"TAXI", A NEW VEHICLE FOR THE TRANSFER OF GENES INTO MONOCOTYLEDONOUS PLANTS

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CERTIFICATION OF SUPERVISOR

In terms of paragraph GP 8 of "General Rules for the degree of Doctor of Philosophy (PhD)" I, supervisor of the candidate Wusi Chen, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

Signed by candidate

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Contents

Abstract

The transfer of foreign genes into cereals followed by their correct expression in a tissue-specific, developmentally regulated manner has become an important research focus. Based on the mechanism of *Agrobacterium tumefaciens* mediated transformation in dicots, a modified method to transform the monocotyledonous rye (*Secale cereale L.*) via *A.tumefaciens* mediated transformation was attempted. The induced bacterium culture was injected into rye seedlings, the transferred reporter gene, *uidA*, was detected by PCR, and the expression of the gene was tested by histochemical assays. However, successful transformation and integration of the transgenes remained doubtful, because the frequency of kanamycin resistance in the progenies (R1 , R2 and R3) did not increase.

To achieve a real transformation and heritable transgenic rye, a new vehicle for gene transfer to plants was developed. A macromolecular complex, termed the TAXI, consisted of histone H1-protected single stranded DNA, containing a selectable marker gene (*npt II*), linked either to a reporter gene (*uidA*) or a glutenin gene. The constructs were transferred by injection of rye seedlings. Molecular analyses demonstrated that all three genes were integrated and expressed in transformed rye and their progenies (R1 and R2).

TAXI mediated gene transfer to rye revealed an important advantage in that single or low numbers of transgenes were inserted into the transformed plant genome. However, the method of TAXI delivery to plants was not efficient. To improve this, a new approach, combining TAXI transformation and the biolistic process, was developed. A rapid regenerable callus line of a grass species, *Digitaria sanguinalis*, was established as a test system. TAXI coated gold particles, carrying a selectable marker gene (*bar*) and a reporter

gene (*uidA*), were used in bombardment experiments. The results of herbicide resistance and molecular analyses demonstrated that single copies or low numbers of the *bar* gene were inserted and expressed in regenerated transformed *D. sanguinalis*. Mendelian segregation in the R1 population was observed in four out of five transgenic lines.

Chapter 1

General Introduction and Literature Review
The methodology of gene transfer into cereals

1.1 Introduction

The improvement of agriculturally important crops is the aim of plant breeding scientists. Until 1982 this improvement relied on sexual recombination of natural or induced mutations followed by selection. The first genetically engineered plants which expressed foreign genes were produced in 1984 using recombinant DNA techniques (Horsch et al., 1984, Hain et al., 1985, Paszkowski et al., 1984). Since then transgenic tobacco plants have been produced using a natural vector, *A.tumefaciens* for the transfer of genes. Transgenic plants of many different species have been produced either by *Agrobacterium* mediated transformation or by one of various chemical and physical methods to deliver DNA directly into plant cells.

The great deal of effort has been focused on the improvement of cereals by genetic engineering because cereals provide more than half of all the world's food. However, they, like most monocotyledonous species, have generally proven to be insensitive to *Agrobacterium* mediated transformation. A recent report of the effective transformation of rice using *A.tumefaciens* to infect scutellar-derived cells suggests that this approach may also be applied to other monocots (Hiei et al., 1994).

In contrast, methodologies of direct gene transfer into protoplasts or callus by-pass the host limitation problem. Microprojectile bombardment is considered the most promising technique for the production of transgenic cereals (for a review see Vasil, 1994). Transgenic cereals, including the agriculturally important species, like wheat, rice and maize, have been produced using the biolistic technique. However, a major problem with direct gene transfer techniques is that frequently multiple copies of transgenes are inserted into the plant genome, implicated in transgene silencing phenomena (Finnegan and McElroy, 1994). In addition, the development of this technology for widespread practical use depends on the development of tissue culture conditions for the production of embryogenic material. Thus there is still a lot of scope for improvement of biolistic gene transfer.

Nevertheless, the transfer of foreign genes into plants by either *Agrobacterium* mediated transformation or direct gene transfer methods has provided a way to improve the quality of plant products, produce disease and insect resistant plants and enabled the use of transgenic plants as bioreactors for the production of biomolecules. In addition, the development of gene transfer methodologies and the study of transient and stable transformation has given considerable insight into basic plant molecular biology, for example, the analysis of gene structure and function, understanding and controlling transgene expression, and understanding and controlling plant development.

In this chapter, I will review various transformation strategies employed in cereal transformation, focussing on two important gene transfer systems, *A.tumefaciens* mediated transformation and microprojectile bombardment.

1.2 A natural gene transfer system, *Agrobacterium* mediated transformation

A.tumefaciens is a plant pathogenic soil bacterium. In nature, when it interacts with plant cells at a wound site, a particular DNA segment from the tumor inducing plasmid (Ti plasmid) is transferred and integrated into the plant genome. The piece of DNA transferred (the T-DNA) contains genes coding for proteins involved in the biosythesis of plant growth factors (the oncogenes), and for bacterial nutrients (the opine sythesis genes) under the control of eukaryotic transcriptional signals. The neoplastic growth of the infected plants thereby creates an ecological niche for the parasitic *Agrobacterium*. The expression of genes carried by this transferred DNA results in a disease called crown gall which can affect many dicot plants (Zambryski, 1988, 1992).

Because the T-DNA of *A.tumefaciens* is the only non-viral nucleic acid known to genetically transform a higher eukaryotic cell in nature, plant molecular biologists have used this natural gene transfer system extensively for plant genome manipulation. A better understanding of the basic principles involved in this transformation process should allow us to find the key to effective transfer and integration of T-DNA, and to identify where the obstacles are which block this transformation process in cereals. This understanding would be helpful in order to modify the process *in vitro* or to construct an artificial T-DNA complex by imitating some of the molecular mechanisms underlying *A.tumefaciens* mediated transformation, in order to achieve the transfer of foreign genes into cereals.

