. U.S.A. 86: 9692-9696.

Brunke, K. J., and Meeusen, R. L. 1991. Insect control with genetically engineered crops. Trends Biot. 9: 197-200.

Bytebier, B., Deboeck, F., De Greve, H., Van Montagu, M., and Hernalsteens, J. -P. 1987. T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. Proc. Natl. Acad. Sci. U.S.A. 84: 5345-5349.

Caboche, M. 1990. Liposome-mediated transfer of nucleic acids in plant protoplasts. Physiol. Plant. 79: 173-176.

Callahan, L. M. 1966. Select herbicides carefully ... turfgrass tolerances do differ. Weeds, Trees and Turf. 5 (11): 6-17.

Callahan, L. M., and Engel, R. E. 1965. Tissue abnormalities induced in roots of colonial bentgrass by phenoxyalkylcarboxylic acid herbicides. Weeds. 13 (4): 336-338.

Cao, J., Wang, Y. -C., Klein, T. M., Sanford, J. C., and Wu, R. 1989. Transformation of rice and maize using the biolistic process. In: Plant Gene Transfer-1989 UCLA Symposium. New York. Alan R. Liss. pp. 21-33.

Cao, J., Zhang, W. G., McElroy, D., and Wu, R. 1991. Assessment of rice genetic transformation techniques. In: Rice Biotechnology. Khush, G. S., and Toenniessen, G. H. (eds.) International Rice Research Institute. Manila. Philippines. pp. 175-198.

Cao, J., Xiaolan, D., McElroy, D., and Wu, R. 1992. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11: 586-591.

Capecchi, M. R. 1980. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell 22: 479-488.

Carrer, H., Staub, J. M., and Maliga, P. 1991. Gentamycin resistance in *Nicotiana* conferred by AAC(3)-I, a narrow substrate specificity a acetyltransferase. Plant Mol. Biol. 17: 301-303.

Casas, A. M., Kononowicz, A. K., Zehr, U. B., Tomes, D. T., Axtell, J. D., Butler, L. G., Bressan, R. A., and Hasegawa, P. M. 1993. Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. U.S.A. 90: 11212-11216.

Castillo, A. M., Vasil, V., and Vasil, I. K. 1994. Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). Bio/Technology 12: 1366-1371.

Catlin, D., Ochoa, O., McCormick, S., and Quiros, C. F. 1988. Celery transformation by *Agrobacterium tumefaciens* : cytological and genetic analysis of transgenic plants. Plant Cell Rep. 7: 100-103

Chabaud, M., Passiatore, J. E., Cannon, F., and Buchanan-Wollaston, V. 1988. Parameters affecting the frequency of kanamycin resistant alfalfa obtained by *Agrobacterium tumefaciens* mediated transformation. Plant Cell Rep. 7: 512-516.

Chan, M. T., Chang, H. H., Ho, S. L., Tong, W. F., and Yu, S. M. 1993. *Agrobacteriun*-mediated production of transgenic rice plants expressing a chimeric alpha-amylase promoter/beta-glucuronidase gene. Plant Mol. Biol. 22: 491-506.

Chang, S. S., Park, S. K., Kim, B. C., Kang, B. J., Kim, D. U., and Nam, H. G. 1994. Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation *in planta*. Plant J. 5 : 551-558.

Charest, P. J., Holbrook, L. A., Gabard, J., Iyer, V. N., and Miki, B. L. 1988. *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. Thero. Appl. Genet. 75: 438-445.

Chee, P. P. 1990. Transformation of *Cucumis sativus* tissue by *Agrobacterium tumefaciens* and regeneration of transformed plants. Plant Cell Rep. 9: 245-248.

Chee, P. P., and Slightom, J. L. 1992. Transformation of cucumber tissues by microprojectile bombardment: identification of plants containing functional and non-functional transferred genes. Gene 118: 255-260.

Chee, P. P., Fober, K. A., and Slightom, J. L. 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. Plant Physiol. 91: 1212-1218.

Cheung, A. Y., Bogorad, L., Van Montagu, M., and Schell, J. 1988. Relocating a gene for herbicide tolerance: a chloroplast gene is converted into a nuclear gene. Proc. Natl. Acad. Sci. U.S.A. 85: 391-395.

Christou, P., McCabe, D. E., and Swain, W. F. 1988. Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiol. 87: 671-674.

Christou, P., McCabe, D. E., Martinell, B. J., and Swain, W. F. 1990. Soybean genetic engineering-commercial production of transgenic plants. Trends Biot. 8: 145-151.

Christou, P., Ford, T. L., and Kofron, M. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9: 957-962.

Christou, P. 1994. Gene transfer to plants via particle bombardment. In: Plant Molecular Biology Manual. Gelvin S. B., and Schilperoort, R. A. (eds.) Kluwer Academic Publishers. Norwell, MA. A2: 1-15.

Chupeau, M. -C., Bellini, C., Guerche, P., Maisonneuve, B., Vastra, G., and Chupeau, Y. 1989. Transgenic plants of lettuce (*Lactuca sativa*) obtained through electroporation of protoplasts. Bio/Technology 7: 503-508.

Church, G. L. 1936. Cytological studies in the *Gramineae*. Amer. J. Bot. 23: 12-15.

Cleveland, T. E., Thornburg, R. W., and Ryan, C. A. 1987. Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol. Biol. 8: 199-207.

Comai, L., Sen, L. C., and Stalker, D. M. 1983. An altered *aroA* gene product confers resistance to herbicide glyphosate. Science 221: 370-371.

Comai, L., Facciotti, D., Hiatt, W. R., Thompson, G., Rose, R. E., and Stalker, D. M. 1985. Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. Nature 317: 741-744.

Comai, L. N., Larson-Kelly, J., Kiser, J. K., Mauss, A. R., Pokalsky, C. K., Shewmaker, K., McBride, A., Jones, A., and Stalker, D. M., 1988. Chloroplast transport of a ribulose bisphosphate carboxylase small subunit-5-enolpyruvyl 3-phosphoshikimate synthase chimeric protein requires part of the mature small subunit in addition to the transit peptide. J. Biol. Chem. 263: 14104-14109.

Comai, L., Larson-Kelly, N., Kiser, J., Jones, A., Stalker, D. M., Moran, P., Kiehne, K., and Koning, A. 1989. Genetic engineering of plants for herbicide resistance. Expression of an RuBP carboxylase small subunit-EPSP synthase chimeric gene, chloroplast transport of the hybrid protein, and tolerance phenotype in transgenic plants. Vortr. Pflanzenzüchtg. 16: 441-454.

Courtney-Gutterson, N., Napoli, C., Lemieux, C., Morgan, A., Firoozabady E., and Robinson, K. E. P. 1994. Modification of flower color in florist's chrysanthemum: production of a white-flowering variety through molecular genetics. Bio/Technology 12: 268-271.

Crossway, A., Oakes, J. V., Irvine, J. M., Ward, B., Knauf, B. C., and Shewmaker, K. 1986. Integration of foreign DNA following micro-injection of tobacco mesophyll protoplasts. Mol. Gen. Genet. 202: 179-185.

Dandekar, A. M., Gupta, P. K., Kurzan, D. J., and Knauf, V. 1987. Transformation and foreign gene expression in micropropagated Douglas-fir (*Pseudotsuga menziesii* ). Bio/Technology 5: 587-590.

Daniell, H., Vivekananda, J., Nielsen, B. L., Ye, G. N., Tewari, K. K., and Sanford, J. C. 1990. Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. Proc. Natl. Acad. Sci. U.S.A. 87: 88-92.

Datta, S. K., Peterhans, A., Datta, K., and Potrykus, I. 1990. Genetically engineered fertile Indica-rice recovered from protoplasts. Bio/Technology 8: 736-740.

Datta, S. K., Datta, K., Soltanifar, N., Donn, G., and Potrykus, I. 1992. Herbicide resistant indica rice plants from IRRI breeding line ER72 after PEG-mediated transformation of protoplasts. Plant Mol. Biol. 20: 619-629.

Davey, M. R., Cocking, E. C., Freeman, J., Pearce, N., and Tudor, I. 1980. Transformation of *Petunia* protoplasts by isolated *Agrobacterium* plasmids. Plant Sci. Lett. 18: 307-313.

Davies, P. A., Larkin, P. J., and Tanner, G. J. 1989. Enhanced protoplast division by media ultrafiltration using dicamba. Plant Sci. 60: 237-244.

Davies, D. R., Hamilton, J., and Mullineaux, P. 1993. Transformation of peas. Plant Cell Rep. 12: 180-183.

Day, A., Debuchy, R., van Dillewijn, J., Purton, S., and Rochaix, J. D. 1990. Studies on the maintenance and expression of cloned DNA fragments in the nuclear genome of the green alga *Chlamydomonas reinhardtii*. Physiol. Planta. 78: 254-260.

Deak, M., Kiss, G. B., Konez, C., and Dudits, D. 1986. Transformation of *Medicago* by *Agrobacterium* mediated gene transfer. Plant Cell Rep. 5: 97-100.

De Block, M. 1988. Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. Theor. Appl. Genet. 76: 767-774.

De Block, M. 1990. Factors influencing the tissue culture and the *Agrobacterium tumefaciens*- mediated transformation of hybrid aspen and poplar clones. Plant Cell Rep. 93: 1110-1116.

De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J., and Zambryski, P. 1984. Expression of foreign genes in regenerated plants and in their progeny. EMBO J. 3: 1681-1689.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Rao Movva, N., Thompson, C., Van Montagu, M., and Leemans, J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6: 2513-2518.

De Block, M., De Brouwer, D., and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. 91: 694-701.

De Greef, W., Delon, R., De Block, M., Leemans, J., and Botterman, J. 1989. Evaluation of herbicide resistance in transgenic crops under field conditions. Bio/Technology 7:61-64.

Dekeyser, R., Claes, B., Marichal, M., Montagu, M. V., and Caplan, A. 1989. Evaluation of selectable markers for rice transformation. Plant Physiol. 90: 217-223.

Dekeyser, R. A., Claes, B., De Rycke, R. M. U., Habets, M. E., Van Montagu, M. C., and Caplan, A. B. 1990. Transient gene expression in intact and organized rice tissues. Plant Cell 2: 591-602.

de la Peña, A., Lörz, H., and Schell, J. 1987. Transgenic rye plants obtained by injecting DNA into young floral tillers. Nature 325: 274-276.

Della-Cioppa, G., Bauer, S. C., Taylor, M. L., Rochester, D, E., Klein, B. K., Shah, D. M., Fraley, R. T., and Kishore, G. M. 1987. Targeting a herbicide-resistant enzyme from *Escherichia coli* to chloroplasts of higher plants. Bio/Technology 5: 579-584.

Denecke, J., Botterman, J., and Devlaere, R. 1990. Protein secretion in plant cells can occur via a default pathway. Plant Cell. 2: 51-59.

Deshayes, A., Herrera-Estrella, L., and Caboche, M. 1985. Liposome-mediated transformation of tobacco mesophyll protoplasts by *Escherichia coli* plasmid. EMBO J. 4: 2731-2737.

D'Halluin, K., Botterman, J., and Greef, W. D. 1990. Engineering of herbicide-resistant alfalfa and evaluation under field conditions. Crop Sci. 30: 866-871.

D'Halluin, K., Bossut, M., Bonne, E., Mazur, B., Leemans, J., and Botterman, J. 1992a. Transformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio/Technology 10: 309-314.

D'Halluin, K., Bonne, E., Bossut, M., De Beuckeleer, M., and Leemans, J. 1992b. Transgenic maize plants by tissue electroporation. Plant Cell 4: 1495-1505.

Dhir, S. K., Dhir, S., Savka, M. A., Belanger, F., Kriz, A. L., Farrand, S. K., and Widholm, J. M. 1992. Regeneration of transgenic soybean (*Glycine max*) plants from electroporated protoplasts. Plant Physiol. 99: 81-88.

Dong, J. - Z., and McHughen A. 1993. An improved procedure for production of transgenic flax plants using *Agrobacterium tumefaciens*. Plant Sci., 88: 61-71.

Draper, J., Davey, M. R., Freeman, J. P., Cocking, E. C., and Cox, B. G. 1982. Ti plasmid homologous sequences present in tissue from *Agrobacterium* plasmid transformed *Petunia* protoplasts. Plant Cell Physiol. 23: 451-458.

Ducrocq, C. Sangwan, R. S., and Sangwan-Norreel, B. S. 1994. Production of *Agrobacterium*-mediated transgenic fertile plants by direct somatic embryogenesis from immature zygotic embryos of *Datura innoxia*. Plant Mol. Biol. 25: 995-1009.

Dulmage, H. T. 1981. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control. In: Microbial control of pests and plant diseases 1970-1980. Burges, H. D. (ed.) Academic Press, New York, pp 193-222.

Dunahay, T. G. 1993. Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers, BioTechniques. 15: 452-460.

Duncan, K., Lewendon, A., and Coggins, J. R. 1984. The complete amino acid sequence of *Escherichia coli* 5-enol-pyruvylshikimate 3-phosphate synthase. FEBS Lett. 170 : 59-63.

Eapen, S., Köhler, F., Gerdemann, M., and Schieder, O. 1987. Cultivar dependence of transformation rates in moth bean after co-cultivation of protoplasts with *Agrobacterium tumefaciens*. Theor. Appl. Genet. 75: 207-210.

Eckes, P., Schmitt, P., Daub, W., and Wengenmayer, F. 1989. Overproduction of alfalfa glutamine synthetase in transgenic tobacco plants. Mol. Gel. Genet. 217: 263-268.

Ebert, P. R., and Clarke, A. E. 1990. Transformation and regeneration of the self-incompatible species *Nicotiana alata* Link & Otto. Plant Mol. Biol. 14: 815-824.

Eichholtz., D. A., Rogers, S. G., Horsch, R. B., Klee, H. J., Hayford, M., Hoffmann, N. L., Braford, S. B., Fink, C., Flick, J., O'Connell, K, M., and Fraley, R. T. 1987. Expression of a mouse dihydrofolate gene confers methotrexate resistance in transgenic petunia plants. Somatic Cell Mol. Genet. 13: 67-76.

Ellis, J. R., Shirsat, A. H., Hepher, A., Yarwood, J. N., Gatehouse, J. A., Croy, R. R. D., and Boulter, D. 1988. Tissue-specific expression of a pea legumin gene in seeds of *Nicotiana plumbaginifolia*. Plant Mol. Biol. 203-214.

Ellis, D. D., McCabe, D. E., Mclnnis, S., Ramachandran, R., Russell, D. R., Wallace, K. M., Martinell, B. J., Roberts, D. R., Raffa, K. F., and McCown, B. H. 1993. Stable transformation of *Picea glauca* by particle acceleration. Bio/Technology 11: 84-89.

Escandón, A. S., and Hahne, G. 1991. Genotype and composition of culture medium are factors important in the selection of transformed sunflower (*Helianthus annuus*) callus. Physiol. Plant. 81: 367-376.

Everett, N. P., Robison, K. E. P., and Mascarenhas, D. 1987. Genetic engineering of sunflower (*Helianthus annuus* L.). Bio/Technology 5: 1201-1204.

Fang, G., and Grumet, R. 1990. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Rep. 9: 160-164.

Feldmann, K. A., and Marks, M. D. 1986. Rapid and efficient regeneration of plants from explants of *Arabidopsis thaliana*. Plant Sci. 47: 63-69.

Felgner, P. L., and Ringold, G. M. 1989. Cationic liposome-mediated transfection. Nature 337: 387-388.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. 1987. Lepofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84: 7413-7417.

Filippone, E., and Lurquin, P. F. 1989. Stable transformation of eggplant (*Solanum melongena* L.) by cocultivation of tissues with *Agrobacterium tumefaciens* carrying a binary plasmid vector. Plant Cell Rep. 8: 370-373.

Fillatti, J. J., Kiser, J., Rose, R., and Comai, L. 1987a. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technology 5: 726-730.

Fillatti, J. J., Sellmer, J., McCown, B., Haissig, B., and Comai, L. 1987b. *Agrobacterium* mediated transformation and regeneration of *Populus*. Mol. Gen. Genet. 206: 192-199.

Finer, J. J., and McMullen, M. D. 1990. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep. 8: 586-589.

Finer, J. J., Vain, P., Jones, M. W., and McMullen, M. D. 1992. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep. 11: 323-328.

Firoozabady, E., Deboer, D. L., Merlo, D. J., Halk, E. L., Amerson, L. N., Rashka, K. E., and Murray, E. E. 1987. Transformation of cotton (*Gossypium hirsutum* L.) by ***Agrobacterium tumefaciens*** and regeneration of transgenic plants. Plant Mol. Biol. 10: 105-116.

Firoozabady, E., Moy, Y., Courtney-Gutterson, N., and Robinson, K. 1994. Regeneration of transgenic rose (***Rosa hybrida***) plants from embryogenic tissue. Bio/Technology 12: 609-613.

Fischhoff, D. A., Bowdish, K. S., Perlak, F. J., Marrone, P. G., McCormick, S. M., Niedermeyer, J. G., Dean, D. A., Kusano-Kretzmer, K., Mayer, E. J., Rochester, D. E., Rogers, S. G., and Fraley, R. T. 1987. Insect tolerant transgenic tomato plants. Bio/Technology 5: 807-813.

Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., and Sanford, J. C. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9: 189-194.

Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., and Sanford, J. C. 1992. Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. Bio/Technology 10: 1466-1472.

Fox, T. D., Sanford, J. C., and McMullin, T. W. 1988. Plasmids can stably transform yeast mitochondria totally lacking endogenous mtDNA. Proc. Natl. Acad. Sci. U.S.A. 85: 7288-7292

Fraley, R. T., Dellaporta, S. L., and Papahadjopoulos, D. 1982. Liposome-mediated delivery of tobacco mosaic virus RNA into tobacco protoplasts: A sensitive assay for monitoring liposome-protoplast interactions. Proc. Natl. Acad. Sci. U.S.A. 79: 1859-1863.

Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S.., Adams, S. P., Bittner, M. L., Brand, L. A., Fink, C. L., Fry, J. S., Gallupi, G. R., and Goldberg, S. B. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. U.S.A. 80: 4803-4807.

Frame, B. R., Drayton, P. R., Bagnall, S. V., Lewnau, C. J., Bullock, W. P., Wilson, H. M., Dunwell, J. M., Thompson, J. A., and Wang, K. 1994. Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. Plant J. 6: 941-948.

Franks, T., and Birch, R. G. 1991. Gene transfer into intact sugarcane cells using microprojectile bombardment. Aust. J. Plant Physiol. 18: 471-480.

Fromm, M., Taylor, L. P., and Walbot, V. 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. U.S.A. 82: 5824-5828.

Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T. M. 1990. Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8: 833-839.

Fry, J., Barnason, A., and Horsch, R. B. 1987. Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vectors. Plant Cell Rep. 6: 321-325.

Funk, C. R., Belanger, F. C., and Murphy, J. A. 1994. Role of endophytes in grasses used for turf and soil conservation. In: Biotechnology of endophytic fungi of grasses. Bacon, C. W., and White, J. F. (eds) CRC Press, Inc., Boca Raton, Florida. pp. 201-209.

Furini, A., Koncz, C. Salamini, F., and Bartels, D. 1994. *Agrobacterium*-mediated transformation of the desiccation-tolerant plant *Craterostigma plantagineum*. Plant Cell Rep. 14: 102-106.

Gasser, C. S., and Fraley, R. T. 1989. Genetically engineering plants for crop improvement. Science 224: 1293-1299.

Gatehouse, A. M. R., and Boulter, D. 1983. Assessment of the anti-metabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the bruchid bettle *Callosobruchus maculatus*. J. Sci. Food Agric. 34: 345-350.

Gatehouse, A. m. R., and Hilder, V. A. 1988. Introduction of genes conferring insect resistance. In: Proceedings of Brighton Crop protection Conference. Vo. 3. Lavenham Press Ltd., Lavenham, Suffolk, UK, pp. 1234-1254.

Gatehouse, A. M. R., Gatehouse, J. A., Dobie, P., Kilminster, A. M., and Boulter, D. 1979. Biochemical basis of insect resistance in *Vigna unguiculata*. J. Sci. Food Agric. 30: 948-958.

Gatehouse, A. M. R., Gatehouse, J. A., and Boulter, D. 1980. Isolation and characterisation of trypsin inhibitors from cowpea. Phytochem. 19: 751-756.

Glaser, A., and Donath, E. 1989. Osmotically-induced surface area expansion in oat protoplasts depends on the transmembrane potential. J. Exp. Bot. 40: 1231-1235.

Goddijn, O. J. M., van der Duyn Schouten, P. M., Schilperoort, R. A., and Hoge, J. H. C. 1993. A chimaeric tryptophan decarboxylase gene as a novel selectable marker in plant cells. Plant Mol. Biol. 22: 907-912.

Goldberg, R. B. 1988. Plants: novel developmental processes. Science 240: 1460-1467.

Golovkin, M. V., Ábrahám, M., Mórocz, S., Bottka, S., Fehér, A., and Dudits, D. 1993. Production of transgenic maize plants by direct DNA uptake into embryogenic protoplasts. Plant Sci. 90: 41-52.

Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R., Willetts, N. G., Rice, T. B., Mackey, C. J., Krueger, R. W., Kausch, A. P., and Lemaux, P. G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2: 603-618.

Gould, S. J., Keller, G. -A., Schneider, M., Howell, S. H., Garrard, L. J., Goodman, J. M., Distel, B., Tabak, H., and Subramani, S. 1990. Peroxisomal protein import is conserved between yeast, plants, insects, and mammals. EMBO J. 9: 85-90.

Gould, J., Devey, M., Hasegawa, O., Ulian, E. C., Peterson, G., and Smith, R. H. 1991. Transformation of *Zea mays* L. using *Agrobacterium* tumefaciens and the shoot apex. Plant Physiol. 95: 426-434.

Grayburn, W. S., and Vick, B. A. 1995. Transformation of sunflower (*Helianthus annuus* L.) following wounding with glass beads. Plant Cell Rep. 14: 285-289.

Green, T. R., Ryan, C. A. 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insect. Science 175: 776-777.

Gritz, L., and Davies, J. 1983. Plasmid-encoded hygromycin-B-resistance: The sequence of hygromycin-B-phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25: 179-188.

Guerche, P., Charbonnier, M., Jouanin, L., Tourneur, C. Paszkowski, J., and Pelletier, G. 1987. Direct gene transfer by electroporation in *Brassica napus*. Plant Sci. 52: 111-116.

Guerineau, F., Brooks, L., Meadow, J., Lucy, A., Robinson, C., and Mullineaux, P. 1990. Sulfonamide resistance gene for plant transformation. Plant Mol. Biol. 15: 127-136.

Guo, Y., Liang, H., and Berns, M. W. 1995. Laser-mediated gene transfer in rice. Physiol. Plant. 93: 19-24.

Guri, A., and Sink, K. C. 1988. *Agrobacterium* transformation of eggplant. J. Plant Physiol. 133: 52-55.

Ha, S. - B., Wu, F. -S., and Thorne, T. K. 1992. Transgenic turf-type tall fescue (*Festuca arundinacea* Schreb.) plants regenerated from protoplasts. Plant Cell Rep. 11: 601-604.

Hagio, T., Hirabayashi, T., Machii, H., and Tomotsune H. 1995. Production of fertile transgenic barley (*Hordeum vulgare* L.) plant using the hygromycin-resistance marker. Plant Cell Rep. 14: 329-334.

Hain, H., Stabel, P., Czernilofsky, A. P., Steinbiss, H. H., Herrera-Estrella, L., and Schell, J. 1985. Uptake, integration, expression and genetic transformation of a selectable chimaeric gene by plant protoplasts. Mol. Gen. Genet. 199: 161-168.

Hartman, C., Lee, L., Day, P. R., and Tumer, N. 1994. Herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation. Bio/Technology 12: 919-923.

Harvey, B. M. R., and Harper, D. B. 1982. Tolerance to bipyridylium herbicides. In Herbicide Resistance in Plants. LeBaron H. M. and Gressel, J. (ed.) Wiley, New York, pp. 215-234.

Hatamoto, H., Boulter, M. E., Shirsat, A. H., Croy, E. J., and Ellis, J. R. 1990. Recovery of morphologically normal transgenic tobacco from hairy roots co-transformed with *Agrobacterium rhizogenes* and a binary vector plasmid. Plant Cell Rep. 9: 88-92.

Haughn, G. W., Smith, J., Mazur, B., and Somerville, C. 1988. Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Genet. 211: 266-271.

Hauptmann, R. M., Vasil, V., Ozias-Atkins, P., Tabaeizadeh, Z., Rogers, S. G., Fraley, R. T., Horsch, R. B., and Vasil, I. K. 1988. Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiol. 86: 602-606.

Hayashimoto, A., Li, Z., and Murai, N. 1990. A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant Physiol. 93: 857-863.

Hayford, M. B., Medford, J. I., Hoffman, N. L., Rogers, S. G., and Klee, H. J. 1988. Development of a plant transformation selection system based on expression of genes encoding gentamycin acetyltransferases. Plant Physiol. 86: 1216-1222.

He, D. G., Mouradov, A., Yang, Y. M., Mouradova, E., and Scot, K. J. 1994. Transformation of wheat (*Triticum aestivum* L.) through electroporation of protoplasts. Plant Cell Rep. 14: 192-196.

Hegarty, T. W. 1978. The physiology of seed hydration and dehydration, and the relation between the water stress and the control of germination: a review. Plant Cell Environ. 1: 109-119.

Hein, M. A. 1958. Registration of Penncross creeping bentgrass. Agron. J. 50: 399.

Herman, P. L., and Marks, M. D. 1989. Trichome development in *Arabidopsis thaliana*. II. Isolation and complementation of the GLABROUSI gene. Plant Cell 1: 1051-1055.

Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J. P., Van Montagu, M., and Schell, J. 1983a. Chimeric genes as dominant selectable markers in plant cells EMBO J. 2: 987-995.

Herrera-Estrella, L,, Depicker, A., Van Montague, m., and Schell, J., 1983b. Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303: 209-213.

Hess, D., Dressler, K., and Nimmrichter, R. 1990. Transformation experiments by pipetting *Agrobacterium* into the spiklets of wheat (*Triticum aestivum* L.). Plant Sci. 72: 233-244.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6 (2): 271-282.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F., and Boulter, D. 1987. A novel mechanism of insect resistance engineered into tobacco. Nature 330: 160-163.