1.2.1 The process of *A.tumefaciens* mediated transformation

A.tumefaciens carries three genetic components required for plant cell transformation : T-DNA and the virulence (*vir*) region, which are located on the Ti plasmid, and chromosomal virulence loci. The T-DNA region is the only DNA fragment to be transferred from *A.tumefaciens* to the plant cell. A set of 25 base pair direct repeat "integration sequences" border the T-DNA region. The *vir* region includes seven operons, *virA* to *virG*, which provide most of the *trans*-acting functions for T-DNA transit. The chromosomal *chv* loci encode products involved in the binding of *A.tumefaciens* to the plant cell during the infection process (Zambryski, 1988, 1992). A brief summary of the transformation process is given below.

(1) Wound signal recognition and transduction

In nature, *A.tumefaciens* only infects wounded plants. This is not only because these plant cells present less of a physical barrier to penetration, but also they also release some chemical compounds which are specific signals for the wound response of *A.tumefaciens*. *Vir* operons of the Ti plasmid are inducible by some of the phenolic compounds and sugars present in plant wound exudate (Stachel et al., 1985, Rogowsky et al., 1987, Alt-Moerbe et al., 1989, Cangilosi et al., 1990). The most prominent inducers are acetosyringone (AS) and hydroxy-acetosyringone (OH-AS).

The *vir* region comprises 35 kb outside the T-DNA. It is organized into seven complementation groups (Figure 1.1). *vir*A, *vir*B, *vir*D, and *vir*G genes are absolutely essential for plant transformation, while the VirC and VirE proteins enhance its efficiency. VirA and VirG activate the expression of the other *vir* loci (Winans, 1992). VirA possesses a transmembrane domain and is localized at the internal membrane of *A.tumefaciens* (Leroux et al., 1987). It directly senses the presence of phenolic inducers in

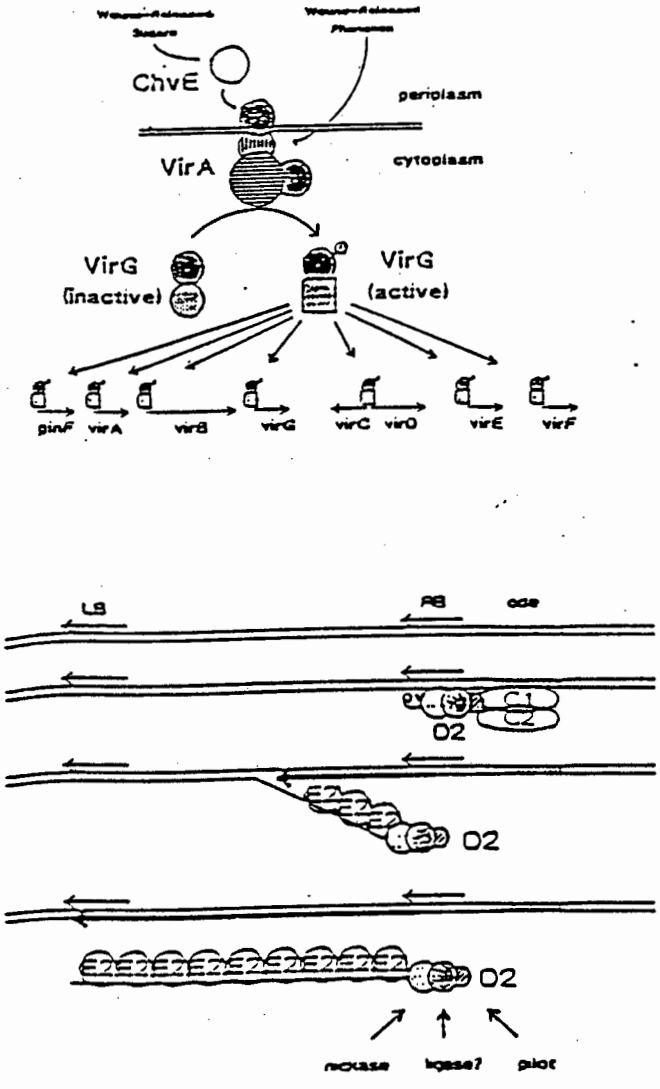


Figure 1.1 Proposed Mechanism of *vir* Gene Induction and T-DNA Formation

Top: The proposed function of the regulatory proteins VirA and VirG. VirA is a transmembrane protein kinase that may directly bind the phenolic inducer. VirG is a target of the VirA kinase and binds to *vir* promoters to activate their transcription.

Bottom: Proposed mechanism of synthesis of single stranded T-DNA. RB: right border; LB: left border; ode: overdrive; C1,C2,D1 and D2: the products of the *vir*C1, *vir*C2, *vir*D1, and *vir*D2 genes. Thick arrows indicate newly synthesized DNA which could displace the bottom strand of T-DNA.

Reprinted from Winans (1992)

the external environment. A periplasmic sugar-binding protein ChvE may be required by VirA to respond to low levels of acetosyringone (Doty et. al., 1996). VirA activates by phosphorylation the VirG protein which is located in the bacterial cytosol. The phosphorylated VirG protein acts as a positive activator of the transcription of all other *vir* genes (Zambryski, 1992, Scheeren-Groot et al. 1994).

(2) T-DNA complex formation

Once the VirA and VirG proteins have transduced the wound signal to induce the *vir* genes, a series of responses is elicited for the generation and transfer of T-strand. The products of the *vir*D operon (VirD1 and VirD2) have an endonuclease activity capable of nicking, by the introduction of ss-breaks the border repeats at a precise site. The nicked sites act as starting points for DNA synthesis in the 5' to 3' direction. The T-strand, which corresponds to the T-DNA bottom strand, will then be released by displacement (Hooykaas et al., 1992). The T-strand retains the VirD2 protein covalently attached to the 5' terminus, and may later function in T-strand transfer and integration (Herrera-Estrella et al., 1988, Ward et al., 1988, Tinland et al., 1995). The 69-kDa single stranded DNA binding proteins, the product of the *vir*E gene, coat the T-strand by cooperative binding, leading to a long and thin nucleoprotein filament. This nucleoprotein structure protects the T-DNA from nuclease attack during the transfer process (Citovsky et al., 1988).

(3) Transfer of the T-DNA complex from *Agrobacterium* to the plant cell

This is a complex process which is not yet fully understood. Research findings suggest that strong similarities exist between the T-DNA transfer process and the bacterial conjugation system (Zambryski, 1988, 1989, 1992., Tinland et al., 1994). In the latter the sequence of the origin of transfer (*oriT*) is strongly homologous to part of the T-DNA border sequence (Waters et al., 1991, Ziegelin et al., 1991). In both systems the transferred DNA appears to be a single stranded molecule and a molecule of a nicking enzyme is covalently attached via a tyrosine residue to the 5' end of the nicked region of transfer. These two proteins, TraI

in the bacterial system and VirD2 in the *vir* system, contain highly homologous sequences in restricted areas and both have mobilization functions in the transfer process (Vogel and Das, 1992). A single stranded DNA binding protein has been implicated in both systems as well (Willetts et al., 1987). VirE2, a ssT-DNA binding protein, plays an important role in T-DNA transfer (Winans, 1992, Zambryski, 1992). The VirE1 protein was found to mediate export of VirE2 from A.tumefaciens into plant cells (Sundberg et al., 1996). Gene products of the *vir*B operon are absolutely essential for T-DNA transfer and, according to the DNA sequence might determine a physical structure (a pore or pilus) through the bacterial membrane, making transfer possible (Christie et al., 1989). It has been reported (Thorstenson et al., 1993) that most of the VirB proteins are located on both the inner and outer bacterial membranes. This suggests that they may form complex pore structures which act as T-DNA export channels. Its gene has clear sequence homology with the *com*G gene of *Bacillus subtilis*, which is involved in ssDNA uptake by competent cells (Albano et al., 1989). Hooykaas et al. (1992) replaced the *trans*-system with the *vir* system and thereby successfully transferred an *incQ* plasmid into recipient *A.tumefaciens* and *E.coli* cells. These results suggest that the T-DNA transfer mechanism may have similarities to the model of DNA transfer during bacterial conjugation.

(4) Nuclear targeting and precise integration of T-DNA in the plant cell

It has been noted that the VirD2 protein may also be responsible for the nuclear targeting of T-DNA in the plant cell and precise integration of T-DNA into the plant genome. Two nuclear localization signals (NLS) are present in both the N- and C-terminal parts of the VirD2 protein (Rossi et al., 1993). It has been reported that VirE also may assist nuclear uptake (Citovsky et al., 1992). Furthermore a recent report shows that VirD2 has an important role in preserving the 5' end of the T-strand during integration. Through an illegitimate recombination process (Gheysen et al., 1991, Mayerhofer et al., 1991), where it may participate in ligation of the 5' end of the T-strand to plant DNA (Tinland et al.,

1995). These findings suggest that a specific functional protein is required for the T-DNA to enter the cell nucleus and be integrated into the plant genome.

1.2.2 The achievement of *Agrobacterium* mediated transformation

The greatest advantage of this system is that it offers the potential to generate transgenic cells at relatively high frequencies without a significant reduction in plant regeneration rates. Moreover, *Agrobacterium* mediated transformation has the following characteristics:

Firstly, the integrated T-DNA is most frequently intact probably due to VirE protection. In contrast the DNA delivered by direct gene transfer methods is naked and easily broken and degraded by nucleases digestion before it integrates into the plant genome. If cyclic plasmids are used, they may be randomly linearized before being inserted into the plant DNA. Therefore, partial integration of transgenes is often observed in transgenic plants produced via direct gene transfer methods.

Secondly, integrated transgenes are normally stable. The transfer and integration of T-DNA in the *Agrobacterium* system is controlled by Vir proteins, the genes of which are outside the T-border sequences. Thus the integration of the T-DNA is, in principle, stable because the *vir* genes, which provide the initial mobility of the T-DNA, do not become integrated into the plant genome.

Thirdly, single or low copies of transgenes are observed in transgenic plants produced via *A.tumefaciens* mediated transformation. Sequencing of the insertion sites of transgenes shows that T-DNA integrated into the plant genome is defined and does not normally undergo any major structural rearrangements (Gheysen et al., 1987). It is also thought that single or low copy number insertion would reduce the homologous inactivation of transgenes expression. In contrast, multiple copies and tandem repeats of inserted foreign

genes have been observed as a common phenomenon in transgenic plants produced by direct gene transfer methods (Finnegan et al., 1994). Such multiple copies and tandem repeat insertions are implicated in transgene silencing phenomena (for reviews see Flavell, 1994, Matzke et al., 1995).

Unfortunately, host limitation appears to be the great weakness of *Agrobacterium* transformation. Research is being carried out to try to explain and/or overcome this difficulty (Cocking et al., 1987, Potrykus, 1990, Hess et al., 1991, Chan et al., 1993, Hiei et al., 1994, Vasil, 1994).

1.2.3 The obstacles to *Agrobacterium* mediated cereal transformation

Which are the obstacles which prevent this transformation process in cereals and can ways be found to modify them? *A.tumefaciens* transformation may be artificially divided into four phases (see section 1.2.1). It is unlikely that the obstacles occur during step 2 or 4. Once the *vir* genes have been induced and expressed in *A.tumefaciens*, the generation of the T-strand, being an intra-bacterial process, should pose no problem (step 2). The same applies to the plant cells to which T-DNA has been transferred, as there is no evidence that the integration process (step 4) is different between monocots and dicots. Once *A.tumefaciens* had been induced and T-DNA had been transferred into rice callus cells which are unwounded and have the ability to divide, a similar transformation efficiency to that of dicots has been obtained (Hiei et al., 1994). This result suggests that the obstacle may rather be in the interaction between *A.tumefaciens* and the plant cell (step 1 and step 3), and may be due to the fact that monocots exhibit no cell proliferation at the wound site, a feature typical of dicots which is required for the formation of transformed cells.