Hilder, V. A., Barker, R. F., Samour, R. A., Gatehouse, A. M. R., Gatehouse, J. A., and Boulter, D. 1989. Protein and cDNA sequences of Bowman-Birk protease inhibitors from the cowpea (*Vigna unguiculata* Walp). Plant Mol. Biol. 13: 701-710.

Hilder, V. A., Gatehouse, A. M. R., and Boulter, D. 1991. Transgenic plants for conferring insect tolerance-protease inhibitor approach. In: Transgenic Plants. Vol. I. Kung S., and Wu, R. (eds.) Academic Press, New York pp. 317-338.

Hille, J., Verheggen, F., Roelvink, P., Franssen, H., van Kammen, A., and Zabel, P. 1986. Bleomycin resistance: A new dominant marker for plant cell transformation. Plant Mol. Biol. 7: 171-176.

Hinchee, M. A. W., Connor-Ward, D. V., Newell, C. A., McDonnell, R. E., Sato, S. J., Gasser, C. S., Fischhoff, D. A., Re, D. B., Fraley, R. T., and Horsch, R. B. 1988.

Production of transgenic soybean plants using *Agrobacterium* mediated DNA transfer. Bio/Technology 6: 915-922.

Hoekstra, F. A., Crowe, J. H. and Crowe, L. M. 1989. Membrane behaviour in drought and its physiological significance. In: Taylorson, R. B. (ed.) Recent Advances in the Development and Germination of Seeds. Plenum. New York. pp. 71-88.

Holt, E. C., and Payne, K. T. 1952. Variation in spreading rate and growth characteristics of creeping bentgrass seedlings. Agron. J. 44: 88-90.

Hooykaas, P. J. J., and Schilperoort, R. A. 1992. *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19: 15-38.

Horn, M. E., Shillito, R. D., Conger, B. V., and Harms, C. T. 1988. Transgenic plants of Orchardgrass (*Dactylis glomerata* L.) from protoplasts. Plant Cell Rep. 7: 469-472.

Horsch, R. B., Fraley, R. T., Rogers, S. G., Sanders, P. R., Lloyd, A., and Hoffmann, N. 1984. Inheritance of functional foreign genes in plants. Science 223: 496-498.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227: 1229-1231.

Hunold, R., Bronner, R., and Hahne, G. 1994. Early events in microprojectile bombardment: cell viability and particle location. Plant J. 5: 593-604.

Iida, A., Seki, M., Kamada, M. Yamada, Y., and Morikawa, H. 1990. Gene delivery into cultured plant cells by DNA-coated gold particles accelerated by a pneumatic particle gun. Theor. Appl. Genet. 80: 813-816.

Iida, A., Yamashida, T., Yamada, Y., and Morikawa, H. 1991. Efficiency of particle bombardment mediated transformation is influenced by cell cycle stage in synchronized cultured cells of tobacco. Plant Physiol. 97: 1585-1587.

Ishida, B. K., Snyder, G. W., and Belknap, W. R. 1989. The use of *in vitro*-grown microtuber discs in *Agrobacterium tumefaciens* mediated transformation of Russet Burbank and Lemhi Russet potatoes. Plant Cell Rep. 8: 325-328.

Iturriaga, G., Jefferson, R. A., and Bevan, M. W. 1989. Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. Plant Cell 1: 381-390.

Jähne, A., Becker, D., Brettschneider, R., and Lörz, H. 1994. Regeneration of transgenic, microspore-derived, fertile barley. Theor. Appl. Genet. 89: 525-533.

James, D. J., Passey, A. J., Barbara, D. J., and Bevan, M. 1989. Genetic transformation of apple (*Malus pumila* Mill.) using a disarmed Ti-binary vector. Plant Cell Rep. 7: 658-661.

James, D. J., Passey, A. J., and Barbara, D. J. 1990. *Agrobacterium* mediated transformation of the cultivated strawberry (*Fragaria* x *Anannassa* Duch.) using disarmed binary vectors. Plant Sci. 69: 79-84.

Jaworski, E. G. 1972. Mode of action of N-phosphonomethyl glycine: inhibition of aromatic amino acid biosynthesis. J. Agric. Food. Chem. 20: 1195-1198.

Jefferson, R. A. 1989. The GUS reporter gene system. Nature 342: 837-838.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Jenes, B., and Pauk, J. 1989. Plant regenation from protoplast derived calli in rice (*Oryza sativa* L.) Plant Sci. 63: 187-198.

Jensen, J. S., Marcker, K. A., Otten, L., and Schell, J. 1986. Nodule-specific expression of a chimaeric soybean leghaemoglobin gene in transgenic *Lotus corniculatus*. Nature 321: 669-674.

Jia, S. -R., Yang, M. -Z., Ott, R., and Chua, N. -H. 1989. High frequency transformation of *Kalanchöe lacinniata*. Plant Cell Rep. 8: 336-340.

Joersbo, M., and Brunstedt, J. 1990. Direct gene transfer to plant protoplasts by mild sonication. Plant Cell Rep. 9: 207-210.

Johnston, S. A., Anziano, P., Shark, K., Sanford, J. C., and Butow, R. 1988. Transformation of yeast mitochondria by bombardment of cells with microprojectiles. Science: 240: 1538-1541.

Johnson, R., Narvaez, J., An, G., and Ryan, C. A. 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* harvae. Proc. Natl. Acad. Sci. U.S.A. 86: 9871-9875.

Jones, J. D. S., Svab, Z., Harper, E. C., Hurwitz, C. D., and Maliga, P. 1987. A dominant nuclear streptomycin resistance marker for plant cell transformation. Mol. Gen. Genet. 210: 86-91.

Jordan, M. C., and McHughen, A. 1988. Glyphosate tolerant flax plants from *Agrobacterium*-mediated gene transfer. Plant Cell Rep. 7: 281-284.

Kado, C. I. 1991. Molecular mechanisms of crown gall tumorigenesis. Crit. Rev. Plant Sci. 10: 1-32.

Kaeppler, H. F., Gu, W., Somers, D. A., Rines, H. W., and Cockburn, A. F. 1990. Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Rep. 9: 415-418.

Kaeppler, H. F., Somers, D. A., Rines, H. W., and Cockburn, A. F. 1992. Silicon carbide fiber-mediated stable transformation of plant cells. Theor. Appl. Genet. 84: 560-566.

Kay, R., Chan, A., Daly, M., and McPherson, J. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299-1302.

Khan, M. R. I., Tabe, L. M., Heath, L. C., Spencer, D., and Higgins, T. J. V. 1994. *Agrobacterium*- mediated transformation of subterranean clover (*Trifolium subterraneum* L.) Plant Physiol. 105: 81-88.

Kiernan, J. M., Goldberg, K. -B., Young, M. J., Schoelz, J. E., and Shepherd, R. J. 1989. Transformation and regeneration of *Nicotiana edwardsonii*. Plant Sci. 64: 67-78.

Kishore, G. M., Brundage, L., Kolk, K., Padgette, S. R., Rochester, D., Huynh, K., and della-Cioppa, G. 1986. Isolation, purification and characterisation of a glyphosate tolerant mutant *E. coli* EPSP synthase. Federal Proceedings 45: 1506.

Klee, H. J., Muskopf, Y. M., and Gasser, C. S. 1987. Cloning of an *Arabidopsis thaliana* gene encoding 5-enol-pyruvylshikimate 3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate tolerant plants. Mol. Gen. Genet. 210: 437-442.

Klein, T. M., Wolf, E. D., Wu, R., and Sanford, J. C. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70-73.

Klein, T. M., Fromm, M. E., Weissinger, A., Tomes, D., Schaaf, S., Sleeten, M., and Sanford, J. C. 1988a. Transfer of foreign genes into intact maize cells using high velocity microprojectiles. Proc. Natl. Acad. Sci. U.S.A. 85: 4305-4309.

Klein, T. M., Gradziel, T., Fromm, M. E., and Sanford, J. C. 1988b. Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Bio/Technology 6: 559-563.

Klein, T. M., Harper, E. C., Svab, Z., Sanford, J. C., Fromm, M. E., and Maliga, P. 1988c. Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. Proc. Natl. Acad. Sci. U.S.A. 85: 8502-8505.

Klein, T. M., Kornstein, L., Sanford, J. C., and Fromm, M. E. 1989. Genetic transformation of maize cells by particle bombardment. Plant Physiol. 91: 440-444.

Klöti, A., Iglesias, V. A., Wünn, J., Burkhardt, P. K., Datta, S. K., and Potrykus, I. 1993. Gene transfer by electroporation into intact scutellum cells of wheat embryos. Plant Cell Rep. 12: 671-675.

Knapp, S., Coupland, G., Uhig, H., Starlinger, P., and Salamini, F. 1988. Transposition of the maize transposable element *Ac* in *Solanum tuberosum*. Mol. Gen. Genet. 213: 285-290.

Kohler, F., Cardon, G., Pohlman, M., Gill, R., and Schielder, O. 1989. Enhancement of transformation rates in higher plants by low-dose irradiation: are DNA repair systems involved in the incorporation of exogenous DNA into the plant genome ? Plant Mol. Biol. 12: 189-199.

Kondo, Y., Shomura, T., Ogawa, Y., Tsuruoka, T., Watanabe, H., Totsukawa, K., Suzuki, T., Moriya, C., and Yoshida, J. 1973. Isolation and physicochemical and biological characterization of SF-1293 substance. Sci. Reports. of Meiji Seika Kaisha 13: 34-41; Chem. Abstr. 1974, 81, 89705b.

Koornneef, M., Hanhart, C., Jongsma, M., Tuma, I., Weide, R., Zabel, P., and Hille, J. 1986. Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci. 45: 201-208.

Kors, F. T. M. 1991. Antibiotics, mode of action, spectrum, resistance, solubility, sterilization, stability. Haarlem: Duchefa Biochemicals B. V.

Koziel, M. G., Beland, G. L., Bowman, C., Carozzi, N. B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwe.., S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M. R., Merlin, E., Rhodes, R., Warren, G. W., Wright, M., and

Evola, S. V. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11: 194-200.

Krans, J. V., Henning, V. T., and Torres, K. G. 1982. Callus induction, maintenance and plantlet regeneration in creeping bentgrass. Crop Sci. 22: 1193-1197.

Krens, F. A., Molendijk, L., Wullems, G. J., and Schilperoort, R. A. 1982. In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature 296: 72-74.

Kuehnle, A. R., and Sugii, N. 1992. Transformation of *Dendrobium* orchid using particle bombardment of protocorms. Plant Cell Rep. 11: 484-488.

Kumar, A., Miller, M., Whitty, P., Lyon, J., and Davie, P. 1995. *Agrobacterium*-mediated transformation of five wild *Solanum* species using *in vitro* microtubers. Plant Cell Rep. 14: 324-328.

Kunitz, M. 1945. Crystallization of a trypsin inhibitor from soybean. Science 101: 668-669.

Landsmann, J., Llewellyn, D., Dennis, E. S. and Peacock, W. J. 1988. Organ regulated expression of the *Parasponia andersonii* haemoglobin gene in transgenic tobacco plants. Mol. Gen. Genet. 214: 68-73.

Langridge, W. H. R., Li, B. J., and Szalay, A. A. 1985. Electric field mediated stable transformation of carrot protoplasts with naked DNA. Plant Cell Rep. 4: 355-359.

LaRossa, R. A. and Falco, S. C. 1984. Amino acid biosynthetic enzymes as targets of herbicide action. Trends Biot. 2: 158-161.

Laursen, C. M., Krzyzek, R. A., Flick, C. E., Anderson, P. C., and Spencer, T. M. 1994. Production of fertile transgenic maize by electroporation of suspension culture cells. Plant Mol. Biol. 24: 51-61.

Lee, K. Y., Townsend, J., Tepperman, J., Black, M., Chui, C. F., Mazur, B., Dunsuir, P., and Bedbrook, J. 1988. The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J. 7: 1241-1248.

Lee, B., Murdock, K., Topping, J., Kries, M., and Jones, G. 1989. Transient gene expression in aleurone protoplasts isolated from developing caryopses of barley and wheat. Plant Mol. Biol. 13: 21-29.

Li, Z., Xie, Q., Rush, R. C., and Murai, N. 1992a. Fertile transgenic rice plants generated via protoplasts from the U.S. cultivar Labelle. Crop Sci. 32: 810-814.

Li, Z., Hayashimoto, A., and Murai, N. 1992b. A sulfonylurea herbicide resistance gene from *Arabidopsis thaliana* as a new selectable marker for production of fertile transgenic rice plants. Plant Physiol. 100: 662-668.

Liener, I. E. 1980. Toxic constituents of plant foodstuffs. 2nd edn. Academic Press, New York.

Lindsey, K., and Jones, M. G. K. 1987. Transient gene expression in electroporated protoplasts and intact cells of sugar beet. Plant Mol. Biol. 10: 43-52.

Lindsey, K., and Gallois, P. 1990. Transformation of sugarbeet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. J. Expt. Bot. 41: 529-536.

Lipke, H., Fraenkel, G. S., and Liener, I. E. 1954. Effect of soybean inhibitors on growth of *Tribolium confusum*. J. Agric. Food Chem. 2: 410-415.

Lloyd, A. M., Barnason, A. R., Rogers, S. G., Byrne, M. C., Fraley, R. T., and Horsch, R. B. 1986. Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. Science 234: 464-466.

Lowe, K. W., and Conger, B. V. 1979. Root and shoot formation from callus cultures of tall fescue. Crop Sci. 19: 397-400.

Luo, Z., and Wu, R. 1988. A simple method for tranformation of rice via the pollen-tube pathway. Plant Mol. Biol. Rep. 6: 165-174.

Lynch, P. T., Finch, R. P., Davey, M. R., and Cocking, E. C. 1991. Rice tissue culture and its application. In: Rice Biotechnology. Khush, G. S., and Toenniessen, G. H. (eds.) CAB International. Wallingford, UK. pp.135-156.

Lyon, B. R., Llewellyn, D. J., Huppatz, J. L., Dennis, E. S., and Peacock, W. J. 1989. Expression of a bacterial gene in transgenic tobacco plants confers resistance to the herbicide 2, 4-dichlorophenoxyacetic acid. Plant Mol. Biol. 13: 533-540.

Machado, A., Puschmann, M., Pühringer, H., Kremen, R. Katinger, H., and Machado, M. 1995. Somatic embryogenesis of *Prunus subhirtella autumno rosa* and regeneration of transgenic plants after *Agrobacterium*-mediated transformation. Plant Cell Rep. 14: 335-340.

Manners, J. M. 1988. Transgenic plants of the tropical pasture legume *Stylosanthes humilis*. Plant Sci. 55: 61-68.

Manners, J. M., and Way, H. 1989. Efficient transformation with regeneration of the tropical pasture legume *Stylosanthes humilis* using *Agrobacterium rhizogenes* and a Ti plasmid-binary vector system. Plant Cell Rep. 8: 341-345.

Masson, J., Lancelin, D., Bellini, C., Lecerf, M., Guerche, P., and Pelletier, G. 1989. Selection of somatic hybrids between diploid clones of potato (*Solanum tuberosum*) transformed by direct gene transfer. Theor. Appl. Genet. 78: 153-159.

Mathews, H., Bharathan, N., Litz, R. E., Narayanan, K. R., Rao, P. S., and Bhatia, C. R. 1990a. The promotion of *Agrobacterium* mediated transformation in *Atropa belladona* L. by acetosyringone. J. Plant Physiol. 136: 404-409.

Mathews, H., Bharathan, N., Litz, R. E., Narayanan, K. R., Rao, P. S., and Bhatia, C. R. 1990b. Transgenic plants of mustard *Brassica juncea* (L.) Czern and Coss. Plant Sci. 72: 245-252.

Mathews, H., Wagoner, W., Cohen, C., Kellogg, J., and Bestwick, R. 1995. Efficient genetic transformation of red raspberry, *Rubus ideaus* L. Plant Cell Rep. 14: 471-476.

May, G. D., Afza, R., Mason, H. S., Wiecko, A., Novak, F. J., and Arntzen, C. J. 1995. Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. Bio/Technology 13: 486-492.

Mazur, B. J., Chui, C. F., and Smith, J. K., 1987. Isolation and characterisation of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85: 1110-1117.

McCabe, D. E., and Martinell B. J. 1993. Transformation of elite cotton cultivars via particle bombardment of meristems. Bio/Technology 11: 596-598.

McCabe, D. E., Swain, W. F., Martinell, B. J., and Christou, P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technology 6: 923-926.

McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., and Fraley, R. 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep. 5: 81-84.

McCown, B. H., McCabe, D. E., Russell, D. R., Robison, D. J., Barton, K. A., and Raffa, K. F. 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep. 9: 590-594.

McGranahan, G. H., Lesile, C. A., Uratsu, S. L., Martin, L. A., and Dandekar, A. M. 1988. *Agrobacterium* mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Bio/Technology 6: 800-804.

McGranahan, G. H., Lesile, C. A., Uratsu, S. L., Martin, L. A., and Dandekar, A. M. 1990. Improved efficiency of the walnut somatic embryo gene transfer system. Plant Cell Rep. 8: 512-516.

McKersie, B. D., Senaratna, T., Bowley, S. R., Brown, D. C. W., Krochko, J. E., and Bewley, J. D.,1989. Application of artificial seed technology in the production of hybrid alfalfa (*Medicago sativa* L.). In Vitro Cell. Dev. Biol. 25: 1183-1188.

Meijer, E. G. M., Schilperoort, R. A., Rueb, S., van Os-Ruygrok, P. E., and Hensgens, L. A. M. 1991. Transgenic rice cell lines and plants: expression of transferred chimeric genes. Plant Mol. Biol. 16: 807-820.

Mendel, R. R., Muller, B., Schulze, J., Kolesnikov, V., and Zelenin, A. 1989. Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theor. Appl. Genet. 78: 31-34.

Mendel, R. R., Clauss, E., Hellmund, R., Schulze, J., Steinbiss, H. H., and Tewes, A. 1990. Gene transfer to barley. In: Progress in Plant Cellular and Molecular Biology. Nijkamp, H. J. J. (ed.) Kluwer Academic Publishers, Dordrecht. pp. 73-78.

Meyer, P., Heidmann, I., Forkmann, G., and Saedler, H. 1987. A new petumia flower colour generated by transformation of a mutant with a maize gene. Nature 330: 677-678.

Michelmore, R., Marsh, E., Seely, S., and Landry, B. 1987. Transformation of lettuce (*Lactuca sativa*) mediated by *Agrobacterium tumefaciens*. Plant Cell Rep. 6: 439-442.

Mickel, C. E., and Standish, J. 1947. Susceptibility of processed soy flour and soy grits in storage to attack by *Tribolium castaneum* (Herbst). Univ. Minn. Agricult. Exp. Stat. Tech. Bull. 178: 1-20.

Miflin, B. J., and Lea, P. J. 1977. Amino acid metabolism. Ann. Rev. Plant Physiol. 28: 299-329.

Miki, B. L., Labbé, H., Hattori, J., Ouellet, T., Gabard, J., Sunohara, G., Charest, P. J., and Iyer, V. N. 1990. Transformation of *Brassica napus* canola cultivars with *Arabidopsis thaliana* acetohydroxyacid synthase genes and analysis of herbicide resistance. Theor. Appl. Genet. 80: 449-458.

Moloney, M. M., Walker, J. M., and Sharma, K. K. 1989. High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. 8: 238-242.

Morikawa, H., Iida, A., Matsui, C., Ikegami, M., and Yamada, Y. 1986. Gene transfer into intact plant cells by electroinjection through cell walls and membranes. Gene 41: 121-124.

Mullins, M. G., Tang, F. C. A., and Facciotti, D. 1990. *Agrobacterium*-mediated genetic transformation of grapevines: transgenic plants of *Vitis rupestris* schule and buds of *Vitis vinifera* L. Bio/Technology 8: 1041-1045.

Murakami, T., Anzai, H., Imai, S., Satoh, A., Nagaoka, K., and Thompson, C. J. 1986. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205: 42-50.

Nagy, F., Morelli, G., Fraley, R. T., Rogers, S. G., and Chua, N. -H. 1985. Photoregulated expression of a pea *rbcS* gene in leaves of transgenic plants. EMBO J. 4: 3063-3068.

Nap, J. P., Bijvoet, J., and Stiekema, W. 1992. Biosafety of kanamycin-resistant transgenic plants. Transgenic Research 1: 239-249.

Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G., and Sala, F. 1987. Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol. Biol. 8: 363-373.

Nehra, N. S., Chibbar, R. N., Kartha, K. K., Dalta, R. S. S., Crosby, W. L., and Stushnoff, C. 1990. Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disk regeneration system. Plant Cell Rep. 9: 292-298.

Nehra, N. S., Chibbar, R. N., Leung, N., Caswell, K., Mallard, C., Steinhauer, L., Baga, M., and Kartha, K. K. 1994. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 5: 285-297.

Neuhaus, G., Spangenberg, G., Mittelsten-Scheid, O., and Schweiger, H. -G. 1987. Transgenic rapeseed plants obtained by microinjection of DNA into microspore-derived embryoids. Theor. Appl. Genet. 75: 30-36.

Newell, C. A., Lowe, J. M., Merryweather, A., Rooke, L. M., and Hamilton, W. D. O. 1995. Transformation of sweet potato (*Ipomoea batatas* (L.) Lam.) with *Agrobacterium tumefaciens* and regeneration of plants expressing cowpea trypsin inhibitor and snowdrop lectin. Plant Sci. 107: 215-227.

Noda, T., Tanaka, N., Mano, Y., Nabeshima, S., Ohkawa, H., and Matsui, C. 1987. Regeneration of horseradish hairy root incited by *Agrobacterium rhizogenes* infection. Plant Cell Rep. 6: 283-286.

Nutter, R., Everett, N., Pierce, D., Danganiban, L., Okubara, P., Lachmansingh, R., Mascarenhas, D., Welch, H., Mettler, I., Pumeroy, L., Johnson, J., and Howard, J.1987. Factors affecting the level of kamamycin resistance in transformed sunflower cells. Plant Physiol. 84: 1185-1192.

Odell, J. T., Knowlton, S., Lin, W., and Mauvais, C. J. 1988. Properties of an isolated transcription-stimulating sequence derived from the cauliflower mosaic virus 35S promoter. Plant Mol. Biol. 10: 263-272.

Ogawa, Y., Tsuruoka, T., Inouye, S., Niida, T. 1973a. Chemical structure of antibiotic SF-1293. Sci. Reports. of Meiji Seika Kaisha 13: 42-48; Chem. Abstr. 1974, 81, 37806r.

Ogawa, Y., Tsuruoka, T., Inouye, S., and Niida, T. 1973b. Synthesis of a new phosphorus-containing amino acid, a component of antibiotic SF-1293. Sci. Reports. of Meiji Seika Kaisha 13: 49-553; Chem. Abstr. 1974, 81, 37788m.

Ohta, Y. 1986. High-efficiency genetic transformation of maize by a mixture of pollen and exogenous DNA. Proc. Natl. Acad. Sci. U.S.A. 83: 715-719.

Omirulleh, S., Abraham, M., Golovkin, M., Stefanov, I., Karabaev, M.K., Mustardy, L., Morocz, S., and Dudits, D. 1993. Activity of a chimeric promoter with doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. Plant Mol. Biol. 21: 415-428.

Ooms, G., Burrell, M. M., Krap, A., Bevan, M., and Hille, J. 1987. Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theor. Appl. Genet. 73: 744-750.

Otani, M. Mii, M., Handa, T., Kamada, H., and Shimada, T. 1993. Transformation of sweet potato (*Ipomoea batatas* (L.) Lam.) plants by *Agrobacterium rhizogenes*. Plant Sci. 94: 151-159.

Ou-Lee, T. -M., Turgeon, R., and Wu, R. 1986. Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter after electroporation of protoplasts of rice, wheat, and sorghum. Proc. Natl. Acad. Sci. U.S.A. 83: 6815-6819.

Parrot, W. A., Hoffman, L. M., Hildebrand, D. F., Williams, E. G., and Collins, G. B. 1989. Recovery of primary transformants of soybean. Plant Cell Rep. 7: 615-617.

Paszkowski, J., Shillito, R. D., Saul, M., Mandák, V., Hohn, T., Hohn, B., and Potrykus, I. 1984. Direct gene transfer to plants. EMBO J. 3: 2717-2722.

Paszkowski, J., Saul, M. W., and Potrukus, I. 1989. Plant gene vectors and genetic transformation: DNA-mediated direct gene transfer to plants. Cell Culture and Somatic Cell Genetics of Plants 6: 51-68.

Pavingerová, D., Dostál, J., Bísková, R., Benetka, V. 1994. Somatic embryogenesis and *Agrobacterium*-mediated transformation of chrysanthemum. Plant Sci. 97: 95-101.

Pechan, P. M. 1989. Successful cocultivation of *Brassica napus* microspores and proembryos with *Agrobacterium*. Plant Cell Rep. 8: 387-390.

Pellegrineschi, A., Damon, J. -P., Valtorta, N., Paillard, N., and Tepfer, D. 1994. Improvement of ornamental characters and fragrance production in lemon-scented geranium through genetic transformation by *Agrobacterium rhizogenes*. 1994. Bio/Technology 12: 64-68.

Peng, J., Lyznik, L. A., Lee, L., and Hodges, T. K. 1990. Co-transformation of Indica rice protoplasts with *gus* A and *neo* genes. Plant Cell Rep. 9: 168-172.

Peng, J., Kononowicz, H., and Hodges, T. K. 1992. Transgenic Indica rice plants. Theor. Appl. Genet. 83: 855-863.

Pereira, L. F., and Erickson, L. 1995. Stable transformation of alfalfa (*Medicago sativa* L.) by particle bombardment. Plant Cell Rep. 14: 290-293.

Perez, P., Tiraby, G., Kallerhoff, J., and Perret, J. 1989. Phleomycin resistance as a dominant selectable marker for plant cell transformation. Plant Mol. Biol. 13: 365-373.

Perl, A., Galili, S., Shaul, O., Ben-Tzvi, I., and Galili, G. 1993. Bacterial dihydrodipicolinate synthase and desensitized aspartate kinase: Two novel selectable markers for plant transformation. Bio/Technology 11: 715-718.