In step 1, the type and quantity of signal molecules released from plant wound sites would influence the initiation of the expression of *vir* genes in *A.tumefaciens*, which is necessary for T-DNA formation. The production of phenolic molecules is one of the crucial events in the wound response of dicot plant cells. These phenolic molecules have been clearly defined as *vir* gene inducers (Stachel et al., 1985). Most cereals are thought not to release these phenolic compounds and they produce *vir* gene inducers either at much lower levels (maize and rice) (Messens et al., 1990), or only in certain tissues (wheat and oats) (Usami et al., 1988).

In step 1 and 3, a specific recognition between *A.tumefaciens* and the plant cell is required to induce the bacterium to attach itself to the surface of plant cells. Without the cell-cell contact, signal transduction and T-DNA transfer is unlikely to occur. The bacterial response system in *A.tumefaciens* limits the host range of the bacterial infection (Yanofsky et al., 1985), because on recognition of the signal compound, the specific attachment of the bacterium to the plant cell requires the activity of a variety of bacterial genes (Thomashow et al., 1987) and the presence of appropriate plant cell wall components which are necessary for the specific recognition (Lippincott et al., 1969). Monocots are thought to lack these cell wall components (Lippincott et al., 1969, 1977).

Though the lack of efficient wound signal molecules and also the specific recognition may block the transforming process, this does not mean that the interaction between *Agrobacterium* and the cereal cells is totally lost. Agroinfection experiments of cereals demonstrate the presence of minute amounts of transferred T-DNA in recipient plants by the viral amplification of a rare event (Grimsley, 1987, 1990). This important discovery indicated for the first time that the transfer of T-DNA from *A.tumefaciens* to cereal cells is possible even without additional inducers. However, agroinfection does not lead to integration of T-DNA.

Another impediment to the transformation may result from the lack of wound proliferation in cereals. This proliferation occurs in other plants as a result of injury. Braun et al. (1952) presented evidence that a window of competence exists at the wound site and is related to the timing of wound cell divisions at this site. Transformation probably occurs only during this time. Moreover, the integration of the T-DNA into the plant genome requires plant DNA synthesis (Gheysen et al., 1987). Thus the ability of wounded plant cells to enter and carry out one or more cell cycles may be absolutely required for successful transformation. Proliferation occurs at the wound site in susceptible dicots in response to wounding (Potrykus, 1990). However, in cereals, this part of the wound response is quite different. Cereal cells around the wound site lignify or sclerify in the absence of apparent cell division (Kahl, 1982). Because of the absence of proliferation, either the minute amount of transferred T-DNA in wound adjacent cells may meet difficulties in the integration into the plant genome or the integrated T-DNA may not be transmitted into progeny cells.

1.2.4 The development of strategies for the use of *A.tumefaciens* transformation system in cereals

Transgenic rice, successfully produced via *A.tumefaciens* mediated transformation (Chan et al., 1993, Hiei et al., 1994) raises the hope again for the genetic engineering of cereals using this natural gene transfer system. The development of strategies to modify this natural system may take the following into account.

Firstly, the lack of efficient signal molecules in wounds of cereals can be remedied by using additional natural wound release compounds of dicots and / or artificial phenolic compounds to induce cultures of *A.tumefaciens*. These inducers initiate the expression of *vir* genes and result in the generation of T-DNA molecules in *A.tumefaciens*, before the bacteria are used to infect cereal cells.

Secondly, modification of attachment function may improve the transfer of T-DNA from *A.tumefaciens* into cereal cells. However, this will be very difficult because the lack of specific recognition between *A.tumefaciens* and cereal cells is determined by their genetic make up. The selection of a suitable *A.tumefaciens* strain following mutation, the use of ligands and the combination of *A.tumefaciens* transformation facilitated by electroporation during infection may improve the interaction of *A.tumefaciens* and cereal cells.

Thirdly, the use of unwounded regenerable meristem calli, e.g. from scutellum as demonstrated by Hiei et al. (1994), as targeting material may avoid lignification and allow the integrated T-DNA to be transmitted to progeny cells through cell division.

An alternative way consists in directly infecting the germline cells *in vivo* (Hess et al., 1991). Although a series of problems with this method need to be overcome, e.g. the difficulty of routinely timing the infection, the inefficient attachment of *A.tumefaciens* to the archesporial cells in the anther, and the low transformation frequency. The further development of this technology may well prove to be worthwhile because it has the advantage of not necessitating tissue culture and will lead to a direct transfer of T-DNA into germline cells.

1.3 Methods of direct gene transfer

Theoretically, direct gene transfer techniques may be used in any plant species. A number of techniques have, therefore, been developed, e.g. PEG induced gene transfer, electroporation, liposome or lipopolyamine mediated gene transfer, microinjection, pollen tube pathway, microlaser method, silicon carbide fiber method and microprojectile bombardment (for reviews see Potrykus, 1990, Vasil, 1994, Walden and Wingender, 1995).

Using these methods, the number of transformed species has greatly increased in the past few years, including some that were otherwise impossible or very difficult to transform. DNA has been directly delivered into protoplasts, cell suspensions, calli, immature embryos, mature embryo parts, seedling meristems, microspores and pollen. These cells or tissues are chosen as the targeting materials for the transfer of foreign genes because they have the capability of regenerating into mature plants, or they are able to differentiate and develop into germ line cells. Transgenic plants covering all the major important cereal species have been produced using various direct gene transfer techniques. Of all the methods, PEG, electroporation and microprojectile bombardment mediated gene transfer have proven the most successful.