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T., and Fischhoff, D. A. 1990. Insect resistant cotton plants. Bio/Technology 8: 939-943.

Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L., and Fischhoff, D. A. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. U.S.A. 88: 3324-3328.

Philipson, W. R. 1937. A revision of the British species of the genus *Agrostis* Linn. J. Linn. Soc. London. 51: 73-151.

Pietrzak, M., Shillito, R. D., Hohn, T., and Potrykus, I. 1986. Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nuc. Acids Res. 14: 5857-5869.

Piper, C. V. 1918. The agricultural species of bent grass. Part I. Rhode Island bent and related grasses. USDA Bulletin No. 692. pp. 1-14.

Plunkett, G., Senear, D. F., Zuroske, G., and Ryan, C. A. 1982. Proteinase inhibitors I and II from leaves of wounded tomato plants: purification and properties. Arch. Biochem. Biophys. 213: 463-472.

Potrykus, I. 1990. Gene transfer to cereals: An assessment. Bio/Technology 8: 535-543.

Potrykus, I. 1991. Gene transfer to plants: Assessment of published approaches and results. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 205-225.

Potrykus, I., Saul, M. W., Petruska, J., Pazkowski, J., and Shillito, R. D. 1985. Direct gene transfer to cells of a graminaceous monocot. Mol. Gen. Genet. 199: 183-188.

Pua, E. -C., Mehra-Palta, A., Nagy, F., and Chua, N. -H. 1987. Transgenic plants of *Brassica napus* L. Bio/Technology 5: 815-817.

Puonti-Kaerlas, J., Eriksson, T., and Engström, P. 1992. Inheritance of a bacterial hygromycin phosphotransferase gene in the progeny of primary transgenic pea plants. Theor. Appl. Genet. 84: 443-450.

Radke, S. E., Andrews, B. M., Moloney, M. M., Crouch, M. L., Kridl, J. C., and Knauf, V. C. 1988. Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Genet. 75: 685-694.

Read, J. W., and Haas, L. W. 1938. Studies on the baking quality of flour as affected by certain enzyme actions. V. Further studies concerning potassium bromate and enzyme activity. Cereal Chem. 15: 59-68.

Ream, L. W., and Gordon, M. P. 1982. Crown gall disease and prospects for genetic manipulation of plants. Science 218: 854-859.

Rech, E. L., Golds, T. J., Husnain, T., Vainstein, M. H., Jones, B., Hammatt, N., Mulligan, B. J., and Davey, M. R. 1989. Expression of a chimaeric kanamycin resistance gene introduced into the wild soybean *Glycine canescens* using a cointegrate Ri plasmid vector. Plant Cell Rep. 8: 33-36.

Redden, R. J., Dobie, P., Gatehouse, A. M. R. 1983. The inheritance of seed resistance to *Callosobruchus maculatus* F. in cowpea (*Vigna unguiculata* L. Walp.). I. Analysis of parental, $F_1$, $F_2$, $F_3$ and backcross seed generations. Aust. J. Agricult. Res. 34: 681-695.

Reich, T. J., Iyer, V. N., and Miki, B. L. 1986. Efficient transformation of alfalfa protoplasts by intranuclear microinjection of Ti-plasmids. Bio/Technology 4: 1001-1004.

Renou, J. P., Brochard, P., and Jalouzot, R. 1993. Recovery of transgenic chrysanthemum (*Dendranthema grandiflora* Tzvelev) after hygromycin resistance selection. Plant Sci. 89: 185-197.

Restrepo, M. A., Freed, D. D., and Carrington, J. C. 1990. Nuclear transport of plant potyviral proteins. Plant Cell 10: 987-998.

Rhodes, C. A., Pierce, D. A., Mettler, I. J., Mascarenhas, D., and Detmer, J. J. 1988. Genetically transformed maize plants from protoplasts. Science 240: 204-207.

Richardson, M. J. 1977. The proteinase inhibitors of plants and microorganisms. Phytochem. 16: 159-169.

Richardson, M. J. 1991. Seed storage proteins: the enzyme inhibitors. In: Methods in Plant Biochemistry. Vol. 5. Rogers, L. J. (ed.) Academic Press, New York. pp. 259-305.

Riggs, C. D., and Bates, G. W. 1986. Stable transformation of tobacco by electroporation: Evidence for plasmid concatenation. Proc. Natl. Acad. Sci. U.S.A. 83: 5602-5606.

Ritala, A., Aspegren, K., Kurtén, U., Salmenkallio-Marttila, M., Mannonen, L., Hannus, R., Kauppinen, V., Teeri, T. H., and Enari, T. -M.1994. Fertile transgenic barley by particle bombardment of immature embryos. Plant Mol. Biol. 24: 317-325.

Robertson, D., Weissinger, A. K., Ackley, R., Glover, S., and Sederoff, R. R. 1992. Genetic transformation of Norway spruce (*Picea abies* (L.) Karst) using somatic embryo explants by microprojectile bombardment. Plant Mol. Biol. 19: 925-935.

Rotino, G. L., and Gleddie, S. 1990. Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens* vector. Plant Cell Rep. 9: 26-29.

Rugini, E., Pellegrineschi, A., Mencuccini, M., and Mariotto, D. 1991. Increase of rooting ability in the woody species kiwi (*Actinidia deliciosa* A. Chev.) by transformation with *Agrobacterium rhizogenes rol* genes. Plant Cell Rep. 10: 291-295.

Russell, J. A., Roy, M. K., and Sanford, J. C. 1992. Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol. 98: 1050-1056.

Russell, D. R., Wallace, K. M., Bathe, J. H., Martinell, B. J., and McCabe, D. E. 1993. Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle acceleration. Plant Cell Rep. 12: 165-169.

Ryan, C. A. 1983. Insect induced chemical signals regulating natural plant protection responses. In: Variable plants and herbivores in natural and managed systems. Denno, R. F., McClure, M. S. (eds) Academic press, New York, pp. 43-60.

Ryan, C. A. 1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. 28: 425-449.

Sacchi, V. F., Parenti, P., Hanozet, G. M., Giordanda, B., Lutly, P., Wolfersberger, M. G., 1986. *Bacillus thuringiensis* toxin inhibits $K^+$-gradient-dependent amino acid transport across the brush border membrane of *Pieris brassicae* midgut cells. FEBS Lett. 204: 213-218.

Sági, L., Panis, B., Remy, S., Schoofs, H., De Smet, K., Swennen, R., and Cammue, B. 1995. Genetic transformation of banana and plantain (*Musa* spp.) vua particle bombardment. Bio/Technology 13: 481-485.

Sanders, G. E. and Pallett, K. E. 1986. Studies into the differential activity of the hydroxybenzonitrite herbicides. I. Photosynthetic inhibition, symptom development and ultratructural changes in two contrasting species. Pestic. Biochem. Physiol. 26: 116-127.

Sanford, J. C., Klein, T. M. Wolf, E. D., and Allen, N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. J. Part. Sci. Tech. 5: 27-37.

Sanford, J. C., Devit, M. J., Russell, J. A., Smith, F. D., Harpending, P. R., Roy, M. K. and Johnston, S. A. 1991. An improved, helium driven biolistic device. Technique 3: 3-16.

Sanford, J. C., Smith, F. D., and Russell, J. A. 1993. Optimizing the biolistic process for different biological applications. Methods Enzymol 217: 483-509.

Sangwan, R. S., Ducrocq, C., Sangwan-Norreel, B. 1993. *Agrobacterium*-mediated transformation of pollen embryos in *Datura innoxia* and *Nicotiana tabacum*: production of transgenic haploid and fertile homozygous dihaploid plants. Plant Sci. 95: 99-115.

Sarria, R., Calderón, A., Thro, A. M., Torres, E., Mayer, J. E., Roca, W. M. 1994. *Agrobacterium*-mediated transformation of *Stylosanthes guianensis* and production of transgenic plants. Plant Sci. 96: 119-127.

Sautter, C., Waldner, H., Neuhaus-Url, G., Galli, A., Neuhaus, G., and Potrykus, I. 1991. Microtargeting: High efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. Bio/ Technology 9: 1080-1085.

Schrammeijer, B., Sijmons, P. C., van den Elzen, P. J. M., and Hoekema, A. 1990. Meristem transformation of sunflower via *Agrobacterium*. Plant Cell Rep. 9: 55-60.

Schreier, P. H. and Schell, J. 1986. Use of chimaeric genes harboring small subunit transit peptide sequences to study transport in chloroplasts. Phil. Trans. R. Soc. Lond. B313: 429-432.

Schroeder, H. E., Schotz, A. H., Wardley-Richardson, T., Spencer, D., and Higgins, T. J. V. 1993. Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). Plant Cell Rep. 101: 751-757.

Schulz, A., Wengenmayer, F., and Goodman, H. M. 1990. Genetic engineering of herbicide resistance in higher plants. CRC Critical Reviews in Plant Sciences. 9: 1-15.

Scorza, R., Ravelonandro, M., Callahan, A. M., Cordts, J. M., Fuchs, M., Dunez, J., and Gonsalves, D. 1995. Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. Plant Cell Rep. 14: 18-22.

Scott, R. J., and Draper, L. J. W. 1987. Transformation of carrot tissues derived from proembryogenic suspension cells: A useful model system for gene expression studies in plants. Plant Mol. Biol. 8: 265-274.

Seki, M., Shigemoto, N., Komeda, Y., Imamura, J., Yamada, Y., and Morikawa, H. 1991. Transgenic *Arabidopsis thaliana* plants obtained by particle bombardment-mediated transformation. Appl. Microbiol. Biotech. 36: 228-230.

Senaratna, T., McKersie, B. D., Kasha, K. J., and Procunier, J. D. 1991. Direct DNA uptake during the imbibition of dry cells. Plant Sci. 79: 223-228.

Serres, R., Stang, E., McCabe, D., Russell, D., Mahr, D., and McCown, B. Gene transfer using electric discharge bombardment and recovery of transformed cranberry plants. J. Amer. Soc. Hort. Sci. 117: 174-180.

Shah, D. M., Winter, J. W., Saunders, P. S., Hironaka, C. M., Steinrucken, H. C., Amrhein, N., and Fraley, R. T. 1985. Gene amplification is the molecular basis for glyphosate resistance in *Petunia* cells. Abstr. OR-09-01. 1st Int. Congr. Plant Molecular Biology, Savannah, GA.

Shah, D. M., Hironaka, C. M., Wiegand, R. C., Harding, E. L., Krivi, G. G. and Tiemeier, D. C. 1986a. Structural analysis of a maize gene coding for glutathione S-transferase involved in herbicide detoxification. Plant Mol. Biol. 6: 203-211.

Shah, D. M., Horsch, R. B., Klee, H. J., Kishore, G. M., Winter, J. A., Tumer, N. E., Hironaka, C. M., Sanders, P. R., Gasser, C. S., Aykent, S., Siegel, N. R., and Rogers, S. G. 1986b. Engineering herbicide tolerance in transgenic plants. Science 233: 478-481.

Shahin, E. A., Spielmann, A., Sukhapinda, K., Simpson, R. B., and Yashar, M. 1986a. Transformation of cultivated alfalfa using disarmed *Agrobacterium tumefaciens*. Crop Sci. 26: 1235-1239.

Shahin, E. A., Sukhapinda, K., Simpson, R. B., and Spivey, R. 1986b. Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes*: transgenic plants with normal phenotypes harbor binary vector T-DNA but no Ri-plasmid T-DNA. Theor. Appl. Genet. 72: 770-777.

Shark, K. B., Smith, F. D., Harpending, P. R., Rasmussen, J. L., and Sanford, J. C. 1991. Biolistic transformation of a prokaryote, *Bacillus megaterium*. Appl. Environ. Microbiol. 57: 480-485.

Sheerman, S., and Bevan, M. W. 1988. A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors. Plant Cell Rep. 7: 13-16.

Shillito, R. D., Saul, M. W., Paszkowski, J., Müller, M., Potrykus, I. 1985. High efficiency direct gene transfer to plants. Bio/Technology 3: 1099-1103.

Shimamoto, K., Terada, R., Izarva, T., and Fujimoto, H. 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338: 274-277.

Shunway, L. K., Rancour, J. M., and Ryan, c. A. 1970. Vacuolar protein bodies in tomato leaf cells and their relationship to storage of chymotrypsin inhibitor I protein. Planta 93: 1-14.

Shumway, L. K., Yang, V. V., Ryan, C. A., 1976. Evidence for the presence of proteinase inhibitor I in vacuolar protein bodies of plant cells. Planta 129: 161-165.

Simon, E. W. 1974. Phospholipid and plant membrane permeability. New Phytol. 73: 377-420.

Skogley, C. R., and Jagschitz, J. A. 1964. The effect of various crabgrass herbicides on turfgrass seeds and seedlings. Proc. Northeast. Weed Control Conf. 18: 523-539.

Slightom, J. L., Jouanin, L., Leach, F., Drong, R. F., and Tepfer, D. 1985. Isolation and identification of TL-DNA/plant junctions in *Convolvulus arvensis* transformed by *Agrobacterium rhizogenes* strain A4. EMBO J. 4: 3069-3077.

Smiley, R. W. 1983. Compendium of turfgrass diseases. The American Phytopathological Society. St. Paul, Minnesota. pp. 11-82.

Smith, F. D., Harpending, P. R., and Sanford, J. C. 1992. Biolistic transformation of prokaryotes: Factors affecting biolistic transformation of very small cells. J. Gen. Microbiol. 138: 239-248.

Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F., and Bushnell, W. R. 1992. Fertile, transgenic oat plants. Bio/Technology 10: 1589-1594.

Songstad, D. D., Halaka, F. G., Deboer, D. L., Armstrong, C. L., Hinchee, M. A. W., Ford-Santino, C. G., Brown, S. M., Fromm, M. E., and Horsch, R. B. 1993. Transient expression of GUS and anthocyanin constructs in intact maize immature embryos following electroporation. Plant Cell Tiss. Organ Cult. 33: 195-201.

Spencer, T. M., Gordon-Kamm, W. J., Daines, R. J., Start, W. G., and Lemaux, P. G. 1991. Bialaphos selection of stable transformants from maize cell culture. Theor. Appl. Genet. 79: 625-631.

Spencer, T. M., O'Brien, J. V., Start, W. G., Adams, T. R., Gordon-Kamm, W. J., and Lemaux, P. G. 1992. Segregation of transgenes in maize. Plant Mol. Biol. 18: 201-210.

Spörlein, B., and Koop, H. -U. 1991. Lipofectin: direct gene transfer to higher plants using cationic liposomes. Theor. Appl. Genet. 83: 1-5.

Sprague, H. B. 1982. Turf management handbook. The Interstate Printers & Publishers, Inc., Danville, Illinois. pp. 197-210.

Srivastava, V., Reddy, A. S., and Guha-Mukherjee, S. 1988. Transformation and regeneration of *Brassica oleracea* mediated by an oncogenic *Agrobacterium tumefaciens*. Plant Cell Rep. 7: 504-507.

Stalker, K. M., Hiatt, W. R., and Comai, L. 1985. A single amino acid substitution in the enzyme 5-enol-pyruvylshikimate 3-phosphate synthase confers resistance to the herbicide glyphosate. J. Biol. Chem. 260: 4724-4728.

Stalker, D. M., McBride, K. E., and Malyj, L. D. 1988. Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science 242: 419-423.

Steffens, R., Fox, f. R., and Kassel, B. 1978. Effect of trypsin inhibitors on growth and metamorphosis of corn borer larvae *Ostrinia nubilalis* (Hubner). J. Agric. Food Chem. 26: 170-174.

Steinrücken, H. C., and Amrhein, N. 1980. The herbicide glyphosate is a protein inhibitor of 5-enol-pyruvylshikimic acid 3-phosphate-synthase. Biochem. Biophys. Res. Commun. 94: 1207-1212.

Steinrücken, H. C., Schulz, A., Amrhein, N., Porter, C. A., and Fraley, R. T. 1986. Overproduction of 5-enol-pyruvylshikimate 3-phosphate synthase in glyphosate-tolerant *Petunia hybrida* cell line. Arch. Biochem. Biophys. 244: 169-178.

Stockhaus, J., Eckes, P., Blau, A., Schell, J., and Willmitzer, L. 1987. Organ-specific and dosage-dependent expression of a leaf/stem specific gene from potato after tagging and transfer into potato and tobacco plants. Nuc. Acids Res. 15: 3479-3491.

Stöger, E., Fink, C., Pfosser, M., and Heberle-Bors, E. 1995. Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep. 14: 273-278.

Stougaard-Jensen, J., Marcker, K. A., Otten, L., and Schell, J. 1986. Nodule specific expression of a chimeric soybean leghaemoglobin gene in transgenic *Lotus corniculatus*. Nature 321: 669-674.

Streber, W. R., and Willmitzer, L. 1989. Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2, 4-D. Bio/Technology 7: 811-816.

Stuckey, I. H., and Banfield, W. G. 1946. The morphological varieties and the occurrence of aneuploids in some species of *Agrostis* in Rhode Island. Amer. J. Bot. 33: 185-190.

Sukhapinda, K., Spivey, R., and Shahin, E. A. 1987. Ri-plasmid as a helper for introducing vector DNA into alfalfa plants. Plant Mol. Biol. 8: 209-216.

Svab, Z., Hajdukiewicz, P., and Maliga, P. 1990. Stable transformation of plastids in higher plants. Proc. Natl. Acad. Sci. U.S.A. 87: 8526-8530.

Sweetser, P. B., Schow, G. S. and Hutchison, J. M. 1982. Metabolism of chlorsulfuron by plants: Biological basis for selectivity of a new herbicide for cereals. Pestic. Biochem. Physiol. 17: 18-23.

Szigeti, Z., Toth, E., and Paless, G. 1982. Mode of action of photosynthesis inhibiting 4-hydroxybenzonitriles containing nitro group. Photosynth. Res. 3: 347-356.

Tachibana, K., Watanabe, T., Sekizawa, Y., and Takematsu, T. 1986. Accumulation of ammonia in plants treated with bialaphos. J. Pestic. Sci. 11: 33-37.

Tada, Y., Sakamoto, M., and Fujimura, T. 1990. Efficient gene introduction into rice by electroporation and analysis of transgenic plants: Use of electroporation buffer lacking chloride ions. Theor. Appl. Genet. 80: 475-480.

Tagu, D., Bergounioux, C., Cretin, C., Perennes, C., and Gadal, P. 1988. Direct gene transfer in *Petunia hybrida* electroporated protoplasts: Evidence for co-transformation with a phosphoenolpyruvate carboxylase cDNA from sorghum leaf. Protoplasma 146: 101-105.

Takeuchi, Y., Dotson, M., Keen, N. T. 1992. Plant transformation: a simple particle bombardment device based on flowing helium. Plant Mol. Biol. 18: 835-839.

Tao, W., Wilkinson, J., Stanbridge, E. J., and Berns, M. W. 1987. Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane. Proc. Natl. Acad. Sci. U.S.A. 84: 4180-4184.

Terakawa, T., Sato, T., and Koike, M. 1992. Plant regeneration from protoplasts isolated from embryogenic suspension cultures of creeping bentgrass (*Agrostis palustris* Huds.). Plant Cell Rep. 11: 457-461.

Thomas, J. C., Guiltinan, M. J., Bustos, S., Thomas, T., and Nessler, C. 1989. Carrot (*Daucus carota*) hypocotyl transformation using *Agrobacterium tumefaciens*. Plant Cell Rep. 8: 354-357.

Thomas, J. C., Wasmann, C. C., Echt, C., Dunn, R. L., Bohnert, H. J., and McCoy, T. J. 1994. Introduction and expression of an insect proteinase inhibitor in alfalfa (*Medicago sativa* L.). Plant Cell Rep. 14: 31-36.

Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M., and Botterman, J. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. 6: 2519-2523.

Thomzik, J. E., and Hain, R. 1990. Transgenic *Brassica napus* plants obtained by cocultivation of protoplasts with *Agrobacterium tumefaciens*. Plant Cell Rep. 9: 233-236.

Tischer, E., DasSarma, S., and Goodman, H. M. 1986. Nucleotide sequence of an alfalfa glutamine synthetase gene. Mol. Gen. Genet. 203: 221-229.

Toki, S., Takamatsu, S., Nojiri, C., Ooba, S., Anzai, H., Iwata, M., Christensen, A. H., Quail, P. H., and Uchimiya, H. 1992. Expression of a maize ubiquitin gene promoter-*bar* chimeric gene in transgenic rice plants. Plant Physiol. 100: 1503-1507.

Tomes, D. T., Weissinger, A. K., Ross, M., Higgins, R., Drummond, B. J., Schaaf, S., Malone-Schoneberg, J., Staebell, M., Flynn, P., Anderson, J., and Howard, J. 1990. Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves. Plant Mol. Biol. 14: 261-268.

Töpfer, R., Gronenborn, B., Schell, J., and Steinbiss, H. H. 1989. Uptake and transient expression of chimeric genes in seed-derived embryos. Plant Cell 1: 133-139.

Toriyama, K., Arimoto, Y., Uchimiya, H., and Hinata, K. 1988. Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technology 6: 1072-1074.

Trinh, T. H., Mante, S., Pua, E. -C., and Chua, N. -H. 1987. Rapid production of transgenic flowering shoots and $F_1$ progeny from *Nicotiana plumbaginifolia* epidermal peels. Bio/Technology 5: 1081-1084.

Trulson, A. J., Simpson, R. B., and Shahin, E. A. 1986. Transformation of cucumber (*Cucumis sativus* L.) plants with *Agrobacterium rhizogenes.* Theor. Appl. Genet. 73: 11-15.

Tsai, C. -J., Podila, G. K., and Chiang, V. L. 1994. *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants. Plant Cell Rep. 14: 94-97.

Tsukakoshi, M., Kurata, S., Nominya, Y., Ikawa, Y., and Kasuya, T. 1984. A novel method of DNA transfection by laser microbeam cell surgery. Appl. Phys. B. 35: 135-140.

Ulian, E. C., Smith, R. H., Gould, J. H., and McKnight, T. D. 1988. Transformation of plants via the shoot apex. In Vitro Cell. Devel. Biol. 24: 951-954.

Umbeck, P., Johnson, G., Barton, K., and Swain, W. 1987. Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Bio/Technology 5: 263-266.

Vaatnou, H. 1967. Responses of five genotypes of *Agrostis* L. to variations in environment. Ph. D. Thesis. Oregan State University. pp. 1-149.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M., and Leemans, J. 1987. Transgenic plants protected from insect attack. Nature 327: 33-37.

Vain, P., McMullen, M. D., and Finer, J. J. 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep. 12: 84-88.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. U.S.A. 85: 5536-5540.

van den Elzen, P. J. M., Townsend, J., Lee, K. Y., and Bedbrook, J. R. A. 1985. Chimaeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol. Biol. 5: 299-302.

Van Eck, J. M., Blowers, A. D., and Earle, E. D. 1995. Stable transformation of tomato cell cultures after bombardment with plasmid and YAC DNA. Plant Cell Rep. 14: 299-304.

Van Rensen, J. J. S. 1982. Molecular mechanisms of herbicide action near photosystem II. Physiol. Plant. 54: 515-521.

Vardi, A., Bleichman, S., and Aviv, D. 1990. Genetic transformation of *Citrus* protoplasts and regeneration of transgenic plants. Plant Sci. 69: 199-206.

Vasil, V., Brown, S. M., Re, D., Fromm, M. E., and Vasil, I. K. 1991. Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. Bio/Technology 9: 743-747.

Vasil, V., Castillo, A. M., Fromm M. E., and Vasil, I. K. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10: 667-674.

Vermaas, W. F. J., Steinback, K. E., and Arntzen, C. J. 1984. Characterization of chloroplast thylakoid polypeptides in the 32kDa region: polypeptide extraction and protein phosphorylation affect binding of photosystem II-directed herbicides. Arch. Biochem. Biophys. 231: 226-232.

Walden, R. 1989. Gene Expression in Transgenic Tissue. In: Genetic Transformation in Plants. Prentice Hall. Englewood Cliffs, New Jersey. pp. 80-100.

Waldron, C., Murphy, E. B., Roberts, J. L., Gustafson, G. D., Armour, S. L., and Malcolm, S. K. 1985. Resistance to hygromycin B, a new marker for plant transformation studies. Plant Mol. Biol. 5: 103-108.

Walker-Simmons, M., Ryan, C. A. 1977. Immunological identification of proteinase inhibitors I and II in isolated tomato leaf vacuoles. Plant Physiol. 60: 61-63.

Walters, D. A., Vetsch, C. S., Potts, D. E., and Lundquist, R. C. 1992. Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. Plant Mol. Biol. 18: 189-200.

Wan, Y., and Lemaux P. G. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104: 37-48.

Wang, Y. C., Klein, T. M., Fromm, M., Cao, J., Sanford, J. C., and Wu, R. 1988. Transformation of rice, wheat, and soybean by the particle bombardment method. Plant Mol. Biol. 11: 433-439.

Wang, Z., Takamizo, T., Iglesias, V. A., Osusky, M., Nagel, J., Potrykus, I., and Spangenberg, G. 1992. Transgenic plants of tall fescue (*Festuca arundinacea scherb.*) obtained by direct gene transfer to protoplasts. Bio/Technology 10: 691-696.

Weber, G., Monajembashi, S., Greulich, K. -O., and Wolfrum, J. 1989. Uptake of DNA in chloroplasts of *Brassica napus* (L.) faciliated by a UV-laser microbeam. Eur. J. Cell Biol. 49:73-79.

Weber, G., Monajembashi, S., Wolfrum, J., and Greulich, K. -O. 1990. Genetic changes induced in higher plant cells by a laser microbeam. Physiol. Plant. 79: 190-193.

Weeks, J. T., Anderson, O. D., and Blechl, A. E. 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol. 102: 1077-1084.

Weide, R., Koornneef, M., and Zabel, P. A. 1989. Simple, nondestructive spraying assay for the detection of an active kanamycin resistance gene in transgenic tomato plants. Theor. Appl. Genet. 78: 169-172.

Wenzler, H., Mignery, G., May, G., and Park, W. 1989. A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. Plant Sci. 63: 79-85.

White, D. W. R., and Greenwood, D. 1987. Transformation of forage legume *Trifolium repens* L. using binary *Agrobacterium* vectors. Plant Mol. Biol. 8: 461-469.

Wilde, H. D., Meagher, R. B., and Merkle, S. A. 1992. Expression of foreign genes in transgenic yellow-poplar plants. Plant Physiol. 98: 114-120.

Williams R. S., Johnston, S. A., Reidy, M., DeVit, M. J., McElligott, S. G., and Sanford, J. C. 1991. Direct transformation of skin and liver tissue in the living mouse. Proc. Natl. Acad. Sci. U.S.A. 88: 2726-2730.

Wohlleben, M., Arnold, W., Broer, I., Hillemann, D., Strauch, E., and Pühler, A. 1988. Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene 70: 25-37.

Wu, L., and Antonovics, J. 1978. Zinc and copper tolerance of *Agrostis stolonifera* L. in tissue culture. Am. J. Bot. 65: 268-271.

Wurtele, E. S., and Bulka, K. 1989. A simple, efficient method for the *Agrobacterium*-mediated transformation of carrot callus cells. Plant Sci. 61: 253-262.

Xavier-Filho, J., Campos, F. A. P., Ary, M. B., Silva, C. P., Carvalho, M.M.M., Macedo, M. L. R., Lemos, F. J. A., and Grant, G.. 1989. Poor correlation between levels of proteinase inhibitors found in seeds of different cultivars of cowpea (*Vigna unguiculata*) and the resistance/susceptibility to predation by *Callosobruchus maculatus*. J. Agric. Food Chem. 37: 1139-1143.

Yamashita, T., Iida, A, Morikawa, H. 1991. Evidence that more than 90% of ß-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus. Plant Physiol. 97: 829-831.

Yang, N. S., Burkholder, J., Roberts, B., Martinell, B., and McCabe, D. 1990. *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. Proc. Natl. Acad. Sci. U.S.A. 87: 9568-9572.

Yao, J. -L., Cohen, D., Atkinson, R., Richardson, K., and Morris, B. 1995. Regeneration of transgenic plants from the commerical apple cultivar Royal Gala. Plant Cell Rep. 14: 407-412.

Ye, G. N., Daniell, H., and Sanford, J. C. 1990 Optimization of delivery of foreign DNA into higher-plant chloroplasts. Plant Mol. Biol. 15: 809-819.

Zambryski, P. 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Ann. Rev. Genet. 22: 1-30.

Zelenin A. V., Titomirov, A. V., and Kolesnikov, V. A. 1989. Genetic transformation of mouse cultured cells with the help of high velocity mechanical DNA injection. FEBS Lett. 244: 65-67.

Zhang, W., and Wu, R. 1988. Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. Theor. Appl. Genet. 76: 835-840.

Zhang, H. M., Yang, H., Rech, E. L., Golds, T. J., Davis, A. S., Mulligan, B. J., Corking, E. C., and Davey, M. R. 1988. Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep. 7: 379-384.

Zhang, W., McElroy, D., and Wu, R. 1991. Analysis of rice 5' region activity in transgenic rice plants. Plant Cell 3: 1155-1165.

Zhang, L. -J., Cheng, L. -M., Xu, N., Zhao, N. -M., Li, C. -G., Yuan, J., and Jia, S. -R. 1992. Efficient transformation of tobacco by ultrasonication. Bio/Technology 9: 996-997.

Zhong, H., Srinivasan, C., and Sticklen, M. B. 1991. Plant regeneration via somatic embryogenesis in creeping bentgrass (*Agrostis palustris* Huds.). Plant Cell Rep. 10: 453-456.

Zhong, H., Bolyard, M. G., Srinivasan, C., and Sticklen, M. B. 1993. Transgenic plants of turfgrass (*Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus. Plant Cell Rep. 13: 1-6.

Zhou, G. Y., Weng, J., Zeng, Y., Huang, J., Qian, s., and Liu, G. 1983. Introduction of exogenous DNA into cotton embryos. Methods. Enzymol. 101: 433-481.

# CHAPTER TWO

# Engineering Herbicide Resistance in Creeping Bentgrass (*Agrostis palustris* Huds.) via Microprojectile Bombardment and Expression of the *bar* Gene in Transgenic Plants

## ABSTRACT

An efficient transformation system to generate large numbers of independent transgenic creeping bentgrasses (*Agrostis palustris* Huds.) by microprojectile bombardment is described. Embryogenic callus was bombarded with a plasmid containing the *bar* gene and cultured on selection medium supplemented with either five mg/l of bialaphos or fifteen mg/l of phosphinothricin (PPT) for twelve weeks. A total of 38 independent bialaphos- or PPT-resistant callus lines were generated and found able to regenerate plants. Except for the plants regenerated from one of the PPT-resistant callus lines, plants from the rest of the resistant callus lines were shown to be resistant to the application of 1.2% Ignite®. All of the resistant callus lines and plants regenerated from them expressed functional phosphinothricin acetyltransferase, the product of *bar*. Integration of *bar* was confirmed by Southern hybridization analysis in the 37 independent resistant plant lines. Transcripts corresponding to the *bar* gene were present in transgenic plants that showed herbicide resistance. Transgenic plants tested were shown to have the ability to decrease the amount of ammonium accumulated after the herbicide application. The magnitudes of herbicide resistance were between 20 and 40 times higher than that of untransformed control plants among tested transgenic creeping bentgrasses.

# Introduction

Allotetraploid creeping bentgrass (*Agrostis palustris* Huds.; 2n=4x=28) is one of the species of bentgrasses that have had the widest usage in the united states. It belongs to the family of *Gramineae* and is a unique cool-season species exhibiting a vigorous stoloniferous growth habit and is the primary cool-season grass used for golf course putting greens, tees, and closely mowed fairways (Beard, 1982).

There exist some major problems, such as multiple applications of herbicides and pesticides, associated with this important turfgrass, both in the golf courses and in residential areas. All of the presently available cultivars of creeping bentgrass are susceptible to a wide range of fungal diseases such as brown patch (*Rhizoctonia solani*), dollar spot (*Sclerotinia homoeocarpa*), and *Pythium* blight. Though most persistent prostrate-growing broadleaf weeds occurring on established creeping bentgrass greens and fairways can be controlled with the use of selective herbicides such as 2,4-D. Grassy weeds, however, are difficult to control since they resemble creeping bentgrass in their tolerance of herbicidal chemicals. For example, a consistently effective herbicide for selective annual bluegrass control in bentgrass greens is lacking. Perennial strains of annual bluegrass are even more difficult to control with selective herbicides than are annual strains (Beard, 1982; Decker and Decker, 1988).

A number of herbicides exhibits good control of annual bluegrass; however, most pose a high risk of injury to bentgrass. Though the application of synthetic herbicides and other pesticides has become an indispensable tool of modern golf course management in that they allow for economical control of weeds and pathogens, it is consuming an increasing share of golf course management expenses, and, at the same time, there are growing concerns about the effects of these chemicals on the environment.

Development of turfgrass varieties which could provide consistent effective control of weeds and pathogens is, therefore, a goal of turfgrass breeding programs. The availability of such varieties could improve weed control by providing flexibility in the timing of applications and in the selection of efficient herbicides, by reducing the number of treatments, and provide the protection against the infection of pathogens. However, the breeding of creeping bentgrass has been limited by the lack of genetic research and its allotetraploid nature.

Plant genetic engineering has undergone a revolution in the past ten years, and advanced approaches in plant biotechnology are now beginning to be used to augment traditional approaches to crop improvement. Development of efficient *in vitro* regeneration systems and novel transformation technologies have opened the way for the engineering of even the most recalcitrant of crops such as maize (Gordon-Kamm *et al.*, 1990), rice (Christou *et al.*, 1991), wheat (Vasil *et al.*, 1992), oats (Casas *et al.*, 1993), and barley (Wan and Lemaux, 1994). Genetic engineering has the potential to improve creeping bentgrass, since it can selectively improve a single trait while still retaining all of the desirable traits in the parental line, a capability not currently available through traditional turfgrass breeding.

The recent development of the biolistic transformation system has furnished us with a useful breeding tool to bypass the obstacles impeding the improvement of creeping bentgrass. The biolistic instrument is capable of accelerating sub-cellular sized microprojectiles coated with foreign DNA over an array of velocities necessary to optimally transform many different cell types. Of the several plant transformation methods available, microprojectile bombardment has the advantage of working with a wider range of cell and tissue types than any other method. Microprojectile bombardment as a method to introduce foreign DNA into plant cells circumvents two major constraints of monocot transformation: the lack of an available natural vector such as *Agrobacterium tumefaciens* and the difficulty of regenerating fertile plants when protoplasts are used for transformation.

Bialaphos (*L* -phosphinothricinyl- *L* -alanyl- *L* -alanine), which is being used as a non-selective, broad-spectrum and postemergent herbicide, is a tripeptide composed of two *L*-alanine residues and an analogue of glutamate known as phosphinothricin (PPT) (Kondo *et al.*, 1973; Ogawa *et al.*, 1973). PPT was initially identified as the active moiety of the herbicidal bialaphos produced by *Streptomyces hygroscopicus* (Kondo *et al.*, 1973). The active ingredient is highly stable as a chemical compound, but its degradation is rapid in a microbiologically active environment such as soil or surface water.

While PPT is a potent inhibitor of the enzyme glutamine synthetase in both plants and bacteria, the intact tripeptide, bialaphos, has little or no inhibitory activity *in vitro* (Bayer *et al.*, 1972; Tachibana *et al.*, 1986a, 1986b). In both plants and bacteria, intracellular peptidases remove the alanine residues and release active PPT. In plants, glutamine synthetase is critical for the assimilation of ammonia as well as for general nitrogen metabolism. It is the major enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid catabolism, and photorespiration. Plants treated with glufosinate-ammonium (ammonium salt of PPT) quickly succumb to the toxic effects of accumulated ammonia (Tachibana *et al.*, 1986a, 1986b).

Transgenic tobacco, potato, and tomato plants, expressing the *bar* gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) were tolerant to 4-10 times the field rate of application of the herbicide (De Block *et al.*, 1987). If the *bar* gene which encodes PPT acetyltransferase (Thompson *et al.*, 1987) could be introduced and employed as a resistant gene in creeping bentgrass, bialaphos could serve as not only a selective agent to facilitate the identification of putative transgenic plants in transformation experiments but also as a selective herbicide on golf courses with bialaphos-resistant creeping bentgrasses. The cultivation of environmentally-sound and bialaphos-resistant turfgrass represents significant economic and environmental advantages for the management of golf courses by reducing the amount and frequency of herbicide application, and by facilitating the weed control process.

In this paper, we describe the stable transformation and regeneration of creeping bentgrass by microprojectile bombardment of embryogenic calli and the use of the *bar* gene as a selectable marker for the efficient recovery of independent bialaphos-resistant callus lines. We show that transgenic plants expressing PAT activity are resistant to high doses of the commercial formulation of PPT. The use of the *bar* gene as reporter gene to analyze plant gene expression is also demonstrated.

# Materials and Methods

## Plant Material

Creeping bentgrass (*Agrostis palustris* Huds.) cultivar "Penncross" which was a synthetic cultivar produced by the random crossing of three vegetatively propagated strains (Hein, 1958) was used as the plant material for the transformation experiment. Friable embryogenic callus was initiated from mature seeds (caryopses) of creeping bentgrass following the method described by Zhong *et al.* (1991). Seeds were first soaked in 70% ethyl alcohol for 15 minutes and then surface-sterilized with 50% commercial grade Clorox solution containing 0.1% Tween 20 for 15 minutes. Finally, the seeds were rinsed with sterilized distilled water three times before being transferred onto embryogenic callus induction medium which contained MS (Murashige and Skoog, 1962) basal medium supplemented with 500 mg/l enzymatic casein hydrolysate, 3 % sucrose, 30 $\mu$M dicamba (3, 6-dicloro-o-anisic acid), 9 $\mu$M BA (6-benzyladenine), and 7 g/l Phytagar.

Cultures were maintained in the dark at 25 °C and subcultured onto fresh-made callus induction medium once per month. Embryogenic callus was observed after approximately one month of culture and selected for multiplication. However, embryogenic calli were used as soon and as fresh as possible and discarded after they had been initiated from seeds for more than four months. Fresh calli were routinely initiated every month to maintain the supply of embryogenic callus.

## Evaluation of Selective Agents

In order to evaluate the selective agents and determine the appropriate concentration of the corresponding selective agent needed to distinguish the cells transformed with the *bar* gene from the nontransformed cells, kill curve tests were performed using various concentrations of bialaphos (kindly provided by Meiji Seika Kaisha of Japan) and phosphinothricin (*D,L*-PPT, glufosinate-ammonium; Riedel-deHaën) (Table 1 and Table 2). Callus initiated from every single seed was maintained separately, since each seed of the cultivar Penncross potentially represented a different genotype. Embryogenic calli were first bombarded with tungsten particles without any plasmid DNA and transferred onto embryogenic callus induction medium.

Three days after the bombardment, bombarded calli were subcultured onto selection medium (callus induction medium without casein hydrolysate) supplemented with various concentrations of bialaphos or phosphinothricin. Bialaphos and phosphinothricin were first dissolved in double distilled water and filter-sterilized (Nalgene filter) before added into the autoclaved medium. Ten small pieces of callus (less than three mm in diameter and approximately five mg in weight) were evenly placed on each Petri dish which contained 35 ml of selection medium. Fifteen dishes which had a total of 150 pieces of callus were used for every selection concentration. Calli were transferred onto fresh-made selection medium every four weeks.

Three durations (four, eight, and twelve weeks) of selection were tested to determine the appropriate length of selection duration. At the end of each selection duration, calli were carefully broken with forceps and separately transferred onto Petri dishes containing 35 ml of callus induction medium (pH of the medium was adjusted to 6.0 and containing same amount of corresponding selective agent) with 50 mg/l of chlorophenol red for four days. The pH indicator chlorophenol red changes from red through orange to yellow as the pH value decreases from 6.0 to 5.0. When subcultured onto chlorophenol red medium adjusted to a pH of 6.0, growing maize callus which survived the PPT selection acidified the surrounding medium, reduced the pH value, and caused the change in color (Kramer *et al.*, 1993). Calli which caused the color change of the medium from deep red to yellow, orange, or red-orange were transferred to callus induction medium and cultured for one more month. Calli which continued to multiply were considered alive, and the number of growing calli was recorded. Culture conditions were all the same as those of callus induction.

### Plasmid Vector

The plasmid used for the biolistic gun transformation method was pTW-a and was kindly provided by Dr. Ray Wu of Cornell University. The schematic map of this plasmid is shown in Figure 1. The plasmid pTW-a contains the *pin* 2 gene downstream of the *pin* 2 promoter and the rice *Act* 1 intron, with the *pin* 2 terminator. In addition, the plasmid contains the *bar* gene downstream of the CaMV35S promoter and is flanked by the nopaline synthase 3' end (*nos* terminator).

### Microprojectile Bombardment

One day prior to the microprojectile bombardment, embryogenic calli were placed onto a 2 $cm^2$ area in the center of a 15 x 60 mm disposable Petri dish containing 35 ml of embryogenic callus induction medium. Prior to bombardment, plasmid DNA was precipitated onto tungsten particles modified from the protocol described by Klein *et al.* (1987). Thirty mg of the dry microcarriers (tungsten M17, average diameter approximately 1.2 $\mu$m) were soaked in 1 ml of 100% ethanol in a microcentrifuge tube. The tungsten particles were then vortexed on high speed for two minutes before they were centrifuged at 15,000 rpm for one minute. After the supernatant was decanted, one ml of sterile distilled water was added into the microcentrifuge tube before the tungsten particles were resuspended and centrifuged.

The deagglomeration process was repeated two times before the microcarriers were resuspended in one ml of sterile distilled water. 100 $\mu$l of the final microcarrier suspension (three mg of the tungsten particles) was transferred into a microcentrifuge tube and vortexed continuously while pipetting aliquots of the suspension to avoid non-uniform sampling.

25 $\mu$g of plasmid DNA, 100 $\mu$l of 2.5 M $CaCl_2$ and 40 $\mu$l of 0.1 M spermidine (free base, tissue culture grade, Sigma) were added into a microcentrifuge tube in the above order, while the microcentrifuge tube was continuously being vortexed. The DNA precipitation mixture was continuously vortexed for ten minutes before the microcarriers were spinned down and the supernatant was removed from the microcentrifuge tube. The DNA precipitation mixture was washed with 250 $\mu$l of 100% ethanol by brief vortexing and then centrifuged at 15,000 rpm for ten seconds. Supernatant was removed and the mixture was resuspended in 100 $\mu$l of 100% ethanol.

10 $\mu$l aliquots of suspension were pipetted onto the center of the macroprojectile for bombardment. After the alcohol was evaporated, each Petri dish with embryogenic calli of creeping bentgrass was bombarded three times with the DNA-tungsten mixture using the Biolistic PDS-1000/He instrument (Bio-Rad Laboratories, Inc.). Following the same protocol, control plates with embryogenic calli were also bombarded with the same tungsten mixture except there was no plasmid DNA.

Physical parameters were optimized to increase numbers of *gus* transient expressing cells per plate. The following conditions were found to be superior and were used as a standard bombardment protocol: rupture disc-pressure: 1550 psi; gap-distance from rupture disc to macrocarrier: 6 mm; macrocarrier travel distance: 16 mm; microcarrier travel distance: 6 cm.

### Selection of Resistant Callus Lines and Plant Regeneration

After the bombardment, calli were separated and transferred onto embryogenic callus induction medium for three days before being exposed to the selective agent.

Three days after the bombardment, calli were subcultured onto selection medium which contained either five mg/l of bialaphos or 15 mg/l of phosphinothricin (ammonium salt) and the same components of embryogenic callus induction medium, except without the enzymatic casein hydrolysate to avoid the possible interference of glutamine or asparagine with selective agent (Wang *et al.*, 1992; Hartman *et al.*, 1994).

The bombarded calli were transferred to fresh-made selection medium every four weeks for twelve weeks, and all cultures were maintained in the dark at 25 °C. During the process of transferring to the second selection plate, individual callus pieces were broken with forceps into several small pieces and maintained separately. After about three months of selection, resistant and viable calli showing evidence of more vigorous growth were transferred onto the same selection medium and maintained under the light for differentiation for two weeks before being transferred onto MS medium containing the same concentration of selective agent (bialaphos or PPT) for regeneration.

All resistant callus tissue developed originally from each piece of callus was defined as a line. Regenerated shoots were maintained and propagated on the MS medium with the same amount of corresponding selective agent for one to two months before being transferred to four-inch plastic pots containing peat and perlite growth medium mixture (Baccto professional planting mix) and placed in the greenhouse.

### Herbicide Application

Putative transgenic creeping bentgrass plants regenerated from resistant callus lines were sprayed with 1.2 % Ignite®, which contained 200 g/l of active ingredient of glufosinate-ammonium (ammonium salt of PPT), in a spray volume of 150 l/ha. Symptoms were evaluated 10 days after the herbicide application. Various rates of Ignite®, ranging from zero to 120 g/l of glufosinate-ammonium, in the same spray volume were then applied to three of the resistant lines, which had showed no damage after the application of 1.2% Ignite®, to determine the magnitude of their herbicide resistance. $LD_{50}$ (lethal concentration that caused 50% of plant damage) was used to represent the magnitude of herbicide resistance. There were ten replications for each application rate.

### DNA Isolation and Southern hybridization Analysis

The method of isolating genomic DNA from resistant and control turfgrass plants was modified from the CTAB method of Rogers and Bendich (1985). 10 grams of fresh turfgrass tissue was ground with mortar and pestle in liquid nitrogen and transferred into an oakridge tube before 30 ml of CTAB extraction buffer [0.1 M Tris-HCl, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB (hexadecyltrimethylammonium bromide), 1% 2-mercaptoethanol] was added. The tube was incubated in a waterbath at 60 °C for one hour with occasional mixing by gentle swirling.

30 ml of phenol/chloroform/isoamyl alcohol solution (25:24:1, v/v/v) was added into the oakridge tube after the extraction buffer was cooled down to room temperature. The tube was then centrifuged at 10,000 rpm for 10 minutes at 10 °C. The top layer of the extraction buffer was pipetted into another oakridge tube, and an equal volume of phenol/chloroform/isoamyl alcohol solution was added into the tube.

The extraction procedure was repeated once before the plant DNA was precipitated with an equal volume of ice cold isopropanol. The precipitated DNA was spinned down at 10,000 rpm for 10 minutes and washed with 100 % ethanol after the supernatant was poured off. The DNA pellet was dried and redissolved in 10 ml of TE buffer (10 mM Tris, 1 mM EDTA). The sample was then extracted three times with chloroform/isoamyl alcohol solution (24:1). The pellet was washed with 70% ethanol twice and dried after the DNA was precipitated with ice cold isopropanol. One mg/ml of

RNase A was added into the DNA solution after the pellet was dissolved in the appropriate amount of TE buffer.

To detect the integration of *bar* gene into the genome of turfgrass, 10 μg of the purified plant DNA was digested with EcoRI, which should release a 0.9 kb fragment containing the *bar* coding region if the integration were successful, and with EcoRV, which has a unique digestion site in the pTW-a. Undigested and digested DNA were electrophoresed in a 0.8% agarose gel and blotted onto a Nytran membrane, following the Product Guide & Methods of the Schleicher & Schuell, Inc.

The plasmid pTW-a was used for the isolation of the 0.6 kb *bar* fragment by digestion with SmaI. The fragments were labeled with $^{32}$P using the $^{T7}$QuickPrime® Kit (Pharmacia Biotech) to generate probes for the detection of sequences containing the *bar* gene. The Nytran membrane was placed in a polyethylene bag containing prehybridization buffer (6x SSPE, 10 x Denhardt's solution, 1.0% SDS, 200 μg/ml fragmented, denatured DNA, 50% formamide pH 7.4, and 10% dextran sulfate 500) for two hours at 42 °C.

After the prehybridization buffer was poured off, the membrane was incubated with the hybridization buffer [6x SSPE, 10 x Denhardt's solution, 1.0% SDS, 50% formamide pH 7.4, and 10% dextran sulfate 500, and $^{32}$P-labeled probe (100 μl per cm$^2$ of membrane)] under continuous agitation overnight at 42 °C. The membrane was then washed in 150 ml of the following solutions: (1) twice in 7 x SSPE/0.5% SDS for 15 minutes at room temperature. (2) twice in 1 x SSPE/1.0% SDS for 15 minutes at 37 °C. (3) once in 0.1 x SSPE/1.0% SDS for 30 minutes at 42 °C.

## RNA Isolation and Northern Hybridization Analysis

Total RNA was isolated from the control and the transformed creeping bentgrasses by the method of Wadsworth *et al*. (1988). 200 mg of leaf tissue samples were ground to a fine powder in liquid nitrogen with a small mortar and pestle. The samples were transferred to 1.5 ml microfuge tubes; 500 $\mu$l of extraction buffer (25 mM sodium citrate, pH 7.0, 4 M guanidinium isothiocyanate, 1.5% (w/v) sodium lauryl sarcosine, 100 mM 2-mercaptoethanol) was added; and the samples were vortexed for 15 seconds. Then 250 $\mu$l of phenol and 250 $\mu$l of chloroform were added; the samples were vortexed for 15 seconds and centrifuged at 12,000 rpm for 15 minutes; and 500 $\mu$l of aqueous phase was transferred to a new tube. The samples were extracted twice more with phenol/chloroform, and, after the final extraction, only 400 $\mu$l of the aqueous phase was transferred to a new microfuge tube. Next, 400 $\mu$l of 6 M lithium chloride was added, and the samples were incubated on ice for one hour.

Following a centrifugation ten minutes at 4 °C, the supernatant was discarded. The pellets were disrupted with a thin glass rod; 1 ml of 3 M lithium chloride was added; and the samples were vortexed until the pellet was evenly suspended. The samples were centrifuged as before, and the supernatants were discarded. The pellets were washed twice in this manner, then resuspended in 400 $\mu$l of 2% potassium acetate, and heated to 55 °C for 10 minutes to dissolve the RNA. The samples were then centrifuged for 5 minutes to remove insoluble matter, and the supernatant was transferred to a new microfuge tube.

The RNA was precipitated by the addition of 1 ml of ethanol and incubated for 15 minutes at -80 °C. The RNA was collected by centrifugation for 15 minutes at 4 °C, dried under a vacuum, and dissolved in 50 $\mu$l of water for 10 minutes at 55 °C. For northern blot hybridization, total RNA (5 $\mu$g) was electrophoretically fractionated through a 1.2% agarose gel containing formaldehyde (Sambrook *et al.*, 1989) and transferred onto Nytran membranes which were probed with the *bar* coding region described above.

## PAT Activity Assay

Phosphinothricin acetyltransferase (PAT) activity was analyzed using a procedure modified from Spencer *et. al.* (1990) and D'Halluin *et al.* (1992). 200 mg of plant tissue or callus was ground in Eppendorf tubes, on ice, with 100 $\mu$l extraction buffer [50 mM Tris-HCl, pH 7.5, 2 mM $Na_2EDTA$, 0.15 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.15 mg/ml leupeptin (Acetyl-Leu-Leu-Arg-al), 0.3 mg/ml bovine serum albumin (BSA), and 0.3 mg/ml dithiothreitol (DTT)] to which 5 mg of polyvinyl polypyrrolidone (PVPP) was added. The Eppendorf tube was centrifuged for ten minutes at 4 °C at 14, 000 rpm to yield a crude extract.

The total protein concentration in the crude extract was measured by employing the Bio-Rad assay with bovine serum albumin as the standard and was adjusted with the extraction buffer to reach a final protein concentration of one mg/ml. To 20 mg total protein, 3 $\mu$l $^{14}C$-acetyl coenzyme A (48.1 mCi/mmol, NEN), and 1 $\mu$l of a 3 mM phosphinothricin (PPT) stock were added.

Reaction mixture was incubated for one hour at 37 °C before 7 $\mu$l of reaction mixture was spotted onto a silica gel thin-layer chromatography (TLC) plate. The plate was then chromatographed in a 3:2 mixture of 1-propanol and $NH_4OH$ (25% $NH_3$, v/v), for six hours under room temperature. The $^{14}C$-labeled acetylated PPT was visualized by autoradiography (XAR-5-Kodakfilm) after a two-day exposure.