1.3.1 PEG and electroporation mediated gene transfer

DNA can be delivered into protoplasts in the presence of polyethylene glycerol (PEG). The first transformed cells (Petunia) were produced by PEG induced direct gene transfer in 1980 (Davey et al., 1980). PEG causes an osmotic swelling in protoplasts as a result of dehydratation of the membranous system by competing for free water and changing the dielectric properties of water. Thus it facilitates the "solvating" of lipid molecules and leads to the creation of bilayer defects (Boni et al., 1987). Thus DNA or macromolecular compounds are allowed to get into cells through a defective membrane.

Almost at the same time the first biological use of electroporation, the formation of holes or pores in the cell membrane by high-voltage electric shock, was used to induce cells to fuse via their plasma membranes (Zimmerman et al., 1976). It was then found that the electropore could be used to introduce macromolecules into cells (Neumann et al., 1982, Potter et al., 1984, Evans et al., 1984). Since then, the technique has become the method for gene transfer in many situations. Electroporation makes use of the fact that the cell

membrane is an electrical capacitor that is unable to pass current (except through ion channels). Subjecting membranes to a high-voltage electric field results in their temporary breakdown and the formation of pores that are large enough to allow macromolecules to enter or leave the cell. This physical breakdown of membranes is associated with an increase in membrane conductivity and permeability (Zimmermann, 1982). DNA fragments or plasmid vectors are then able to enter the cell. Because these effects are reversible, the original membrane resistance and impermeability are restored after a short time.

Using these techniques of direct DNA delivery into protoplasts, transgenic maize (Rhodes et al., 1988, Golovkin et al., 1993, Omirulleh et al., 1993, Sukhapinda et al., 1993) and rice (Shimamoto et al., 1989, Datta et al., 1990, 1992, Hayakawa et al., 1992, Uchimiya et al., 1993, Fujimoto et al., 1993) have been produced in several laboratories. Protoplasts are chosen as the target, because they have several advantages over other acceptor cells :

(1) The absence of a cell wall exposes the membrane and facilitates the entry of DNA.
(2) The enzymatic isolation procedure may trigger a response similar to that caused by wounds which stimulates the cell into the competent state. This state is necessary for transformation and regeneration (Potrykus et al., 1985).
(3) The foreign genes reach many cells, thus increasing the chance for recovery of transgenic plants.
(4) Cereal protoplasts prepared from embryogenic cell suspensions reveal totipotency (Vasil et al., 1990, 1992).

Plants have been regenerated from embryogenic protoplasts of all major species of cereals (Vasil and Vasil, 1992), however, the difficulties in the establishment and maintenance of embryogenic cell suspension cultures of cereals as the source of totipotent protoplasts and the inefficiency in regeneration of plants from transformed protoplasts limits the wide application of these techniques. A new procedure of electroporation has therefore been

developed to deliver DNA into intact cultured cells (D'Halluin et al., 1992), immature embryos (Songstad et al., 1993), bisected mature embryos (Xu and Li, 1994) and young inflorescences (Barcelo et al., 1994). These approaches may improve the electroporation technique for cereal genetic engineering. At the present time, however, the technique has limited use.

1.3.2 Microprojectile bombardment

The biolistic technique for the transfer of genes into plants was introduced in the late 1980's (Sanford, 1987, Klein et al., 1987, Christou, 1988). This technique uses high velocity small metal particles, microprojectiles, covered with DNA to deliver the desired gene to target cells via a suitable particle gun (Sanford et al., 1987, Klein et al., 1992). Compared with other transformation techniques, the biolistic process has a number of fundamental advantages :

(1) The technique appears to be effective regardless of species and tissue or cell types. There is no apparent difference in the efficiency of biolistic transformation between monocots and dicots. The desired genes can be delivered into intact cells and explants, such as cell suspensions, embryogenic callus, immature embryos, meristem tissue, leaf pieces, microspores and young inflorescence tissues.

(2) The biolistic process first made possible the transformation of organelle genomes, i.e. chloroplasts (Svab et al., 1990, Ye et at., 1990) and mitochondria (Johnston et al., 1988, Fox et al., 1988).

(3) Microprojectiles are small enough to enter a cell or explant in a non-lethal manner. Thus recovery from injury and survival of transformed cells or explants is possible.

(4) Plants can be regenerated from these transformed cells and explants either directly or with a short callus phase.

(5) One shot yields many hits, thus improving the yield of transformants.

These achievements have greatly improved the genetic engineering of cereals. Fertile transgenic plants encompassing all the major cereal species have been produced via biolistics in several laboratories: transgenic rice (Christou et al., 1991, Li et al., 1993), maize (Fromm et al., 1990, Gordon-Kamm et al., 1990, Koziel et al., 1993, Murry et al., 1993), rye (Castillo et al., 1994), barley (Wan et al., 1994) and wheat (Vasil et al., 1992, 1993, Weeks et al., 1993, Becker et al., 1994, Nehra et al., 1994). These successes all demonstrate that biolistics appears to be the most efficient and promising approach for the transfer of genes to plants.

On the other hand, however, this technique is still in the development stage and some aspects require improvement. The efficiency of transformation is not as high as in electroporation or *A.tumefaciens* mediated transformation in dicots (Binns, 1990). This may be influenced by several factors: the process of coating DNA onto particles has not yet been optimized; particle aggregation often occurs during DNA coating; particle delivery, to reach deep cell layers where the meristemic cells are located may be difficult (Protrykus, 1990). Furthermore, the biolistic process as such has no direct effect on the chromosomal integration mechanism as the *A.tumefaciens* system apparently does. In addition, the phenomenon of transgene silencing associated with direct gene transfer has been noted as a new problem in plant genetic engineering (Finnegan et al., 1994).