## Detection of Ammonia in Plant Extracts

Six-month-old nontransgenic control creeping bentgrasses as well as three transgenic plant lines used to test their magnitudes of herbicide resistance were treated with 1.2% Ignite®, as described in a previous section on 'Herbicide Application'. Two hours after the herbicide spraying, 250 mg young leaf material was extracted in one ml of water containing 50 mg polyvinyl polypyrrolidone (PVPP; Sigma). Insoluble material was pelletted by centrifuging for five minutes in an Eppendorf centrifuge. 200 $\mu$l of the supernatant was transferred to a new Eppendorf tube and diluted with 800 $\mu$l water.

The ammonia content was determined by the method of De Block *et al.* (1989) and D'Halluin *et al.* (1992). 1.5 ml of reagent A (5 g phenol and 25 mg sodium nitroprusside in 500 ml water) was added to 20 $\mu$l of the diluted plant extract, followed by 1.5 ml of reagent B (2.5 g sodium hydroxide, 1.6 ml of sodium hypochlorite with 13% available chlorine, to 500 ml of water). The reaction mixture was incubated for 15 minutes at 37 °C. The absorbance was measured at room temperature at 625 nm.

The concentration of ammonia ($NH_4^+$-N) was determined on a standard curve ($\mu$g $NH_4^+$-N/g fresh weight = $\mu$g determined $NH_4^+$-N x 450). The standard curve was made using $NH_4Cl$ in concentrations ranging from 0 to 100 $\mu$g $NH_4^+$-N (3.82 g $NH_4Cl$ = 1 g $NH_4^+$-N), with 20 $\mu$l leaf extract from an untreated control plant added to the reaction mixtures. The measurement of ammonium concentration was taken at several times after the herbicide spraying (as indicated in Table 4). There were five replications in each measurement.

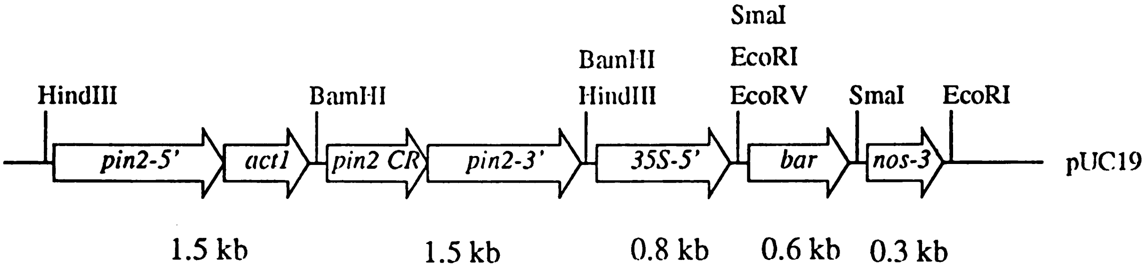


**Figure 1.** Schematic representation of the plasmid pTW-a.

# Results

## Evaluation of Selective Agents

Embryogenic calli of creeping bentgrass cultivar Penncross were bombarded with tungsten particles which had no plasmid DNA and exposed to various concentrations of selective agents for three selection durations to evaluate the efficiency of bialaphos and phosphinothricin in the killing of nontransgenic calli. Before being transferred onto the selection medium, embryogenic calli were first transferred onto callus induction medium and given three days after they were bombarded to recover from the damage caused by the biolistic bombardment.

At the end of each selection duration, calli were separately transferred onto Petri dishes containing callus induction medium (with the same amount of corresponding selective agent) with 50 mg/l of chlorophenol red for four days. Calli which caused the color change of the medium were transferred to callus induction medium. Calli which continued to multiply and grow were considered alive, and the number of surviving calli was recorded one month after they had been on callus induction medium.

Calli of creeping bentgrass were not very sensitive to either bialaphos or phosphinothricin (PPT) after they had been exposed to the selection medium for four weeks (Table 1, Table 2, and Figure 2). The concentration of bialaphos (17.5 mg/l) and of PPT (25 mg/l) to achieve 95% of selection efficiency were relatively high after the first selection duration. Both bialaphos and PPT were not very effective in the killing of embryogenic callus, especially at lower concentrations. However, the number of growing calli which survived the selection decreased as the duration of selection increased for both selective agents. For instance, after four weeks of selection on 15 mg/l of PPT, there were still 32 growing calli on the selection medium. The selection efficiency was less than 80% at this concentration. However, as the selection time increased to eight weeks, the number of growing calli dropped to eight. After twelve weeks of selection on 15 mg/l of PPT, there were only two calli surviving at this concentration of PPT. More than 95% of the tested calli were killed. This phenomenon was also observed with the other concentrations for both bialaphos and PPT.

Bialaphos was found to be a more effective selective agent in killing control calli of creeping bentgrass. Not only was the necessary concentration of bialaphos to kill the same percentage of calli lower than that of PPT, but also the duration needed for bialaphos to achieve the same efficiency was less than that of PPT. After four weeks of selection, bialaphos was able to kill more than 95% of the tested calli at the concentration of 17.5 mg/l (54.0 $\mu$M). However, the concentration needed for PPT to obtain the same efficiency after the same duration of selection was 25 mg/l (126.3 $\mu$M). The fact that bialaphos was a better selective agent than PPT was even more significant when the duration of selection increased from four weeks to twelve weeks. There were only seven growing calli on bialaphos selection medium at the concentration of 5 mg/l (15.4 $\mu$M); however, there were still nine calli surviving the PPT selection at the concentration of 12.5 mg/l (63.1 $\mu$M).

Both bialaphos and PPT were employed as selective agents to distinguish calli transformed with the *bar* gene from those nontransformed calli. The concentration of bialaphos and of PPT used in the selection medium was 5 and 15 mg/l, respectively, which could achieve 95% of selection efficiency after three transfers on the selection medium and twelve weeks of selection.

### Selection of Transformed Callus Lines

To test whether the expression of the phosphinothricin acetyltransferase (PAT) encoded by the bialaphos resistant gene (*bar*) from *Streptomyces hygroscopicus* allowed for the selection of transformed plant cells, embryogenic calli of creeping bentgrass cv.

**Table 1.** Effects of the duration of selection and the concentration of phosphinothricin on the callus growth of creeping bentgrass

| | Number of callus growing on callus induction medium after each duration of selection on callus induction medium containing various concentrations of phosphinothricin* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration of phosphinothricin (mg/l) | | | | | | | | | | | | |
| Weeks of selection# | 0 | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 17.5 | 20 | 25 | 30 | 35 | 40 |
| 4 | 150 | 88 | 64 | 62 | 51 | 41 | 32 | 19 | 14 | 6 | 4 | 1 | 0 |
| 8 | 150 | 67 | 43 | 30 | 23 | 14 | 8 | 3 | 2 | 0 | 0 | 0 | 0 |
| 12 | 150 | 48 | 37 | 22 | 14 | 9 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

*There were fifteen Petri dishes in every concentration, and each Petri dish contained ten small pieces of callus. At the end of each selection duration, calli were carefully broken into small pieces and separately transferred onto Petri dishes containing 35 ml of callus induction medium (with the same amount of PPT) with 50 mg/l of chlorophenol red for four days. Calli which caused the color change of the medium from deep red to yellow, orange, or red-orange were transferred to callus induction medium and cultured for one month. Calli which continued to multiply on callus induction medium were considered alive, and the number of growing calli was recorded.

#Calli were transferred to fresh-made selection medium every four weeks.

**Table 2.** Effects of the duration of selection and the concentration of bialaphos on the callus growth of creeping bentgrass

Number of callus growing on callus induction medium after each duration of selection on callus induction medium containing various concentrations of bialaphos*

| | Concentration of bialaphos (mg/l) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weeks of selection# | 0 | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 17.5 | 20 | 25 | 30 | 35 | 40 |
| 4 | 150 | 48 | 36 | 30 | 12 | 11 | 8 | 6 | 4 | 3 | 0 | 0 | 0 |
| 8 | 150 | 29 | 17 | 14 | 11 | 7 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 150 | 21 | 7 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*There were fifteen Petri dishes in every concentration, and each Petri dish contained ten small pieces of callus. At the end of each selection duration, calli were carefully broken into small pieces and separately transferred onto Petri dishes containing 35 ml of callus induction medium (with the same amount of bialaphos) with 50 mg/l of chlorophenol red (CR) for four days. Calli which caused the color change of the medium from deep red to yellow, orange, or red-orange were transferred to callus induction medium and cultured for one month. Calli which continued to multiply on callus induction medium were considered alive, and the number of growing calli was recorded.

#Calli were transferred to fresh-made selection medium every four weeks.

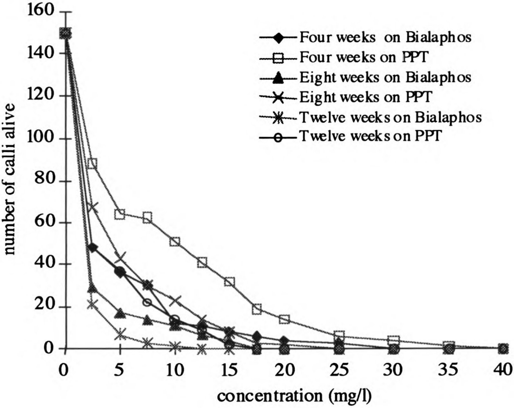


**Figure 2**. Callus responses of creeping bentgrasses to various concentrations of bialaphos and phosphinothricin and to three durations of selection.

Penncross were bombarded with tungsten particles coated with the *bar*-containing plasmid pTW-a. Calli were transferred onto callus induction medium and given three days after the bombardment to allow them to multiply and recover from the damage caused by the bombardment of tungsten particles before they were exposed to selection medium containing either 5 mg/l of bialaphos or 15 mg/l of PPT.

Calli that did not turn brown or black and survived the selection were transferred onto fresh-made selection medium every four weeks. After twelve weeks of selection and three transfers onto fresh-made selection medium, no growth was observed on calli from the control plates. Thus, the low dose of 5 mg/l of bialaphos and 15 mg/l of PPT were both sufficient to inhibit the growth of sensitive callus.

Resistant callus lines appeared four to six weeks after the bombardment, as they could be easily distinguished from those non-growing or dead calli. In order to avoid the possible problems of chimerism and escape, those resistant calli were still maintained on selection medium and transferred every four weeks for a total of twelve weeks. During the process of transfer, resistant calli were carefully broken into small pieces with forceps and evenly distributed on selection medium.

Results from transformation experiments are shown in Table 3. The number of callus lines surviving the selection decreased as the duration of selection increased for both selective agents. A large percentage of the bombarded calli were killed during the first selection period on 5 mg/l of bialaphos (four weeks of selection). However, it took a little longer for PPT (15 mg/l) to distinguish resistant callus lines from sensitive ones. After four weeks on the selection medium containing bialaphos or PPT, there were 170 and 163 resistant callus lines, respectively.

It was not until the end of the second selection duration that the resistant callus lines could be easily discriminated from the sensitive lines for both selective agents. There were still 84 callus lines showing resistance to bialaphos and 92 to PPT at the end of the second selection duration.

**Table 3**. Results from transformation experiments of creeping bentgrass

| Selective agent (concentration) | No. of plates bombarded | No. of resistant callus lines after each selection duration | | | No. of resistant callus lines regenerating plants after the last selection | No. of plant lines resistant to herbicide application (2.4 g/l glufosinate ammonium) |
|---|---|---|---|---|---|---|
| | | 4 weeks | 8 weeks | 12 weeks | | |
| Bialaphos (5 mg/l) | 25 | 47 | 20 | 7 | 7 | 7 |
| | 25 | 32 | 9 | 1 | 1 | 1 |
| | 25 | 29 | 14 | 3 | 3 | 3 |
| | 25 | 39 | 24 | 6 | 6 | 6 |
| | 25 | 23 | 17 | 5 | 5 | 5 |
| Subtotal | 125 | 170 | 84 | 22 | 22 | 22 |
| PPT (15 mg/l) | 25 | 43 | 19 | 2 | 2 | 2 |
| | 25 | 38 | 26 | 4 | 4 | 4 |
| | 25 | 47 | 33 | 6 | 6 | 5 |
| | 25 | 24 | 11 | 3 | 3 | 3 |
| | 25 | 11 | 3 | 1 | 1 | 1 |
| Subtotal | 125 | 163 | 92 | 16 | 16 | 15 |
| Total | 250 | 333 | 176 | 38 | 38 | 37 |

However, the number of resistant callus lines dropped dramatically during the last selection duration. Twenty-two and sixteen bialaphos and PPT-resistant callus lines, respectively, were recovered from 250 plates (125 plates for each selective agent) of bombarded calli after about three months of selection on either bialaphos or PPT. Resistant callus lines from both selection schemes were able to multiply and grow vigorously on selection medium during the last two weeks of selection.

Though it was difficult to keep track of each bombarded callus lines, there were two reasons to suspect that most of these resistant callus lines probably arose from independent integration events. Firstly, bombarded calli were separated before being transferred onto selection medium, and resistant calli were collected and maintained as individual cell lines. Secondly, very few callus pieces from the same plate showed resistance to either selective agent after three months of selection.

### Plant Regeneration

After three months of selection in the dark, embryogenic resistant callus lines were placed under the light for two weeks before they were transferred onto regeneration medium (MS medium) containing the same level of bialaphos or PPT and grown under the light. A high proportion of calli from each resistant line remained viable, and shoot regeneration was observed one to two weeks after they were transferred to the regeneration medium. All of the resistant callus lines from both selection schemes were able to form shoots and roots without the addition of any growth regulator (Table 3).

Most of the creeping bentgrass plants regenerated from resistant callus lines were morphologically similar to seed-derived plants, and their growth was indistinguishable from nontransformed control plants (either seed-derived or regenerated from calli bombarded without plasmid DNA of pTW-a). The total period of time from initiation of the cultures to acclimation of the plants in the greenhouse was about six months.

## Herbicide Resistance of Transgenic Creeping Bentgrasses

To evaluate whether the level of PAT activity in putative transgenic creeping bentgrasses was sufficient to confer resistance to the herbicide Ignite®, which contained 200 g/l of glufosinate-ammonium, transgenic and nontransformed control plants were first sprayed with 1.2 % Ignite (2.4 g/l glufosinate-ammonium) in a spray volume of 150 l/ha. Ten days after the herbicide application, the results showed that this application rate was able to kill all of the control plants (Figure 3).

There was almost no plant damage observed on most of the transgenic plants at that screening rate. A few of them did show some damage; however, they were all able to recover from the damage by two weeks after spraying. Plants regenerated from one of the PPT-resistant callus lines were all killed by the herbicide at the application rate of 2.4 g/l glufosinate-ammonium.

Plants of three resistant lines showing no damage after the screening application of herbicide were multiplied and tested for their tolerance to various concentrations of Ignite® to evaluate the magnitude of herbicide resistance. The level of PAT activity for transgenic plants regenerated from three independent resistant callus lines appeared to be different, as reflected by the extent of plant damage caused by various rates of herbicide application and their values of $LD_{50}$ (Figure 4).

The $LD_{50}$ values of tested transgenic plants were about 20 to 40 times higher than that of nontransgenic plants (Figure 4). Among tested plants, transgenic plants from transformation event A (resistant callus line A) showed the highest level of herbicide resistance and transgenic B plants the lowest. These three clones were also tested for their concentrations of ammonia after they had been sprayed with 1.2% Ignite® to determine whether there was a correlation between their magnitudes of herbicide resistance and the abilities to detoxify the herbicide.

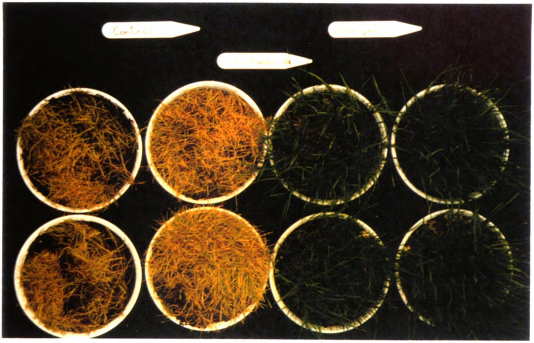


**Figure 3**. Effects of herbicide application on transgenic plants (on the right) and untransformed control plants (on the left) at an application rate of 1.2 % Ignite® (2.4 g/l of the active ingredient glufosinate-ammonium). Plants were photographed two weeks after the herbicide spraying.

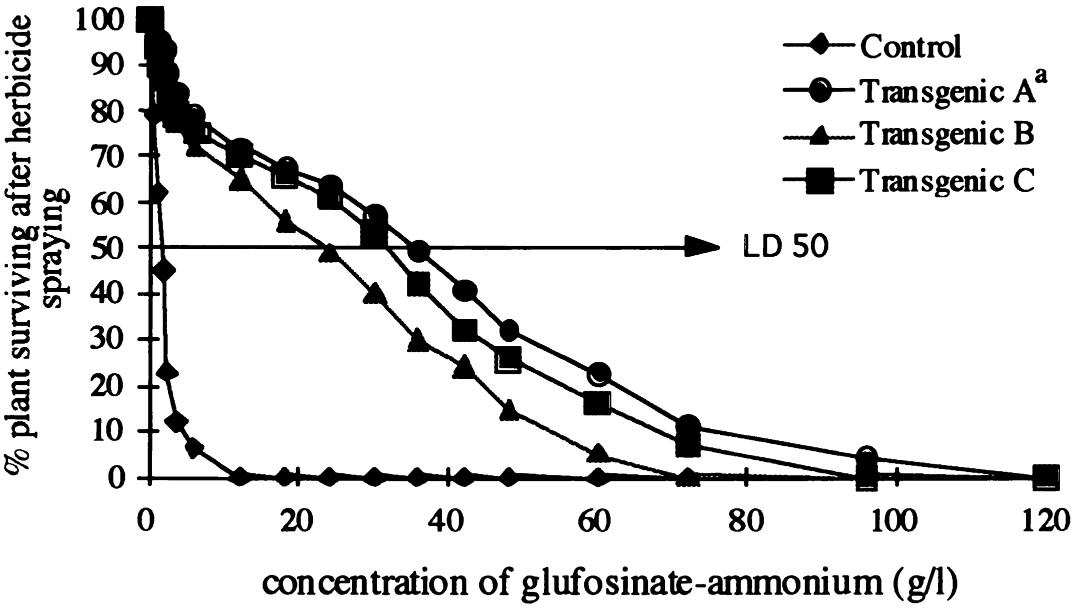


**Figure 4.** The magnitudes of herbicide resistance of transgenic creeping bentgrasses regenerated from three independent resistant callus lines.

[a] : A, B, and C indicate three independent transgenic lines.

### Analysis of PAT Activity

Evidence for the expression of the *bar* gene was obtained by the analysis for the phosphinothricin acetyltransferase (PAT) activity by thin layer chromatography (TLC). Resistant callus lines, as well as transgenic plants regenerated from them which had shown resistance to the herbicide application were tested for their PAT activity. The assays were performed using a mixture of *D*- and *L*-phosphinothricin, in which *L*-PPT is the active moiety of bialaphos and is generated in plant cells through cleavage of bialaphos by intracellular peptidases. *L*-PPT, a potent inhibitor of glutamine synthetase in plants and bacteria (De Block *et al.*, 1987), is acetylated by the *bar* gene product in the presence of acetyl-CoA to yield an inactive product, *N*-acetyl-PPT.

Protein extracts from independent resistant callus lines and young leaves of regenerated transgenic plants from each resistant callus line all showed PAT activity. The expression of the *bar* gene was observed in the enzymatic assay in which $^{14}$C-labeled acetylated PPT was detected after separation by TLC (Figure 5). Minor differences were found in the levels of PAT activity between independent resistant lines of callus and plant, and there was a very low background activity detected in the controls. The resistant callus lines were maintained on the corresponding selection medium and repeatedly tested positive for PAT activity during a six-month period (data not shown).

### Southern Hybridization Analysis

Southern hybridization analyses were performed on control plants as well as transgenic creeping bentgrasses obtained by bialaphos or phosphinothricin selection for the presence of the introduced *bar* gene. A $^{32}$P-labeled purified DNA fragment containing the coding sequence of the *bar* gene from pTW-a was used as the probe. Undigested genomic DNA from transgenic plants was hybridized with the *bar* probe, and

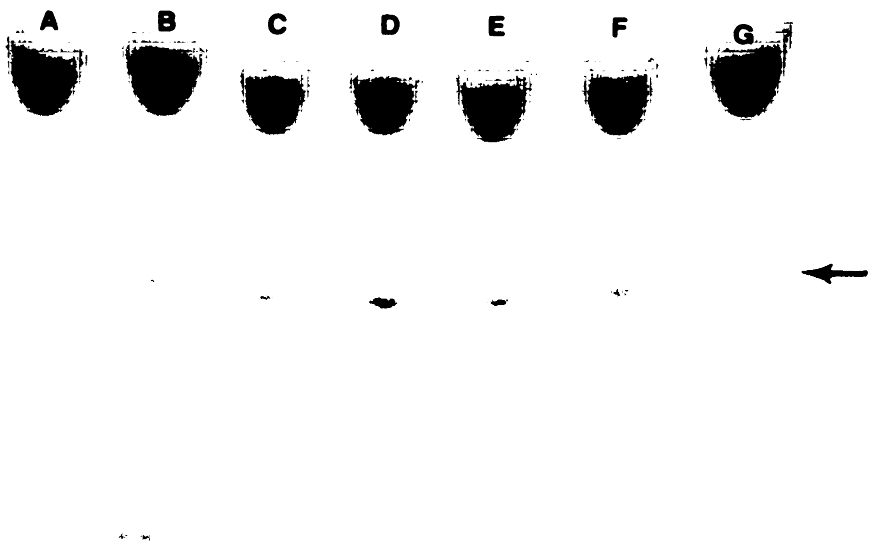


**Figure 5**. Detection of phosphinothricin acetyltransferase (PAT) activity by thin layer chromatography. Lane A: untransformed control plant; Lane B through G: transgenic creeping bentgrasses regenerated from independent resistant callus lines. Position of the *N*-acetyl-PPT is indicated by the arrow.

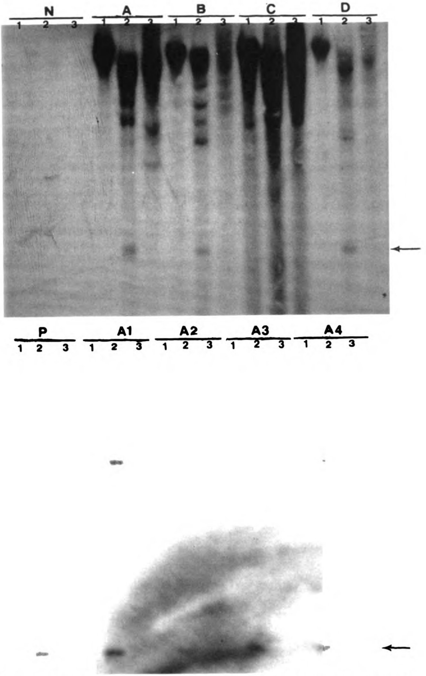


**Figure 6**. **Top**: Southern hybridization analysis of transgenic creeping bentgrass plants from independent resistant callus lines. Lane A, B, C, and D indicate different transgenic lines and lane N indicates nontransgenic control plant. Lane 1: undigested genomic DNA; Lane 2: EcoRI digested genomic DNA; Lane 3: EcoRV digested genomic DNA.
**Bottom**: Southern hybridization analysis of transgenic creeping bentgrasses from same transgenic resistant callus line A. P: positive plasmid control; Lane A1 through A4 indicate transgenic plants from same transgenic resistant callus line A. Lane 1: undigested genomic DNA; Lane 2: EcoRI digested genomic DNA; Lane 3: EcoRV digested genomic DNA. The fragment of *bar* coding region is indicated by the arrow.

hybridization was observed only in the region of high molecular weight DNA, providing evidence that the *bar* gene was integrated into genomic DNA, as exemplified in Figure 6.

Genomic DNA was digested with EcoRI, which releases a 0.9-kb fragment from pTW-a that contains the coding sequence of the *bar* gene (0.6 kb) and the *nos* 3' end (0.3 kb), and with EcoRV, which has a unique digestion site in the pTW-a. Genomic DNA from resistant plants was shown to contain *bar*-hybridizing sequences. The variation in the banding pattern and copy numbers was exemplified in Figure 6. Hybridization patterns varied from line to line. All transgenic lines contained an expected 0.9-kb fragment, although most also contained extra hybridization fragments of different sizes that could represent rearranged copies of the *bar* expression unit or be the result of partial digestion of the genomic DNA.

Furhtermore, the hybridization banding pattern of genomic DNA digested with EcoRV suggested that there were multiple integrations in some of the transgenic lines. The hybridization patterns of transgenic lines were unique, consistent with the expectation that all these lines were from independent transformation events. However, the hybridization patterns could not be used to determine whether a resistant callus line arose from more than one independently transformed cell. There was no apparent correlation between the copy number of the intact or rearranged *bar* genes and the level of expression (PAT activity and herbicide resistance) in transgenic plants.

Southern analysis with the *bar* gene as the probe was also performed on genomic DNA isolated from plants regenerated from the same resistant callus line, since the polymorphisms of the multiple restriction fragments hybridizing to the *bar* gene can provide us with more accurate information to determine whether they regenerated from the same transformation event. The banding patterns were identical and suggested that analyzed transgenic plants were regenerated from the same unique transformation event.

### Northern Hybridization Analysis

Northern analysis was performed to determine if *bar* transcripts accumulated in transgenic plants. Total RNA was isolated from young leaves of untransformed creeping bentgrasses and of transgenic plants showing resistance to herbicide application, then separated on a formaldehyde agarose gel, and probed with the SmaI fragment corresponding to the *bar* coding sequence (Figure 1). PAT-specific mRNA was detected in the analysis of total RNA, as shown in Figure 7.

No signal was detected in the untransformed control plants, while resistant transgenic plants contained expected size transcripts of around 600 bp. Although all of the analyzed transgenic plants which regenerated from independent transformation events contained the same *bar* gene, they showed some variation in the accumulation of PAT-specific mRNA, as shown by their different densities on the autoradiogram (Figure 7).

### Determination of Ammonium Levels in Herbicide-treated Plants

For a more sensitive indicator of glutamine synthetase inhibition, we measured the accumulation of ammonium in transgenic and untransformed plants treated with 1.2% of Ignite®. In order to determine whether there was a correlation between their magnitudes of herbicide resistance and the ability to detoxify glufosinate-ammonium, transgenic plants tested for their concentrations of ammonia after the herbicide spraying were from the same transgenic lines which had been tested for their magnitudes of herbicide resistance.

Ammonia accumulated rapidly in treated control plants and increased 30-fold only two hours after the herbicide spraying (Table 4 and Figure 8). The concentration of ammonium in treated control plants continued to increased sharply during the two-day testing period. Two days after the herbicide application, the ammonium level reached

4,380 μg $NH_4^+$-N per gram of fresh leaf sampl,e and there was substantial necrosis observed on control plants.

The levels of ammonium in the tested transgenic plants did increase after the herbicide treatment. The levels of ammonium increased 10, 15, and 11 times for transgenic plants from A, B, and C transformation events, respectively, two hours after the spraying of the herbicide. However, transgenic plants were able to detoxify the glufosinate-ammonium and decrease the amount of ammonium, as shown in Table 4.

Transgenic plants from independent transformation lines showed differential responses to the herbicide application and different ability to detoxify the herbicide. Four hours after the herbicide application, transgenic A plants were able to start decreasing the amount of ammonium accumulated after the spraying (Table 4). Though it was a little slower, transgenic C plants showed the same ability to decrease the amount of ammonium as transgenic A plants. However, the ability of transgenic B plants to detoxify the herbicide was not as good as those of transgenic A or C plants. The level of ammonium in transgenic B plants continued to rise for six hours after the spraying and began to decrease 12 hours after the herbicide application.

The abilities for independent transgenic plants to detoxify glufosinate-ammonium corresponded to their magnitudes of herbicide resistance (compare Figure 4 and Table 4). Transgenic A plants had the highest level of herbicide resistance and were able to decrease the accumulation of ammonium in a more efficient way. Transgenic B plants, which showed the lowest level of herbicide resistance, were found to be the fastest in the accumulation and the slowest in the decreasing of ammonium among the three tested independent transgenic lines.

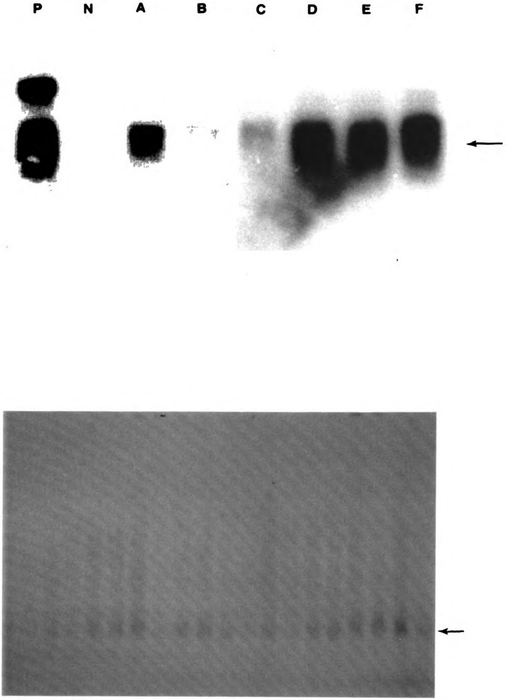


**Figure 7**. Northern hybridization analysis of the *bar* transcript in transgenic creeping bentgrasses. **Top**: Lane P: positive control; Lane N: untransformed control plants; Lanes A through F: independent transgenic plants. **Bottom**: mRNA isolated from additional independent transgenic plants. Position of the 600 bp *bar* transcript is indicated by the arrow.

**Table 4.** Ammonium concentration in transgenic and untransformed creeping bentgrasses after the application of 2.4 g/l glufosinate-ammonium

| | Concentration of ammonium (μg $NH_4^+$-N / g fresh weight)[♠] | | | | |
|---|---|---|---|---|---|
| | Control plants | | Treated transgenic plants[Δ] | | |
| Hours after application | Untreated[*] | Treated | A | B | C |
| 0[#] | 13 | 16 | 18 | 16 | 20 |
| 2 | 14 | 450 | 180 | 250 | 225 |
| 4 | 13 | 720 | 155 | 280 | 180 |
| 6 | 12 | 855 | 80 | 295 | 90 |
| 12 | 15 | 1350 | 55 | 230 | 68 |
| 18 | 14 | 2115 | 43 | 168 | 45 |
| 24 | 12 | 2700 | 22 | 110 | 23 |
| 30 | 17 | 3250 | 14 | 80 | 17 |
| 36 | 12 | 3750 | 14 | 50 | 14 |
| 42 | 15 | 4160 | 15 | 35 | 12 |
| 48 | 18 | 4380 | 11 | 26 | 15 |

[♠] There were five replications in each measurement of ammonium concentration.

[Δ] A, B, and C indicate transgenic plants from three independent transgenic events.

[*] Untreated means there was no herbicide application on untransformed control plants.

[#] Ammonium concentrations of transgenic and untransformed plants measured before the herbicide application.

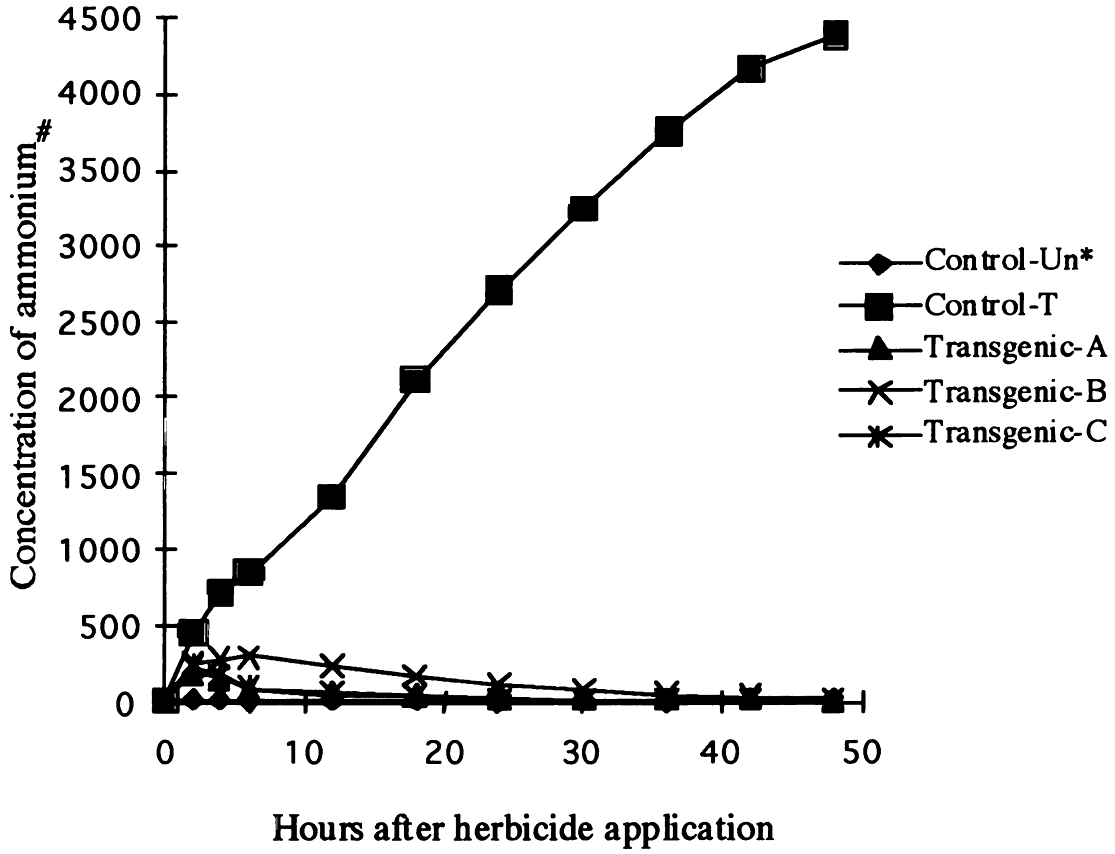


**Figure 8.** The accumulation of ammonium in transgenic and untransformed creeping bentgrasses after spraying with 1.2% Ignite®. Samples were taken before the herbicide application and at the times indicated in Table 4.

#: The concentration of ammonium is $\mu$g $NH_4^+$-N per gram of fresh leaf samples.

*: Un : Untreated untransformed control creeping bentgrasses; T: treated untransformed control plants. Transgenic A, B, and C indicate transgenic plants from three independent resistant callus lines.

## Discussion

These results demonstrated that both bialaphos and phosphinothricin (PPT) can be used as an efficient selective agent in the selection of callus which had been transformed with the *bar* gene by microprojectile bombardment. After three transfers and twelve weeks of culture on selection medium containing 5 mg/l of bialaphos, it was possible to select out calli which had been transformed with the *bar* gene. There was no escape detected in this selection scheme, and all of the resistant callus lines were able to regenerate transgenic plants expressing the enzyme phosphinothricin acetyltransferase (PAT) which detoxifies PPT by acetylation. However, 15 mg/l of PPT was necessary to distinguish transformed calli from nontransgenic ones under the same selection scheme.

The reason that a higher concentration of PPT was necessary to achieve the same selection efficiency than of bialaphos might be partially due to the application of *D*, *L*-PPT in the selection medium. Bialaphos was a tripeptide antibiotic, which consisted of *L*-PPT and two *L*-alanine residues. Upon removal of these residues by peptidases, *L*-PPT was released and was the potent inhibitor of glutamine synthetase. The *D*-isomer of *D*, *L*-PPT was not active in the inhibition of glutamine synthetase (Kondo *et al.*, 1973; Hoerlein, 1994). The exact percentage of *D*-isomer in the mixture of *D*, *L*-PPT was unknown, so it was unsure that the reason for this phenomenon was solely the presence of *D*-PPT, which reduced the percentage of active components in the mixture. With the availability of both pure *D*-PPT and *L*-PPT, it should be possible to access the role of the *D*-isomer related to the *L*-isomer in the inhibition of glutamine synthetase.

Several factors must be refined to develop a routine transformation system for creeping bentgrasses. The length of selection duration and the concentration of selective agent appear to be important in the selection of transgenic callus lines. Concentration of selective agents need to be carefully chosen to avoid being either too low and thereby allowing an undesirable number of escapes to develop, or too high so that transformants expressing low or moderate levels of resistance are lost. The length of selection, another factor in the development of a transformation system, has to be judiciously determined. It should not be too long, since the chance of somaclonal variation will increase with culture age, and cultures may loose their regeneration capacity due to prolonged culture. However, if the length of selection is not long enough, and the selection pressure is not strong, there may still be a lot of escapes.

Hartman *et al.* (1994) reported the transformation of creeping bentgrasses with the *bar* gene after suspension cells were bombarded with vectors containing the gene and placed under selection for eight weeks without transfer. Initially, a concentration of 2 mg/l of bialaphos was used in the selection medium, but it was subsequently raised to 4 mg/l to reduce the number of escapes in their experiment. However, the percentages of recovered plants after selection that survived the herbicide application (2 mg/ml of Herbiace® which contains bialaphos) ranged from 0-13.7%. In our selection scheme, though embryogenic callus was employed as the bombarded material instead of suspension cells, 5 and 15 mg/l of bialaphos and PPT, respectively, were used in the selection medium, and the bombarded embryogenic calli were under selection for twelve weeks. All of the recovered plants which regenerated from bialaphos-resistant callus lines were able to survive the application of glufosinate-ammonium at the rate of 2.4 g/l. There was only one clone from the PPT selection scheme unable to survive the herbicide spraying. The other advantage of our transformation strategy is that callus tissues are easier to initiate and are more likely to regenerate plants compared to suspension cultures.

It was apparent that the length of selection duration was also a crucial factor in the selection of transgenic callus lines in our experiments. The number of resistant callus lines decreased as the length of the selection duration increased for both bialaphos and

PPT selection schemes (Table 3). The number of resistant callus lines decreased dramatically during the last selection duration (between nine and twelve weeks). Some callus lines of creeping bentgrass cultivar Penncross sensitive to bialaphos or PPT did not turn brown until after five to six weeks of exposure to the selective agent, so it was difficult to discriminate transformed callus lines from nontransformed ones. Twelve weeks of selection appeared to be the appropriate length of selection duration, since it maintained the regeneration capacity of resistant callus lines, and all them were able to regenerate plants.

These results also suggested that the frequency of transfer after callus lines had been bombarded might play an important role in the success of discriminating transgenic callus lines from untransformed ones. Bombarded suspension cells were maintained on the same selection plate for eight weeks without transfer in the experiment of Hartman *et al.* (1994). Embryogenic callus tissues were transferred every four weeks in our selection scheme, and only one escape was detected after regenerated plants were sprayed with herbicide. The frequent transfer onto fresh-prepared selection medium provided some advantages to the selection of resistant callus lines with the desired transgene sequence over the selection scheme with no transfer. On the one hand, it may avoid problems associated with cross-feeding. Transgenic callus tissue might provide neighboring nontransgenic tissue with glutamine and detoxify the selective agent for them. On the other hand, toxic substances such as excess amounts of ammonium accumulated in sensitive tissue might diffuse to transgenic tissue and cause difficulty in the identification of transformed callus lines. Another possible advantage is that the frequent transfer and separation of bombarded callus into small pieces may reduce the possibility of chimerism, one of the common problems found in transformation research, and provide a more even selection pressure on all of the callus lines. The frequent transfer can also reduce the possibility of the degradation of PPT and bialaphos and increase the selection efficiency.

The phenomenon of heterogeneous responses of callus lines initiated from mature seeds of cultivar Penncross was possibly due to the breeding history of this cultivar. Penncross is a first generation synthetic cultivar produced by the random crossing of three vegetatively propagated strains selected by H. B. Musser (Hein, 1958). The synthetic cultivar is genetically heterogeneous, and each seed of Penncross possibly represents a different genotype. The results from the evaluation of selective agents reflected the heterogeneity of callus lines initiated from Penncross (Table 1 and Table 2). The decision concerning the concentration of the selective agent and the length of the selection duration again played an important role in the selection of transgenic resistant callus lines of Penncross, since the selection strategy should provide a dependable and repeatable scheme to select transgenic callus lines from a heterogeneous population.

Kramer *et al.* (1993) used a successful selection scheme with PPT as the selective agent and with the pH indicator chlorophenol red for the identification of transformed protoplast-derived colonies of maize. Chlorophenol red also proved to be a useful tool in determinating the appropriate concentrations of selective agents needed to kill embryogenic callus of creeping bentgrasses and in shortening the time necessary to discriminate surviving colonies from dead ones in the evaluation of selective agents. We also employed chlorophenol red to distinguish transgenic callus lines from nontransgenic ones; however, the identification frequencies were not satisfactory, especially during the early stages of selection (data not shown).

PPT- or bialaphos-resistance conferred by the *bar* gene was a useful selectable marker for the isolation of transformed creeping bentgrasses. By using the Southern hybridization analysis to detect stably integrated *bar* gene sequences, we showed that all of the plants regenerated from resistant callus lines, except plants from one PPT-resistant callus line, were transformed. The *bar* gene also proved to be a good reporter gene in creeping bentgrasses. PAT activity and PPT-specific mRNA were detected in all of the transgenic plants, and three of independent resistant plant lines tested were able to decrease the accumulation of ammonium within a short period of time. The *bar* gene can not only successfully confer resistance to transformed plants but can also be used as a selectable marker in the transformation of creeping bentgrasses with other useful genes.

Penncross, the cultivar used in this experiment, is widely used on golf greens and has been the most popular creeping bentgrass cultivar. The transgenic plants showed a high level of resistance to glufosinate-ammonium, which is the active ingredient of commercial herbicide Ignite®, Rely®, Liberty™ and Finale™, and, therefore, have great commercial potential. The utilization of a PPT-resistant cultivar on golf courses can not only reduce the amount of the toxic herbicide 2,4-D applied on golf courses, but can also be more environment-friendly, since PPT was degraded rapidly by microbes in soil or surface water to 3-methyl phosphinicopropionic acid and ultimately to $CO_2$ (Humburg *et al.*, 1994). With transgenic creeping bentgrasses expressing the *bar* gene, the spraying of PPT or bialaphos on golf courses cultivated with resistant creeping bentgrasses can kill both monocot and dicot weeds and provide a more efficient control of weed problems.

# BIBLIOGRAPHY

Bayer, E., Gugel, K. H., Haebele, K., Hagenmaier, H., Jessipow, S., Koenig, W. A., and Zaehner, H. 1972. Phosphinothricin und phosphinothricyl-alanin. Helv. Chim. Acta 55: 224-239.

Beard, J. B. 1982. Turf management for golf courses. Macmillan Publishing Company, New York. pp. 95-176.

Casas, A. M., Kononowicz, A. K., Zehr, U. B., Tomes, D. T., Axtell, J. D., Butler, L. G., Bressan, R. A., and Hasegawa, P. M. 1993. Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA 90: 11212-11216.

Christou, P., Ford, T. L., and Kofron, M. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9: 957-962.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, N. R., Thompson, C., Van Montagu, M., and Leemans, J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6: 2513-2518.

De Block, M., De Brouwer, D., and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. 91: 694-701.

Decker, H. F. and Decker, J. M. 1988. Lawn care: a hansbook for professionals. Prentice Hall, New Jersey. pp. 119-173.

D'Halluin, K., De Block, M., Denecke, J., Janssens, J., Leemans, J., Reynaerts, A., and Botterman, J. 1992. The *bar* gene as selectable and screenable marker in plant engineering. In: Methods in enzymology. 216: 415-426. Wu, R. (ed.) Academic Press, Inc. C. A.

Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R., Willetts, N. G., Rice, T. B., Mackey, C. J., Krueger, R. W., Kausch, A. P., and Lemaux, P. G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2: 603-618.

Hartman, C. L., Lee, L., Day, P. R., and Tumer, N. E. 1994. Herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation. Bio/Technology 12: 919-923.

Hein, M. A. 1958. Registration of Penncross creeping bentgrass. Agron. J. 50: 399.

Hoerlein, G. 1994. Glufosinate (phosphinothricin), a natural amino acid with unexpected herbicidal properties. In: Reviews of Environmental Contamination and Toxicology. 138: 73-145. Ware, G. W. (ed.) Springer-Verlag, Inc. N. Y.

Humburg, N. E., Colby, S. R., Hill, E. R., Kitchen, L. M., Lym, R. G., McAvoy, W. J., and Prasad, R. 1994. Herbicide handbook of the weed science society of America. 7th edithio. Weed Science Society of America. Champaign, IL. pp.147-149.

Klein, T. M., Wolf, E. D., Wu, R., and Sanford, J. C. 1987. High velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70-73.

Kondo, Y., Shomura, T., Ogawa, Y., Tsuruoka, T., Watanabe, H., Totsukawa, K., Suzuki, T., Moriya, C., and Yoshida, J. 1973. Isolation and physico-chemical and biological characterization of SF-1293 substance. Sci. Reports of Meiji Seika Kaisha 13: 34-41; Chem. Abstr. 1974, 81, 89705b.

Kramer, C., DiMaio, J., Carswell, G. K., and Shillito, R. D. 1993. Selection of transformed protoplast-derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol red. Planta 190: 454-458.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Ogawa, Y., Tsuruoka, T., Inouye, S., and Niida, T. 1973. Chemical structure of antibiotic SF-1293. Sci. Reports of Meiji Seika Kaisha 13: 42-48; Chem. Abstr. 1974, 81, 37806r.

Rogers, S. O. and Bendich, A. J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. 5: 69-76.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning-a Laboratory Manual, Second edition. Cold Spring Harbor Laboratory Press, N. Y.

Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F., and Bushnell, W. R. 1992. Fertile, transgenic oat plants. Bio/Technology 10: 1589-1594.

Spencer, T. M., Gordon-Kamm, W. J., Daines, R. J., Start, W. G., and Lemaux, P. G. 1990. Bialaphos selection of stable transformants from maize cell culture. Theor. Appl. Genet. 79: 625-631.

Tachibana, K., Watanabe, T., Sekizawa, Y., and Takematsu, T. 1986a. Inhibition of glutamine synthetase and quantitative changes of free amino acids in shoots of bialaphos treated Japanese barnyard millet. J. Pest. Sci. 11: 27-31.

Tachibana, K., Watanabe, T., Sekizawa, Y., and Takematsu, T. 1986b. Action mechanism of bialaphos. II. Accumulation of ammonia in plants treated with bialaphos. J. Pest. Sci. 11: 33-37.

Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M., and Botterman, J. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. 6: 2519-2523.

Vasil, V., Castillo, A. M., Fromm, M. E., and Vasil, I. K. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/technology 10: 667-674.

Wadsworth, G. J., Redinbaugh, M. G., and Scandalios, J. G. 1988. A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Analyt. Biochem. 172: 279-283.

Wan, Y. and Lemaux, P. G. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104: 37-48.

Wang, Z., Takamizo, T., Iglesias, V. A., Osusky, M., Nagel, J., Potrykus, I., and Spangengerg, G. 1992. Transgenic plants of tall fescue (*Festuca arundinacea* Schreb.) obtained by direct gene transfer to protoplasts. Bio/Technology 10: 691-696.

Zhong, H., Bolyard, M. G., Srinivasan, C., and Sticklen, M. B. 1993. Transgenic plants of turfgrass (*Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus. Plant Cell Rep. 13: 1-6.

# CHAPTER THREE

## Evaluation of Bialaphos on the Prevention of Fungal Diseases in Transgenic Bialaphos-resistant Creeping Bentgrasses (*Agrostis palustris* Huds.)

## ABSTRACT

Bialaphos showed a higher level of *in vitro* antifungal activity against the brown patch disease pathogen, *Rhizoctonia solani*; the dollar spot disease pathogen, *Sclerotinia homoeocarpa*; and the *Pythium* blight pathogen, *Pythium aphanidermatum*, than phosphinothricin (PPT). While PPT, an inhibitor of glutamine synthetase, suppressed the mycelial growth of *R. solani* and *S. homoeocarpa*, it had no inhibitory effect on *Pythium aphanidermatum* up to the highest concentration (600 mg/l) in our testing regimes. Whereas bialaphos, the precursor of PPT and also an inhibitor of glutamine synthetase, was significantly effective in the inhibition of growth of *R. solani* and *S. homoeocarpa*, it was less so in that of *Pythium aphanidermatum*. Mean $ED_{50}$ values for inhibition of mycelia growth of *R. solani* and *S. homoeocarpa* were 5.54 and 33.04 mg/l (17.10 and 101.98 $\mu$M), respectively, for bialaphos, compared with 292.18 and 270.06 mg/l (1475.66 and 1363.94 $\mu$M), respectively, for PPT.

Various concentrations of bialaphos solutions were applied to transgenic bialaphos-resistant creeping bentgrasses either three hours before or two days after plants were inoculated with mycelia of *R. solani*, *S. homoeocarpa* and *Pythium aphanidermatum*. Bialaphos application was able to significantly reduce the

symptomatic infection by *R. solani* and *S. homoeocarpa.* Though the inhibitory effect of bialaphos spraying on the suppression of *Pythium aphanidermatum* was not as effective as that of *R. solani* and *S. homoeocarpa*, disease development was still reduced to a significant extent. Better control of the disease development of dollar spot and *Pythium* blight also occured when bialaphos was applied three hours before the pathogen inoculation. These results also indicated that bialaphos may be used simultaneously and efficaciously as a herbicide for weed control and as a fungicide for the control of diseases caused by fungal pathogens in turf areas with transgenic bialaphos-resistant creeping bentgrasses.

# Introduction

Turfgrasses have been recognized for their importance to our quality of life for a long time and are cultured in nearly all inhabited regions of the world. Bentgrass (*Agrostis* spp.) is the cool-season turfgrass used commonly on greens in the cool and transitional climatic regions and in the cooler portions of the warm climatic region. Creeping bentgrass (*Agrostis palustris* Huds.) is the most commonly used *Agrostis* species on golf putting greens and similar closely cut turf areas in North America (Beard, 1982). Among the existing cultivars of creeping bentgrass, Penncross is the major cultivar employed today and is being used increasingly on greens of many golf courses.

Turfgrass plants are affected by numerous diseases, insects, and physiologic disorders resulting from environmental stresses. Although diseases and the costs of controlling them are difficult to estimate, the science of turfgrass pathology, management, and breeding have frequently focused on disease control as a primary research objective (Smiley, 1983). Turfgrass diseases are a greater problem on golf courses than on most other types of turf use. This is due to close mowing, heavy fertilization, intense irrigation, and constant bruising from traffic and divoting. Disease problems are most severe on putting greens, especially those composed of bentgrass or annual bluegrass in contrast to bermudagrass turfs. The creeping bentgrasses are susceptible to a variety of diseases such as brown patch (*Rhizoctonia* spp.), dollar spot (*Sclerotinia homoeocarpa*), *Pythium* blight (*Pythium* spp.), *Fusarium* blight (*Fusarium roseum* and *F. tricinctum*), and takeall patch (*Gaeumannomyces graminis*) (Smiley, 1983).

Bialaphos, the active ingredient of the commercial formulation herbicide Herbiace® (Meiji Seika Kaisha) which is now being used in agriculture as a nonselective and broad-spectrum contact herbicide, is a tripeptide composed of two *L*-alanine residues and an analogue of glutamate known as phosphinothricin (PPT) (Ogawa *et al.*, 1973; Kondo *et al.*, 1973). PPT inhibits the amino acid biosynthetic enzyme glutamine synthetase (GS) of bacteria and plants (Bayer *et al.*, 1972; Leason *et al.*, 1982). Bialaphos is a precursor of PPT produced by some strains of *Streptomyces*, and the intact tripeptide has little or no inhibitory activity *in vitro* (Bayer *et al.*, 1972; Tachibana *et al.*, 1986a). In both plants and bacteria, the active PPT moiety is released by intracellular peptidases which remove the alanine residues from bialaphos.