1.3.3 Strategies for the improvement of direct gene transfer techniques

Reviewing plant transformation experiments, advances in two areas are considered to contribute to their success: the efficiency of DNA delivery techniques and the culture conditions required for producing highly embryogenic material. Therefore strategies for the improvement of cereal genetic engineering may need to focus on these two aspects.

(1) The improvement of the efficiency of DNA delivery techniques

In almost all direct gene transfer processes, naked plasmids are used as vectors of the transgenes. Comparison with the T-DNA complex transferred to plant cells in *Agrobacterium* mediated transformation reveals several disadvantages of using naked plasmid DNA.

a) The naked plasmid can be easily degraded by nucleases. In contrast, T-DNA is protected by VirE proteins.

b) Before integration, random linearization of plasmids may result in breakage of transgenes. In contrast, a precisely linearized T-DNA fragment carries intact transgenes.

c) Integration of double stranded DNA is considered to be less successful than that of single stranded DNA (Rondenburg et al., 1989).

d) Naked plasmids have no nuclear targeting function as the T-DNA complex does.

e) Multiple copy insertions and tandem repeats of transgenes in the plant genome often occur when using direct gene transfer methods, which is not frequently the case in *Agrobacterium* mediated transformation.

Considering these disadvantages, strategies for improving the transformation efficiency in direct gene transfer systems should aim to modify the form of transferred DNA, e.g. by imitating the T-DNA complex and reducing the copy number of transgenes in the transformed plant cells.

(2) Optimizing the culture conditions for the successful regeneration of transformed cells

A regenerable cell or callus line is the essential prerequisite for direct gene transfer methods in the genetic engineering of cereals. This dependence constitutes a problem because many plants are difficult to regenerate from single cells. Various factors, including

tissue physiology, development and specific culture requirements, have made these plants notoriously difficult to handle in culture for highly embryogenic materials. In addition, somaclonal variation presents problems with the routine recovery of a new species of transgenic cereal. Methods which do not rely so heavily on tissue culture manipulations must be developed. There are no routine ways which can be used to overcome these difficulties and an investigation has to be done in every specific cases, which makes this a time consuming and painstaking procedure.

1.3 The aim of this investigation

This project is aimed at exploring alternative methodologies for the transfer of genes into cereals, towards improving the transformation efficiency and reducing homologous inactivation of transgenes in transgenic plants.

The investigation was in three stages :

(1) An attempt to transform a cereal plant via *Agrobacterium* mediated transformation.

a) Is it possible to achieve gene transfer by inducing the expression of *vir* genes through the artificial addition of inducers ?

b) Which types of tissues and which developmental stages of the cereal plants are optimal for the transfer and integration of foreign genes ?

In the investigation presented here, successful transformation via *A.tumefaciens*, induced *in vitro* prior to injection into plants, appeared to be doubtful. The possible reasons for this were not investigated further.

(2) The establishment of an artificial T-DNA transfer system imitating *Agrobacterium* transformation

The great advantage of *Agrobacterium* mediated transformation is its efficiency. However, host specificity is a problem. In the second stage of this study this was investigated using an artificial T-DNA macromolecular complex *in vitro*. A linear single stranded form of DNA was adopted and a nuclear targeting protein, histone H1, was used to protect the ssDNA and facilitate its entry into the cell nucleus. These measures may increase the chance of T-DNA entering the targeting plant cell nucleus and integrating into plant genome. This artificial macromolecular complex is termed the TAXI.

Rye was chosen as a candidate to produce a transgenic cereal. because it is the most adaptable species of all cereal crops. It can be grown on poor soils and in cold temperatures and it tolerates soil acidity and alkalinity. Aside from the selectable marker gene (*nptII*), the GUS gene and a wheat glutenin gene were chosen as candidates for the transfer and integration. High molecular weight (HMW) glutenin subunits are thought to provide the elasticity of the dough prepared from wheat flour and thus determine bread-baking quality (Halford et al.,1987). Therefore, the transfer and expression of a wheat glutenin gene in rye may improve the quality of rye flour. An additional reason was that a glutenin cDNA library had been made in our laboratory, partially sequenced at both 5' and 3' ends, and mapped in the central repeat region. These results supplied a number of clues that would allow the identification of the expression of a glutenin gene in rye. Figure 1.2 demonstrates the proposed secondary structure of glutenin subunits.

(3) The combination of TAXI transformation with the biolistic process

As discussed above, naked plasmids used in direct gene transfer processes may reduce the transformation efficiency. We wondered whether this could be improved by combining the

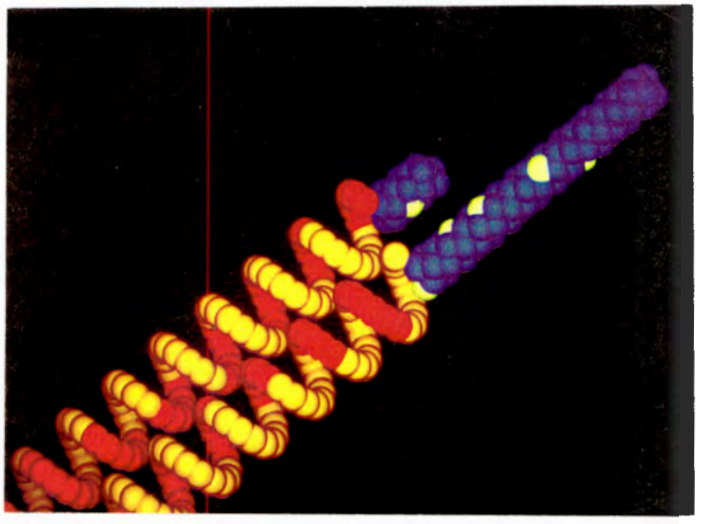


Figure 1.2 The Proposed Secondary Structure of High Molecular Weight Glutenin Subunits

HMW glutenin subunits have molecular weights ranging from 60000 to 80000. The proteins consist of three domains: a repetitive central domain flanked by non-repetitive N- and C-terminal domains. The central domain contains two consensus repeat motifs, PGQGQQ and GYYPTSLQQ. The N- and C-terminal domains contain most or all of the cysteine residues : 3 or 5 in the N-terminal domain, and 1 in the C-terminal domain.Each amino acid residue is depicted as a sphere.