Glutamine synthetase plays an important role in the assimilation of ammonia both in plants and bacteria (Miflin and Lea, 1977; Joy, 1988). Inhibition of GS by these compounds causes a rapid buildup of intracellular ammonia levels and an associated disruption of chloroplast structure, resulting in the inhibition of photosynthesis and plant cell death (Tachibana *et al.*, 1986b). Bayer *et al.* (1972) reported that the tripeptide bialaphos has a certain antibiotic effect in *Escherichia coli* and *Bacillus subtilis*, which is neutralized by the addition of glutamine. Experiments with GS isolated from *E. coli* confirmed that PPT inhibits the GS enzyme in competition with glutamate as varying substrate. Leason *et al.* (1982) determined GS inhibition by PPT in peas and demonstrated that the mode of action is the same in bacteria and plants.

PPT resistance has been achieved by introducing a gene that codes for a detoxifying enzyme (PAT: phosphinothricin acetyltransferase) into several plant species (De Block *et al.*, 1987, 1989; Spencer *et al.*, 1990; Christou *et al.*, 1991; Somers *et al.*, 1992; Casas *et al.*, 1993; Wan and Lemaux, 1994; Akama *et al.*, 1995). The gene, designated *bar*, was found in strains of *Streptomyces* that produce bialaphos and isolated from *S. hygroscopicus* by Murakami *et al.* (1986). The *bar* gene product protects these strains from the action of their own antibiotic by metabolizing PPT to an inactive, acetylated derivative (Thompson *et al.*, 1987).

We have previously generated transgenic creeping bentgrasses showing high levels of *bar* gene expression, which resulted in herbicide (either PPT or bialaphos) resistance. It has been reported that bialaphos treatment of transgenic rice plants expressing the *bar* gene could prevent infection by the sheath blight fungal pathogen (*Rhizoctonia solani*) (Uchimiya *et al.*, 1993). Since *R. solani* is the etiologic agent of brown patch, one of the severe fungal diseases of creeping bentgrass, we were prompted to assess its application to bialaphos-resistant transgenic creeping bentgrasses as a means of protecting against fungal diseases. If the application of bialaphos could be shown to reduce the plant damage due to fungal infection, a novel and economical usage for the herbicide would be to provide weed control in turf areas with bialaphos-resistant creeping bentgrasses while simultaneously and cumulatively reducing levels of fungal pathogens.

In this communication, we present the evidence that the application of bialaphos on transgenic bialaphos-resistant creeping bentgrasses can prevent or alleviate the infection by several fungal pathogens, including *Rhizoctonia solani* (brown patch), *Sclerotinia homoeocarpa* (dollar spot), and *Pythium aphanidermatum* (*Pythium* blight). This experiment indicates that it may, therefore, be possible to provide an opportunity for the simultaneous control of weeds and fungal diseases in golf courses of transgenic creeping bentgrasses expressing the *bar* gene.

## Materials and Methods

### *In Vitro* Test

The sensitivity of different pathogens to phosphinothricin (PPT; glufosinate-ammonium from Riedel-deHaën of German) and bialaphos (kindly provided by Meiji Seika Kaisha of Japan) was first tested *in vitro* by culturing mycelium of fungi on PPT- or bialaphos-supplemented medium. Fungus isolates of *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, and *Pythium aphanidermatum* were kindly provided by Dr. J. M. Vargas of Michigan State University and cultured on potato dextrose agar medium (PDA, 39 g/l; Difco Laboratories, Detroit, MI) as stock plates.

PPT and bialaphos were dissolved in double-distilled water and filter-sterilized (Nalgene filter) before adding into molten PDA (60 $^{o}$C) medium after sterilizing at 121 $^{o}$C for 15 minutes. A dilution series was used to obtain the needed concentrations. The amended medium was mixed thoroughly and poured into 10-cm-diameter Petri dishes.

Inoculum for the study, which consisted of seven-mm-diameter agar plugs with mycelium of each fungus isolates, was taken from stock plates and transferred onto freshly prepared PDA medium. After the plates were incubated for at least one week under the light at 25 $^{o}$C and the growths of mycelium had completely covered the plates, plugs were taken aseptically from plates with actively growing mycelium of *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, and *Pythium aphanidermatum* on PDA medium. Inverted plugs were then transferred to the center of test plates (one plug per plate) containing 20 ml PDA medium supplemented with various concentrations of PPT or bialaphos ranging from 0 to 600 mg/l. Test plates with inoculum were wrapped with parafilm and placed under the light at 25 $^{o}$C. There were 15 replications in each treatment.

The growth of different fungi determined by measuring the radial length (mm) of the colonies on corresponding plates after incubation for four days was used as an indicator of the sensitivity of the fungus to the addition of PPT or bialaphos into the PDA medium. Mycelial growth on bialaphos- and PPT-supplemented medium was compared with that on non-amended medium. Percent inhibition was plotted as a function of the bialaphos or PPT concentration. Linear regression was used to fit a line to the points and determine the concentration causing a 50 % reduction in growth ($ED_{50}$). Tukey's Honestly Significant Difference Test at $P = 0.05$ was employed to perform the statistical analysis to detect differences in the mean values of radial length.

## Greenhouse Test

Twelve grams of dry wheat seeds were autoclaved twice for 30 minutes in a 125 ml glass flask containing 25 ml of distilled water. Five 7-mm-diameter PDA plugs with actively growing mycelium of *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, or *Pythium aphanidermatum* were put into the flask and mixed with the autoclaved wheat seeds. The fungal culture was incubated under the light at 25 $^{o}$C for one week after the inoculation.

In order to have uniform plants for fungal inoculation, three-month old healthy transgenic (all from the same transgenic event) and nontransgenic control creeping bentgrasses (*Agrostis palustris* Huds.) cultivar Penncross which were grown in four-inch plastic pots were trimmed (about one inch in height) then fertilized with Peter's 20-20-20 one week before the pathogen inoculation. To inoculate creeping bentgrasses with different pathogens, about 500 mg of wheat seeds with mycelium of the corresponding fungus were preweighed and evenly distributed on top of each plant.

Various concentrations of bialaphos solution ranging from 200 to 2,400 mg/l were prepared before the application and sprayed on the creeping bentgrasses with a hand sprayer either three hours before or two days after the fungus inoculation. After the bialaphos application, plants were wrapped with plastic bags with holes to raise the humidity. Transgenic and nontransgenic control plants which were not treated with bialaphos were subject to the same pathogen infection procedures as well. There were ten replications in each treatment.

Disease rating on a zero to ten scale with the smallest increment of 0.5 was recorded on the basis of the percentage of plant damage due to fungal infection one week after the initial pathogen inoculation, for example, 0: no damage; 5: 50 % plant damage; 10: death. Differences in mean disease rating values between two spraying times were detected using F test at $P = 0.05$ and Tukey's Honestly Significant Difference test at $P = 0.05$ were employed to perform the statistical analysis to detect differences in mean disease rating values between different concentrations of bialaphos spraying with the same spraying time for each fungal pathogen.

# Results

## *In Vitro* Test

Fungal pathogens *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, and *Pythium aphanidermatum*, the etiologic agents of brown patch, dollar spot, and *Pythium* blight diseases, respectively, of creeping bentgrasses were inoculated and cultured on PDA medium supplemented with various concentrations of bialaphos or PPT to assess their responses to these two inhibitors of glutamine synthetase.

*Rhizoctonia solani* was very sensitive to the addition of bialaphos into the PDA medium (Table 1 and Figure 1). Even at the lowest concentration of one mg/l, the mycelial growth of *Rhizoctonia solani* was significantly suppressed as compared to that on PDA medium with no bialaphos supplement. Only about five mg/l ($ED_{50}$ = 5.54 mg/l) of bialaphos amendment was needed to reduce the fungal growth by 50 % (Table 1 and Table 6). There was almost no fungal growth observed four days after the inoculation when the bialaphos concentration of PDA medium was 60 mg/l. There was still no significant growth of *Rhizoctonia solani* even two weeks after the initial inoculation when the concentration of bialaphos was 60 mg/l or higher (data not shown).

PPT was also very effective in the suppression of the mycelial growth of *Rhizoctonia solani* (Table 2 and Figure 1). However, the presence of PPT was not as effective as that of bialaphos in suppressing the growth of *Rhizoctonia solani* on PDA medium . Bialaphos inhibited mycelial growth of *R. solani* more than PPT did, as reflected by their values of $ED_{50}$ (Table 6). The growth of mycelium was significantly reduced at the concentration of 25 mg/l PPT. More PPT amendment (292.18 mg/l or 1475.66 17.10 μM), as compared to the amount of bialaphos supplement (5.54 mg/l or 17.10 μM), was necessary to reduced the growth of *Rhizoctonia solani* by 50 %. There was still some mycelial growth of *R. solani* observed even when 600 mg/l of PPT was amended into the PDA medium. The same trend was also evident for *Sclerotinia homoeocarpa* and *Pythium aphanidermatum*, where the $ED_{50}$ values for *S. homoeocarpa* and *Pythium aphanidermatum* were higher for PPT than for bialaphos (Table 6).

The mycelial growth of *S. homoeocarpa* was also sensitive to the presence of bialaphos and PPT (Table 3, Table 4 and Figure 2), though its responses were apparently different from those of *R. solani* (Table 6 and Figure 4). Higher concentrations of bialaphos and PPT were necessary to significantly reduce the mycelial growth of *S. homoeocarpa* than that of *R. solani*. The $ED_{50}$ value of *S. homoeocarpa* for bialaphos was higher than that of *R. solani* (33.04 and 5.54 mg/l, respectively) (Table 6). More than 150 mg/l of bialaphos amendment was necessary to completely suppressed the mycelial growth of *S. homoeocarpa* on PDA medium. However, *S. homoeocarpa* responded a little more sensitively to higher concentrations of PPT (between 400 and 600 mg/l) than *R. solani* (Table 2, Table 4 and Figure 4). The $ED_{50}$ value for PPT of *S. homoeocarpa* was lower than that of *R. solani* (270.06 and 292.18 mg/l, respectively) (Table 6). In general, the effect of bialaphos or PPT amendment into PDA medium on the inhibition of mycelial growth of *R. solani* and *S. homoeocarpa* was effective with the highest concentration resulted in the least growth of mycelium.

Compared with *R. solani* and *S. homoeocarpa*, *Pythium aphanidermatum* was the least sensitive fungus to both bialaphos and PPT (Table 5, Table 6, Figure 3 and Figure 4). At least 500 mg/l of bialaphos supplement was needed to significantly reduce the mycelial growth on the PDA medium (Table 5) and the whole plate was covered with the mycelium of *Pythium aphanidermatum* one week after the initial inoculation. The presence of PPT had no effect on the inhibition of *Pythium aphanidermatum* up to the highest concentration (600 mg/l) amended in PDA medium.

**Table 1.** Sensitivity of *Rhizoctonia solani* to bialaphos on potato dextrose agar medium

| concentration of bialaphos (mg/l) | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0 | 1 | 5 | 10 | 20 | 40 | 60 | 80 |
| 40.0 ± 0.0[#] | 31.6 ± 0.7 | 12.5 ± 0.7 | 7.3 ± 0.5 | 4.4 ± 0.2 | 1.5 ± 0.2 | 0.4 ± 0.1a[*] | 0 ± 0.0a |

[#] The radial length (mm) of the colony growing on various concentrations of bialaphos-supplemented potato dextrose agar medium four days after inoculation was used as an indicator to measure the sensitivity of *Rhizoctonia solani* to bialaphos.

[*] Mean ± S.E. (standard error) for 15 replications. Means with the same letter were not significantly different according the Tukey's test at P = 0.05.

**Table 2.** Sensitivity of *Rhizoctonia solani* to phosphinothricin on potato dextrose agar medium

| concentration of phosphinothricin (mg/l) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 0 | 25 | 50 | 100 | 200 | 300 | 400 | 500 | 600 |
| 40.0±0.0[#] | 35.0±1.2a[*] | 30.8±1.0a | 25.4±1.8b | 20.2±1.0bc | 16.8±1.0cd | 13.0±0.8de | 12.0±1.1e | 6.8±0.5 |

[#] The radial length (mm) of the colony growing on various concentrations of phosphinothricin-supplemented potato dextrose agar medium four days after inoculation was used as an indicator to measure the sensitivity of *Rhizoctonia solani* to phosphinothricin.

[*] Mean ± S.E. (standard error) for 15 replications. Means with the same letter were not significantly different according the Tukey's test at $P = 0.05$.

**Table 3.** Sensitivity of *Sclerotinia homoeocarpa* to bialaphos on potato dextrose agar medium

| concentration of bialaphos (mg/l) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 20 | 40 | 60 | 80 | 100 | 150 | 200 | 250 | 300 |
| 40.0±0.0[#] | 26.8±1.3 | 20.0±1.4 | 15.0±0.5a[*] | 13.2±0.4a | 10.8±0.5ab | 7.0±0.7b | 1.8±0.4c | 0.6±0.2c | 0±0.0c |

[#] The radius length (mm) of the colony growing on various concentrations of bialaphos-supplemented potato dextrose agar medium four days after inoculation was used as an indicator to measure the sensitivity of *Sclerotinia homoeocarpa* to bialaphos.

[*] Mean ± S.E. (standard error) for 15 replications. Means with the same letter were not significantly different according the Tukey's test at $P = 0.05$.

**Table 4.** Sensitivity of *Sclerotinia homoeocarpa* to phosphinothricin on potato dextrose agar medium

| concentration of phosphinothricin (mg/l) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 50 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 500 | 600 |
| 40.0±0.0a[#*] | 37.8±0.6a | 33.0±1.0 | 29.0±0.7 | 23.8±1.1 | 17.6±0.6b | 15.2±0.8bc | 12.0±0.9cd | 10.2±0.6d | 3.8±0.7e | 2.4±0.7e |

[#] The radial length (mm) of the colony growing on various concentrations of phosphinothricin-supplemented potato dextrose agar medium four days after inoculation was used as an indicator to measure the sensitivity of *Sclerotinia homoeocarpa* to phosphinothricin.

[*] Mean ± S.E. (standard error) for 15 replications. Means with the same letter were not significantly different according the Tukey's test at $P = 0.05$.

**Table 5.** Sensitivity of ***Pythium aphanidermatum*** to bialaphos on potato dextrose agar medium

| concentration of bialaphos (mg/l) | | | | | |
|---|---|---|---|---|---|
| 0 | 100 | 200 | 300 | 400 | 500 |
| 40.0 ± 0.0 a[#] [*] | 40.0 ± 0.0 a | 40.0 ± 0.0 a | 39.0 ± 0.4 a | 38.0 ± 0.5 a | 31.0 ± 1.0 |

[#] The radial length (mm) of the colony growing on various concentrations of bialaphos-supplemented potato dextrose agar medium four days after inoculation was used as an indicator to measure the sensitivity of ***Pythium aphanidermatum*** to bialaphos.

[*] Mean ± S.E. (standard error) for 15 replications. Means with the same letter were not significantly different according the Tukey's test at $P = 0.05$.

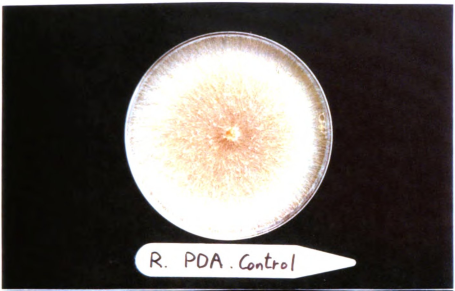
**Table 6.** *In vitro* inhibition of *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, and *Pythium aphanidermatum* by bialaphos or phosphinothricin

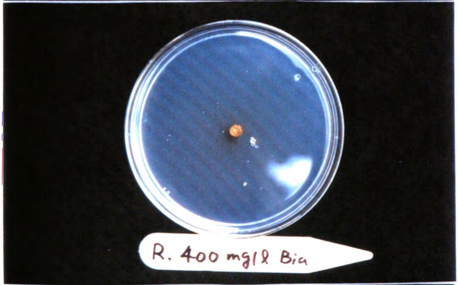
| Fungus | Amending ingredient | Linear regression[#] | Correlation coefficient | $ED_{50}$ (mg/l) |
|---|---|---|---|---|
| *Rhizoctonia solani* | bialaphos | Y = 0.91X + 44.96 | 0.72 | 5.54 |
| | PPT | Y = 0.11X + 17.86 | 0.97 | 292.18 |
| *Sclerotinia homoeocarpa* | bialaphos | Y = 0.28X + 40.75 | 0.88 | 33.04 |
| | PPT | Y = 0.17X + 4.09 | 0.98 | 270.06 |
| *Pythium aphanidermatum* | bialaphos | Y = 0.037X - 4.28 | 0.79 | 1467.18 |
| | PPT | ---------------------* | ----- | ------- |

[#] Percent inhibition [Y] was plotted as a function of bialaphos or phosphinothricin (PPT) concentration [X].

[*] There was no inhibition of *Pythium aphanidermatum* by phosphinothricin up to the highest concentration (600 mg/l) amended in PDA medium.

**Figure 1.** The inhibition of bialaphos and phosphinothricin on the mycelium of *Rhizoctonia solani* four days after the initial inoculation.
Top: the growth of mycelium on PDA medium.
Middle: the growth of mycelium on PDA medium contained 400 mg/l of bialaphos.
Bottom: the growth of mycelium on PDA medium contained 400 mg/l of phosphinothricin.


R. PDA. Control


R. 400 mg/l Bia

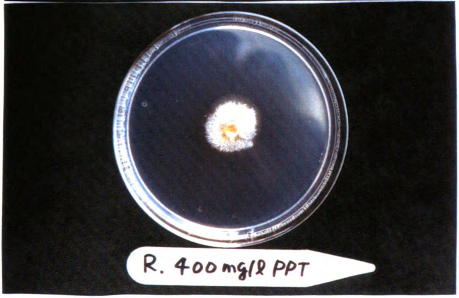
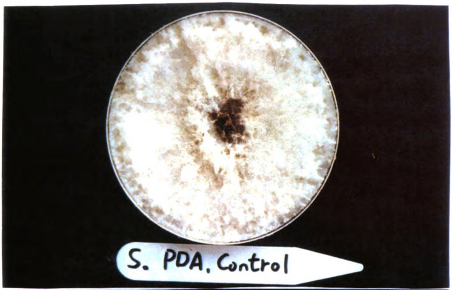

R. 400 mg/l PPT

**Figure 2.** The inhibition of bialaphos and phosphinothricin on the mycelium of *Sclerotinia homoeocarpa* four days after the initial inoculation.
Top: the growth of mycelium on PDA medium.
Middle: the growth of mycelium on PDA medium contained 400 mg/l of bialaphos.
Bottom: the growth of mycelium on PDA medium contained 400 mg/l of phosphinothricin.


S. PDA, Control


S. 400 mg/l Bia

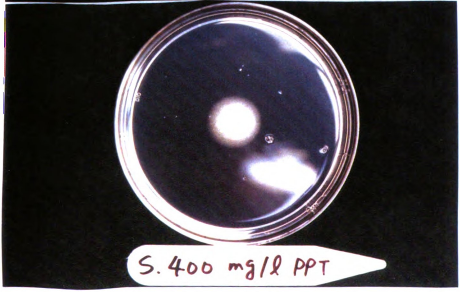
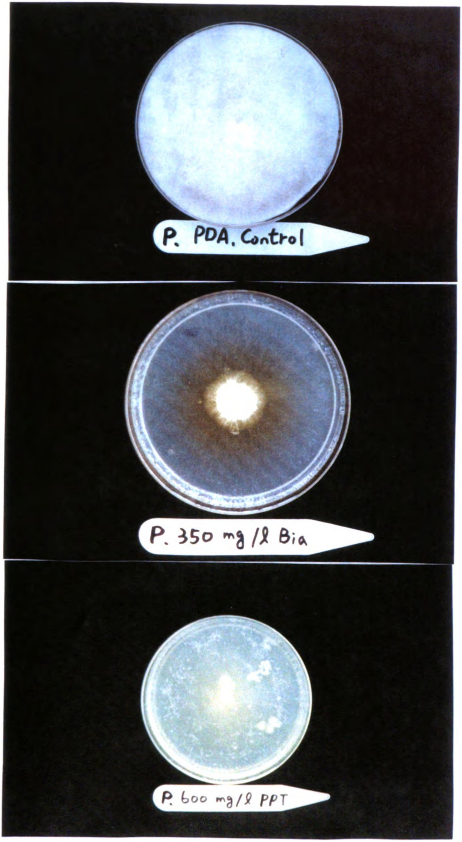

S. 400 mg/l PPT

**Figure 3.** The inhibition of bialaphos and phosphinothricin on the mycelium of ***Pythium aphanidermatum*** four days after the initial inoculation.
Top: the growth of mycelium on PDA medium.
Middle: the growth of mycelium on PDA medium contained 350 mg/l of bialaphos.
Bottom: the growth of mycelium on PDA medium contained 600 mg/l of phosphinothricin.


P. PDA. Control
P. 350 mg/l Bia
P. 600 mg/l PPT

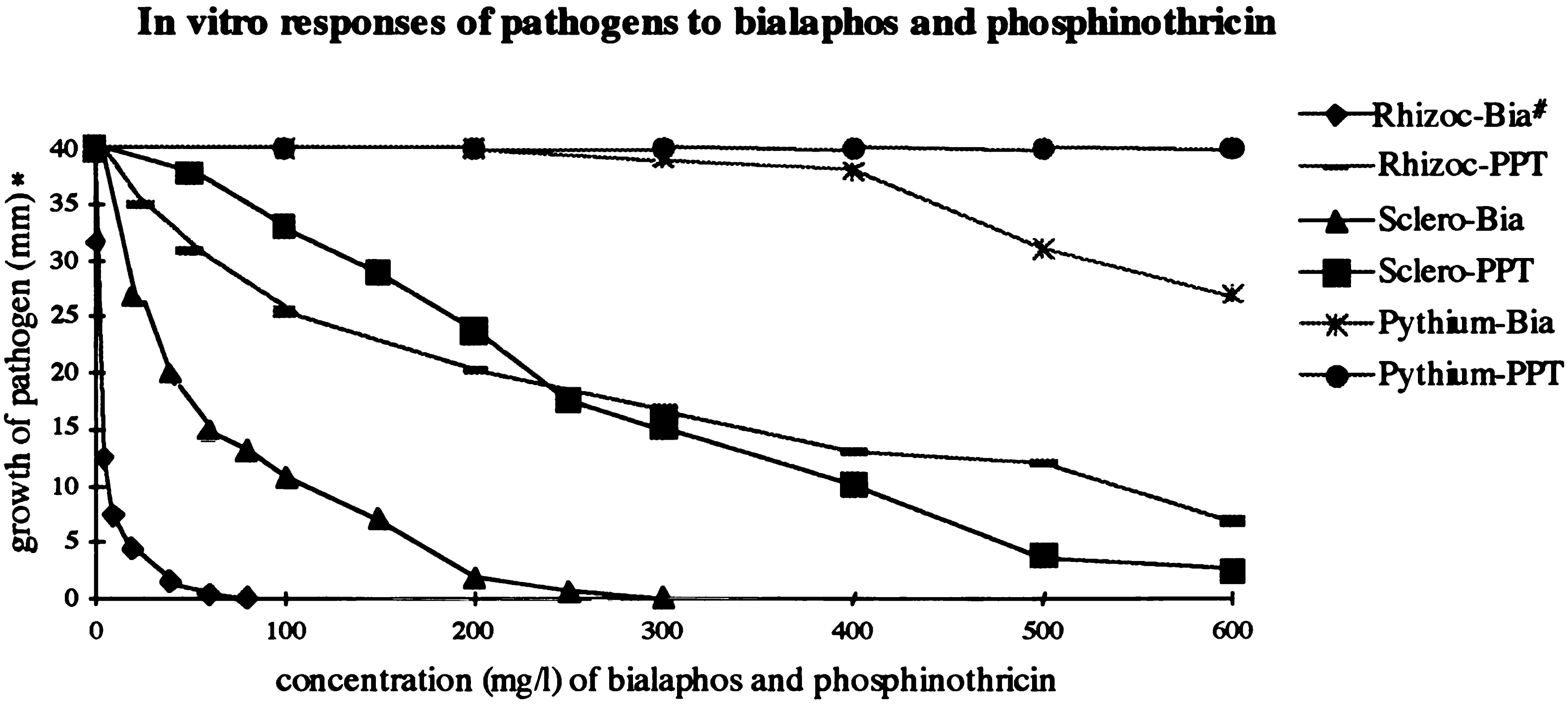


**Figure 4.** *In vitro* responses of *Rhizoctonia solani*, *Sclerotinia homoeocarpa* and *Pythium aphanidermatum* to bialaphos- or phosphinothricin-amended potato dextrose agar medium.
*The radial length (mm) of the colony was used to represent the mycelial growth of the pathogen for 15 replications.
#Rhizoc: *Rhizoctonia solani* ; Sclero: *Sclerotinia homoeocarpa* ; Bia: bialaphos; PPT: phosphinothricin.

However, the amendment of bialaphos and PPT did show some inhibitory effect on the growth of *Pythium aphanidermatum* when the amount of mycelium, instead of the measurement of radial length of mycelium, was employed as the indicator to represent the growth of *Pythium aphanidermatum*. (Figure 3).

## Greenhouse Test

Various concentrations of bialaphos solution were applied on transgenic creeping bentgrasses expressing the *bar* gene to assess the effects of bialaphos spraying on the development of the three different pathogens (Table 7). The application of bialaphos had a very significant effect on the suppression of brown patch disease development when the disease rating was taken one week after the fungus inoculation. When bialaphos application was executed three hours before the inoculation of *Rhizoctonia solani* on transgenic plants, disease symptoms were rarely observed, and there was only minimal plant damage due to the infection of *Rhizoctonia solani*. At the concentration of 200 mg/l of bialaphos solution, about one-tenth of the recommended herbicide spraying rate to kill untransformed creeping bentgrasses, the application significantly reduced the plant damage due to pathogen infection.

Transgenic plants that were not treated with bialaphos showed typical symptoms of brown patch disease and a significant amount of plant damage. Even two days after the pathogen inoculation, when the disease began to develop, the application of bialaphos could still significantly restrain the growth of mycelium and the development of brown patch disease. The untreated control plants, either transgenic or nontransgenic, were severely damaged by the infection of *R. solani*. The grass blades became water soaked and dark at first but soon became dry, wither, and turned light brown. The disease was able to continuously develop even after plastic bags were taken off and a lot of the untreated plants were dead three weeks after the pathogen inoculation (data not shown).

There was no significant difference observed between two different timings of bialaphos application ($F = 0.29 < F_{.05\,(1,63)} = 4.00$).

The bialaphos application, either three hours before or two days after the pathogen inoculation, was also very effective in preventing the disease development of *S. homoeocarpa*, the etiologic agent of dollar spot, on transgenic creeping bentgrasses. The plant damages on transgenic bialaphos-resistant creeping bentgrasses after the bialaphos application were significantly less than those on transgenic plants not treated with bialaphos. The difference between the two application times was significant ($F = 8.23 > F_{.05\,(1,81)} = 3.98$), and there was more plant damage caused by the infection of *S. homoeocarpa* when the bialaphos spraying on transgenic plants was done two days after the pathogen inoculation.

The development of disease symptoms of dollar spot on transgenic and nontransgenic control plants which were not applied with any bialaphos solution was not as rapid and severe as that of brown patch, and plant damage of untreated plants caused by the fungal infection was also less severe than that of *R. solani* in our testing system. Most untreated control plants showed small, circular, sunken white patches when covered with plastic bags and a few of them were able to recover from the damage caused by the infection of *S. homoeocarpa* when plastic bags were taken off two week after the data of disease rating had been collected.

The bialaphos application was more effective in protecting against the infection of *R. solani* than against the infection of *S. homoeocarpa*. There were more plant damages observed on transgenic bialaphos-resistant creeping bentgrasses due to the disease development of the dollar spot pathogen than those of the brown patch pathogen after the application of bialaphos. However, the disease development was significantly restrained and most plants were able to completely recover from the infection and grew normally.

Though the spraying of bialaphos, applied either three hours before or two days after the pathogen inoculation, was effective in the prevention of disease development as reflected by the results that the increases in the concentration of bialaphos did reduce the plant damage caused by either brown patch or dollar spot, the treatment means were not significantly different. The lowest applied rate of 200 mg/l of bialaphos was high enough to suppress the disease development of both fungal pathogens.

The results also showed that a single application of bialaphos could suppress the disease development of *Pythium* blight, though not as effectively as with brown patch and dollar spot (Figure 5). When 200 mg/l of bialaphos was applied three hours before the pathogen inoculation, it significantly restrained the infection of *Pythium aphanidermatum* and reduced the amount of plant damage one week after the initial inoculation. Better disease control was achieved when higher rate of bialaphos spraying was applied on transgenic plants. Higher concentration of bialaphos (at least 800 mg/l) was needed to significantly reduce the plant damage due to the infection of *Pythium* blight when bialaphos was applied two days after the inoculation. Bialaphos application on transgenic plants before the pathogen inoculation provided a little better protection against the infection by *Pythium aphanidermatum* ($F = 25.57 > F_{.05\,(1,\,153)} = 3.96$). However, no matter which application time and concentration of bialaphos were employed in this study, the infection of *Pythium* blight was severe and caused more plant damage than the other tested pathogens when disease symptoms were examined two weeks after the initial inoculation (data not shown).

**Table 7**. Effects of bialaphos application on the development of brown patch, dollar spot, and *Pythium* blight diseases in transgenic creeping bentgrasses

| | | Disease rating[⊗] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | concentration of bialaphos (mg/l) | | | | | | | | Untreated[*] | |
| Pathogen | Time of spraying[Δ] | 200 | 400 | 600 | 800 | 1200 | 1600 | 2000 | 2400 | Trans- | Nontrans- |
| *Rhizoctonia solani* | Three hours before | 0.4±0.2a[#] | 0.3±0.2a | 0.2±0.1a | -----[Ψ] | ----- | ----- | ----- | ----- | 7.8±0.4 | 8.2±0.4 |
| | Two days after | 0.5±0.2a | 0.5±0.2a | 0.2±0.1a | ----- | ----- | ----- | ----- | ----- | | |
| *Sclerotinia homoeocarpa* | Three hours before | 0.6±0.2a | 0.4±0.2a | 0.2±0.1a | 0.1±0.1a | ----- | ----- | ----- | ----- | 5.2±0.4 | 6.3±0.5 |
| | Two days after | 1.3±0.4a | 0.9±0.2a | 0.7±0.2a | 0.5±0.2a | ----- | ----- | ----- | ----- | | |
| *Pythium aphanidermatum* | Three hours before | 4.3±0.6bcd | 3.6±0.8bcd | 3.4±0.6bcd | 3.2±0.6bcd | 2.8±0.7bcd | 2.4±0.7cd | 2.2±0.6d | 2.1±0.5d | 8.3±0.3a | 9.0±0.2 |
| | Two days after | 5.8±0.5ab | 5.6±0.3abc | 5.1±0.3abcd | 4.8±0.4bcd | 4.4±0.6bcd | 3.8±0.9bcd | 3.4±0.7bcd | 3.1±0.6bcd | | |

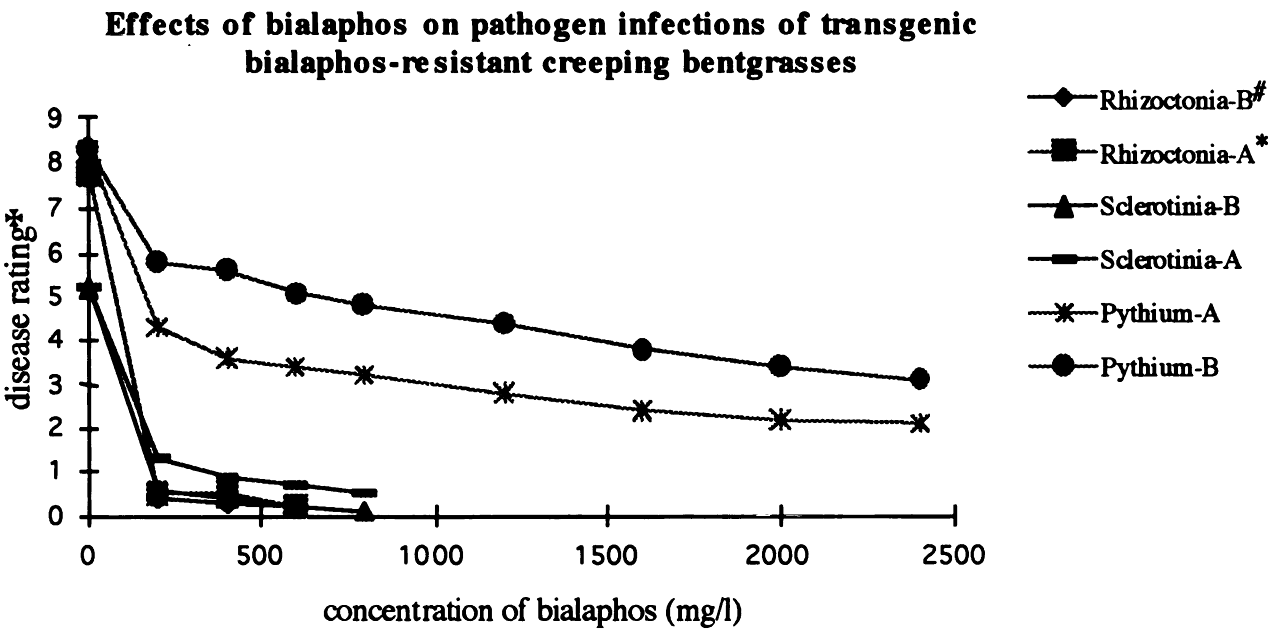
[⊗] Disease rating on a zero to ten scale with the smallest increment of 0.5 was recorded on the basis of the percentage of plant damage due to pathogen infection one week after the pathogen inoculation. 0: no damage; 5: 50 % plant damage; 10: dead.

[Δ] Various concentrations of bialaphos were applied either three hours before or two days after the pathogen inoculation

[*] Untreated indicated there was no bialaphos application either before or after the pathogen inoculation. Trans-: transgenic; Nontrans-: nontransgenic. When 200 mg/l bialaphos was sprayed on control plants (data not shown) which were not inoculated with any pathogen, nontransgenic plants were severely damaged after two weeks while transgenic plants were not affected by the application.

[#] Mean ± S.E. (standard error) for ten replications. Means of the same spraying time for each pathogen with the same letter were not significantly different according to the Tukey's test at P = 0.05. Data of untreated-nontransgenic plants were not included in the statistical analysis.

[Ψ] There was no bialaphos application at this concentration for the corresponding pathogen inoculation.



Effects of bialaphos on pathogen infections of transgenic bialaphos-resistant creeping bentgrasses

**Figure 5.** Effects of bialaphos application on fungal infections of transgenic bialaphos-resistant creeping bentgrasses.

*Disease rating was based on the percentage of plant damage caused by the corresponding pathogen infections one week after the pathogen inoculation.

#B indicates that the application of bialaphos was three hours before the pathogen inoculation.
*A indicates that the application of bialaphos was two days after the pathogen inoculation.

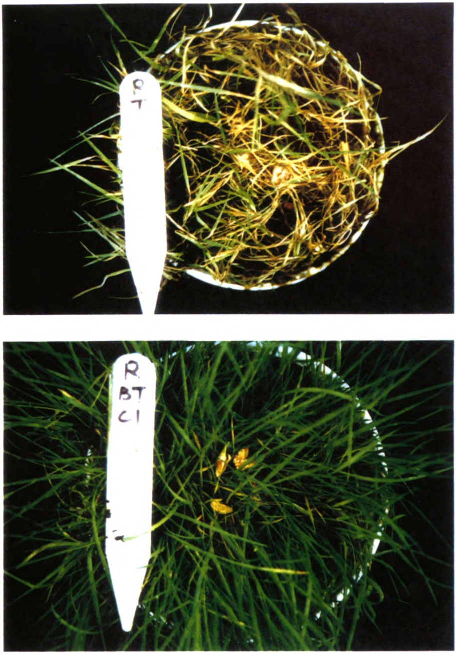


**Figure 6.** The application of bialaphos on the prevention of pathogen infection by *Rhizoctonia solani* in transgenic bialaphos-resistant creeping bentgrasses.
Top: transgenic plant which was not sprayed with bialaphos showed severely damaged by the infection of *R*. solani.
Bottom: transgenic plant which was sprayed with 200 mg/l of bialaphos three hours before the pathogen inoculation showed little damage by the infection of *R. solani*.

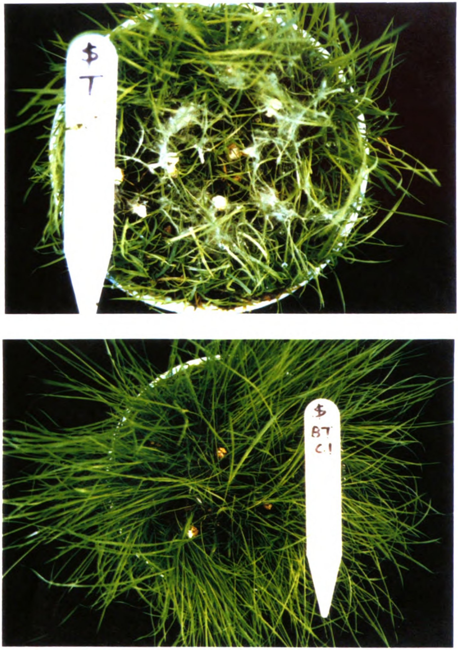


**Figure 7.** The application of bialaphos on the prevention of pathogen infection by *Sclerotinia homoeocarpa* in transgenic bialaphos-resistant creeping bentgrasses.
Top: transgenic plant which was not sprayed with bialaphos showed severely damaged by the infection of *S. homoeocarpa*.
Bottom: transgenic plant which was sprayed with 200 mg/l of bialaphos three hours before the pathogen inoculation showed almost no damage by the infection of *S. homoeocarpa*.

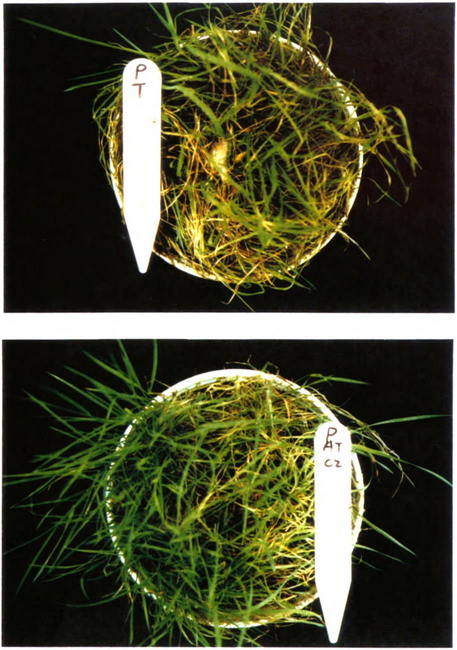


**Figure 8.** The application of bialaphos on the prevention of pathogen infection by *Pythium aphanidermatum* in transgenic bialaphos-resistant creeping bentgrasses.
Top: transgenic plant which was not sprayed with bialaphos showed severely damaged by the infection of *Pythium aphanidermatum*.
Bottom: transgenic plant which was sprayed with 400 mg/l of bialaphos one day after the pathogen inoculation showed some damage by the infection of *Pythium aphanidermatum*.

# Discussion

Bialaphos exhibited inhibitory activity *in vitro* against the growth of *R. solani*, *S. homoeocarpa*, and *Pythium aphanidermatum* that was superior to PPT, as reflected by their $ED_{50}$ values. Increasing concentrations of bialaphos and PPT were of greater effectiveness, with the highest rates resulting in the smallest colonies of mycelium. When working with mean values of $ED_{50}$ for *R. solani* and *S. homoeocarpa*, the $ED_{50}$ values for PPT were higher than those for bialaphos. It is surprising that the mycelial growth of *R. solani* was significantly inhibited at the concentration of one mg/l bialaphos (21 % reduction).

The same trend was also evident in the case of *Pythium aphanidermatum*, though the inhibition of the mycelial growth due to the inclusion of bialaphos in PDA medium was not as significant as with *R. solani* and *S. homoeocarpa.* There was no significant inhibition of the mycelium of *Pythium aphanidermatum* detected up to the highest concentration of supplement (600 mg/l) in our treatment regimes of PPT when radial length was used to measure the growth of mycelium. However, both bialaphos and PPT did show certain degree of inhibitory effect on the amount of mycelial growth of *Pythium aphanidermatum*. (Figure 3). The inhibition by bialaphos of growth of *Pythium aphanidermatum* was corroborated by the protection that the application of bialaphos on transgenic plants provided against the infection by *Pythium aphanidermatum* and by the reduction on the plant damage due to *Pythium* blight.

It is intriguing to observe that different fungal pathogens showed the same trend in differential *in vitro* responses toward bialaphos and PPT. Bialaphos is a tripeptide precursor of PPT, an analogue of glutamate, in which two alanine residues are linked to the PPT. While PPT is an inhibitor of glutamine synthetase in both plant and bacteria, the intact tripeptide has little or no inhibitory activity ***in vitro*** (Bayer *et al.*, 1972; Tachibana *et al.*, 1986a). In both bacteria and plants, intracellular peptidases remove the alanine residues and release active PPT (*L*-phosphinothricin).

Though the PPT used in this study was a mixture of *D*- and *L*-phosphinothricin (ammonium-*DL*-homoalanin-4-ylmethylphosphinat), in which the *D*-isomer is the inactive inhibitor of glutamine synthetase and the *L*-isomer is the active moiety of tripeptide bialaphos, it is still difficult to explain the significant differences shown in the magnitude of $ED_{50}$ values where the sensitivities of *R. solani* and *S. homoeocarpa* for bialaphos were higher than those for PPT. Though it has not been reported, we speculated, however, that the *D*-isomer, the inactive inhibitor of glutamine synthetase, might have interfered with the *L*-isomer, the active moiety of tripeptide bialaphos, in the binding of glutamine synthetase and reduced the inhibition efficiency of *L*-PPT.

It is even more surprising to know that the bialaphos provided a better selection efficiency in killing nontransgenic callus of creeping bentgrass than the PPT did (Chapter Two of the dissertation). Though it has been remarked that the inefficiency of PPT selection was due to the interference of glutamine or asparagine in the culture medium with herbicide activity (Wang *et al.*, 1992), it seems to us that it is more or less due to the nature of PPT. The composition of selection media used to establish the kill curves for bialaphos and PPT were identical except for the selective agent in our experiment. Compared to PPT, bialaphos was able to kill nontransgenic callus more effectively, at a lower concentration, and within a shorter period of selection. It would be interesting to see whether the same trend could be applicable to other plant materials and fungus species.

Certainly, the relative sensitivity to bialaphos and PPT was significant, but the basic difference in the values of $ED_{50}$ for the three different fungi was significant as well. In our *in vitro* test, the three pathogens also showed different responses to bialaphos and

PPT. *R. solani* was most sensitive to the presence of either bialaphos or PPT and *Pythium aphanidermatum* was the least. It has been noticed for some time that the application of one of several fungicides, such as Benomyl, Chlorothalonil, Cycloheximide+Thiram, PCNB, and Triadimefon, could provide an efficient control of brown patch and dollar spot diseases at the same time. However, they could not effectively control *Pythium* blight in most cases. Most the fungicides designated to control *Pythium* blight, such as Chloroneb, Ethazole, Metalaxyl, and Propamocarb, were not able to prevent infection by either *R. solani* or *S. homoeocarpa* (Beard, 1982).

The *in vitro* sensitivity data help explain some of the efficacy trends observed in the greenhouse study. Application of bialaphos on transgenic bialaphos-resistant creeping bentgrasses, even at the lowest rates, showed universal effects in suppressing disease development and reducing plant damage due to fungal infection. Bialaphos spraying was most significant in restraining the disease symptoms of *R. solani*. Both spraying times showed significant effectiveness in the suppression of the disease development of brown patch.

*S. homoeocarpa* was also significantly sensitive to the *in vivo* application of bialaphos; however, the application before the pathogen inoculation provided a better control of dollar spot. But even if bialaphos was applied two days after the pathogen inoculation when the pathogen had started to develop and spread, it still provided good plant protection and was able to significantly restrain the disease symptoms of *S. homoeocarpa*.

Though it was not as effective as in the cases of *R. solani* and *S. homoeocarpa*, bialaphos spraying still limited plant damage due to infection by *Pythium aphanidermatum*. The timing of bialaphos application was also important in suppressing the disease symptoms of *Pythium* blight. Better results in reducing plant damage were obtained when bialaphos was applied three hours before the pathogen inoculation.

Interactions between herbicides and plant pathogens have been well documented (Altman, 1985; Ben-Yephet *et al.*, 1991). The main cause of this phenomenon is that the biological activity of pesticides may extend beyond its effects on the target organisms. Upon treatment with herbicides, plant diseases caused by fungal pathogens have been reported to increase (El-Khadem *et al.*, 1979 ; Altman, 1981) or decrease (Grinstein *et al.*, 1979, 1984; Cohen *et al.*, 1986). More research needs to be done not only to assess the applicability of the antifungal activity of bialaphos toward other fungi, but also to investigate the mechanism of its inhibitory effect so that we might better understand the interactions between bialaphos and fungal pathogens and explain the differing reactions of the various fungi to the application of bialaphos.

Bialaphos has mainly been used as a broad-spectrum contact herbicide and as a selective agent in plant transformation experiments. However, it has been reported that it could be used as an effective selective agent to improve the transformation frequencies of *Cercospora kikuchii*, a fungal pathogen of soybean (Upchurch *et al.*, 1994). Their report and the results of our *in vitro* study, where the bialaphos showed significant inhibitory effects toward *R. solani* and *S. homoeocarpa*, suggest that bialaphos could be used as an efficient fungicide for a variety of fungal pathogens.

The application of 200 mg/l of bialaphos, which is about one tenth of the recommended concentration to kill untransformed turfgrass plants, was enough to significantly reduce plant damage due to the infection of both *R. solani* and *S. homoeocarpa*. The low rates at which bialaphos was effective present a novel and economical means for the control of some fungal pathogens. These facts coupled with the results presented in this paper which show that the application of bialaphos could prevent or suppress the infection by several fungal diseases indicate that it may, therefore, be possible to combat fungal infections and weed infestation simultaneously in fields of bialaphos-resistant creeping bentgrasses by a judicious choice of concentration, frequency, and time of application.

# BIBLIOGRAPHY

Akama, K., Puchta, H., and Hohn, B. 1995. Efficient *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the *bar* gene as selectable marker. Plant Cell Rep. 14: 450-454.

Altman, J. 1981. Effect of trifluralin on *Rhizoctonia* development in pinto beans. (Abstr). Phytopathol. 71: 199.

Altman, J. 1985. Impact of herbicides on plant diseases. In: Ecology and management of soilborne plant pathogens. Parker, C. A., Rovira, A. D., Moore, K. J., Wong, P. T. W. and Kollmorgen, J. F. (eds.) American Phytopathological Society, St. Paul, MN. pp: 227-231.

Bayer, E., Gugel, K. H., Haebele, K., Hagenmaier, H., Jessipow, S., Koenig, W. A., and Zaehner, H. 1972. Phosphinothricin und phosphinothricyl-alanin. Helv. Chim. Acta 55: 224-239.

Beard, J. B. 1982. Turf management for golf courses. Macmillan Publishing Company, New York. pp. 119-124.

Ben-Yephet, Y., Siti, E., and Frank, Z. R. 1983. Control of *Verticillium dahliae* by metamsodium in loessial soil and effect on potato tuber yields. Plant Dis. 67: 1223-1225.

Casas, A. M., Kononowicz, A. K., Zehr, U. B., Tomes, D. T., Axtell, J. D., Butler, L. G., Bressan, R. A., and Hasegawa, P. M. 1993. Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA 90: 11212-11216.

Christou, P., Ford, T. L., and Kofron, M. 1991. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9: 957-962.

Cohen, R., Riov, J., Lisker, N., and Katan, J. 1986. Involvement of ethylene in herbicide-induced resistance to *Fusarium oxysporum* f. ssp. *melonis*. Phytopathol. 76: 1281-1285.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, N. R., Thompson, C., Van Montagu, M., and Leemans, J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6: 2513-2518.

De Block, M., De Brouwer, D., and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. 91: 694-701.

El-Khadem, M., Zahran, M., and El-Kassaz, M. K. 1979. Effect of the herbicides trifluralin, dinitramine and fluometuron on Rhizoctonia disease in cotton. Plant Soil 51: 463-470.

Grinstein, A., Elad, Y., Katan, J., and Chet, I. 1979. Control of *Sclerotium rolfsii* by means of a herbicide and *Trichoderma harzianum*. Plant Dis. Rep. 63: 823-826.

Grinstein, A., Lisker, N., Katan, J., and Eshel, Y. 1984. Herbicide-induced resistance to plant wilt diseases. Physiol. Plant Pathol. 24: 347-356.

Joy, K. W. 1988. Ammonia, glutamine, and asparagine: a carbon-nitrogen interface. Can. J. Bot. 66: 2103-2109.

Kondo, Y., Shomura, T., Ogawa, Y., Tsuruoka, T., Watanabe, H., Totsukawa, K., Suzuki, T., Moriya, C., and Yoshida, J. 1973. Isolation and physico-chemical and biological characterization of SF-1293 substance. Sci. Reports of Meiji Seika Kaisha 13: 34-41; Chem. Abstr. 1974, 81, 89705b.

Leason, M., Dunliffe, D., Parkin, D., Lea, P. J., and Miflin, B. J. 1982. Inhibition of pea leaf glutamine synthetase by methionine sulphoximine, phosphinothricin and other glutamate analogues. Phytochem. 21: 855-857.

Miflin, B. J., and Lea, P. J. 1977. Amino acid metabolism. Ann. Rev. Plant Physiol. 28: 299-329.

Murakami, T., Anzai, H., Imai, S., Satoh, A., Nagaoka, K., and Thompson, C. J. 1986. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205: 42-50.

Ogawa, Y., Tsuruoka, T., Inouye, S., and Niida, T. 1973. Chemical structure of antibiotic SF-1293. Sci. Reports of Meiji Seika Kaisha 13: 42-48; Chem. Abstr. 1974, 81, 37806r.

Smiley, R. W. 1983. Compendium of turfgrass diseases. The American Phytopathological Society, St. Paul, MN. pp. 11-72.

Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F., and Bushnell, W. R. 1992. Fertile, transgenic oat plants. Bio/Technology 10: 1589-1594.

Spencer, T. M., Gordon-Kamm, W. J., Daines, R. J., Start, W. G., and Lemaux, P. G. 1990. Bialaphos selection of stable transformants from maize cell culture. Theor. Appl. Genet. 79: 625-631.

Tachibana, K., Watanabe, T., Sekizawa, Y., and Takematsu, T. 1986a. Inhibition of glutamine synthetase and quantitative changes of free amino acids in shoots of bialaphos treated Japanese barnyard millet. J. Pest. Sci. 11: 27-31.

Tachibana, K., Watanabe, T., Sekizawa, Y., and Takematsu, T. 1986b. Action mechanism of bialaphos. II. Accumulation of ammonia in plants treated with bialaphos. J. Pest. Sci. 11: 33-37.

Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M., and Botterman, J. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. 6: 2519-2523.

Uchimiya, H., Iwata, M., Nojiri, C., Samarajeewa, P. K., Takamatsu, S., Ooba, S., Anzai, H., Christensen, A. H., Quail, P. H., and Toki, S. 1993. Bialaphos treatment of transgenic rice plants expressing a *bar* gene prevents infection by the sheath blight pathogen (*Rhizoctonia solani*). Bio/Technology 11: 835-836.

Upchurch, R. G., Meade, M. J., Hightower, R. C., Thomas, R. S., and Callahan, T. M. 1994. Transformation of the fungal soybean pathogen *Cercospora kikuchii* with the selectable marker *bar*. Appl. Environ. Microbiol. 60: 4592-4595.

Wan, Y. and Lemaux, P. G. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104: 37-48.

Wang, Z., Takamizo, T., Iglesias, V. A., Osusky, M., Nagel, J., Potrykus, I., and Spangenberg, G. 1992. Transgenic plants of tall fescue (*Festuca arundinacea* Schreb.) obtained by direct gene transfer to protoplasts. Bio/Technology 10: 691-696.