α-Helix region: blue, highly conserved sequences of N-terminal and C-terminal regions
Cysteine: yellow-green
ß-spiral region: domain of repeated sequences
concensus of hexapeptide: red
concensus of nonapeptide: orange

(Computer graphics : Dr. D. Maeder, based on model proposed by Miflin et al., 1976)

techniques of TAXI transformation with microprojectile bombardment. Therefore this formed the third stage of this investigation. *Digitaria sanguinalis* was chosen as the candidate monocot because of its relative ease and speed in callus induction and plant regeneration. The *bar* gene was used instead of the NPT II gene as the selectable marker because of its low level of natural resistance in cereals, making the selection of transformed material more sensitive (D'Halluin et al., 1991). Instead of using the CaMV 35S promoter, the maize ubiquitin promoter, known to promote good gene expression in monocots (Christensen et al., 1992), was used to drive the GUS gene. The presence of the ubiquitin gene intron-1 upstream of the GUS gene could eliminate the expression of this gene in bacteria.

In addition, a useful technique which is essential to remove contaminating plasmids carrying the target genes was developed in this investigation. Using this technique single copies of transgenes can be easily detected in the plant genome, and transgenic plants can be quickly identified from a large number of plants without false positive signals produced by plasmid contamination. This technique may be applicable in many experimental situations.

In summary, the goal of this project was to improve the transfer of genes into cereals through a combination of advantages in both *Agrobacterium* transformation and the biolistic process.

Chapter 2

Elimination of Plasmid Contamination from Genomic DNA Prior to PCR Amplification of genes

Summary

At an early stage in my investigation, I realized that one of the major obstacles to proving the presence of transgenes in the plant genome was contamination of the genomic DNA with non-integrated vector. Therefore, a technique has been developed to remove traces of plasmid DNA from the large excess of genomic DNA. The technique described in this chapter removes plasmid DNA from an excess of genomic DNA in the order of 10^6 - 10^7. It may be applicable in many experimental situations in which it becomes necessary to remove contaminating plasmids from genomic DNA samples. In my investigation the development of this methodology was an indispensable prerequisite for the evaluation of the transgenes produced by the various transfer techniques.

2.1 Introduction

Detection of single copy genes in a genome is of fundamental importance in molecular genetics. Southern blot transfer is the main technique used for this purpose, by means of which transgenes can be detected not only qualitatively, but also quantitatively. The limits of detection are determined by the size of the respective genome. To detect single copy genes within a large sized genome, the Southern blot method may not be sensitive enough in some cases. Even with the use of intensive radioactively labelled probes, a long exposure time is required. The genome size in higher plants is usually larger than that in animals and in particular rye has the second largest genome size in major cereals. This makes it difficult to detect single copy foreign genes in putatively transformed rye plants. Therefore, a specific and sensitive method was required for screening putative transformants.

PCR is a quick and sensitive technique to detect single copy genes in plants, although it will not determine whether they are integrated into the genome. However, its high sensitivity also makes the method prone to false positives due to contamination by minute amounts of plasmid containing the genes to be investigated.

In this investigation, the main contamination source was the plasmid carried by *A.tumefaciens* which is difficult to remove from plant genomic DNA isolated from leaves. The GUS and glutenin 43 genes were the reporter genes in this study. The principles of the technique to remove contaminating DNA are described as follows :

A polybiotinylated ssDNA, derived from the parent plasmid, which does not contain the target genes, was produced and used as a means to trap contaminating plasmid. Prior to the execution of PCR, this ssDNA was added to the genomic DNA sample for hybridization with the contaminating plasmid. The annealed plasmid DNA/ssDNA complex and excess

ssDNA were subsequently removed using a streptavidin solid matrix. The purified genomic DNA was then used as a template for specific PCR amplification. The procedure is outlined in Figure 2.1

2.2 Materials and Methods

2.2.1 ssDNA preparation

The original plasmid used in the construction of pMKG and pKG43 was pBluescript SK. To eliminate contamination by these two plasmids, pBluescript SK was used to produce specific ssDNA. During hybridization, this ssDNA will anneal to contaminating pMKG and pKG43. *Eco*RI-linearized pBluescript SK was digested with exonuclease III (10 units enzyme for 0.4 - 1 ug DNA) in 50mM Tris-HCl pH 7.0, 5mM dithiothreitol (DTT), 5mM $MgCl_2$, 0.05 mg/mL BSA, at 37°C for 20 minutes. The reaction was stopped by heating at 75°C for 10 minutes. Exonuclease III is a 3' to 5' double-strand specific exonuclease that catalyzes release of 5' nucleotides from the 3'-hydroxy end of double-stranded DNA. This digestion will produce two ssDNA fragments with different sequences, approximately half the length of the linearized pBluescript SK (1.45 kb).

pBI121 is derived from pBin19 (Jefferson, 1987). For eliminating this plasmid contamination, pBI121 was digested by *Eco*RI, *Sst*II and *Bam*HI. The 5.1-kb vector fragment, from which the T-DNA region has thus been excised, was isolated and purified from a 1% agarose gel by using a GENECLEAN II kit. Before digestion by exonuclease III, this fragment was tested via PCR amplification for the absence of the GUS fragment, an essential prerequisite for its application as a trap. To prepare ssDNA the procedure described above was used.

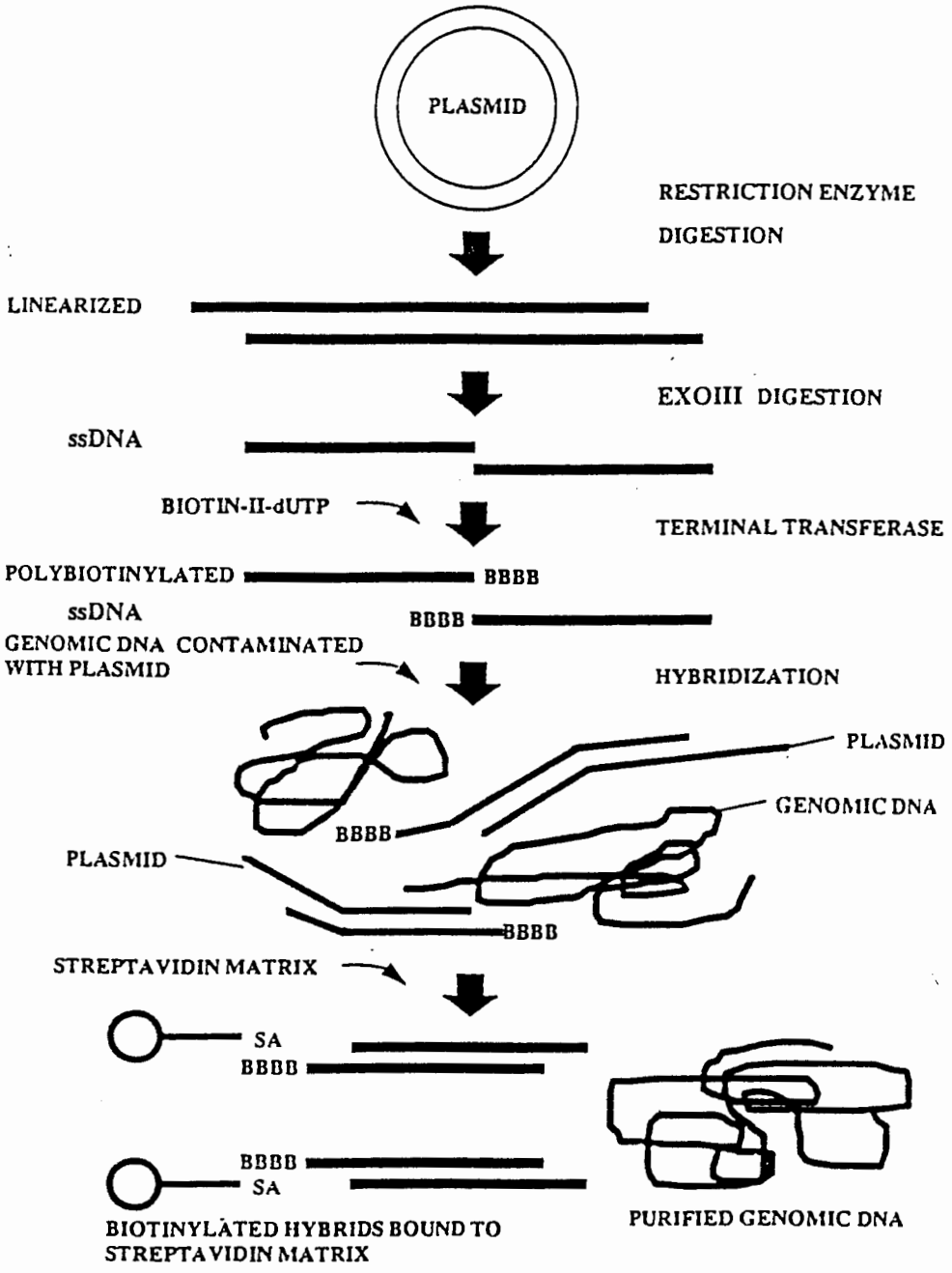


Figure 2.1 Diagram of the Method

2.2.2 Biotinylation

A poly[biotin-11-dUMP] tail was added to the ssDNA using terminal transferase and biotin-11-dUTP (Vincent et al., 1982). A typical 50 uL reaction mixture included:
200 ng ssDNA, 20 M biotin-11-dUTP, 0.05 mM bovine serum albumin [BSA], 1 mM $CoCl_2$, 100 mM sodium cacodylate pH 7.0, 10 units terminal transferase. After 30 minutes at 37°C, the reaction was stopped by adding 1 uL 0.5 M EDTA. Unincorporated biotin-11-dUPT was removed by passage through a Sephadex G-50 spin column.

2.2.3 Hybridization

Because the polybiotinylated ssDNA contains the original plasmid vector sequences, it should be able to anneal to the denatured contaminating plasmid strands during hybridization.
Genomic DNA was isolated from plant tissues using a modified CTAB method (see Appendix B). After RNase treatment, genomic DNA was precipitated with ethanol. In order to establish the magnitude of contamination, 10 samples of 250 ng each of genomic DNA from untransformed plants were used as templates in ^{32}P-labelled PCR amplification. Comparing the amplification from a series of dilutions of the plasmid templates, the average plasmid contamination is approximately between 0.05-0.5 pg (data not shown). The hybridization ratio between contaminating plasmid and polybiotinylated ssDNA was determined by titration. At a ratio of 1 : 10000, plasmid contamination can be completely eliminated under certain conditions of hybridization.

Hybridization was performed in a 50 uL volume : 250 ng rye genomic DNA containing approximately 0.5 pg contaminating plasmid (1.7×10^{-19} single strand moles in the case of pMKG and pKG43; 1.1×10^{-19} single strand moles in the case of pBI121), and 1 ng

polybiotinylated ssDNA (1.9 x 10^{-15} moles). This solution was heated in a water bath at 95°C for 5 minutes and then allowed to cool to room temperature slowly.

2.2.4 Removal of Hybrids

Streptavidin and the homologous protein avidin bind noncovalently up to four molecules of *d*-biotin with a binding constant in the order of 10^{15} M, making the reaction virtually irreversible. Biotinylated macromolecules are bound with similar binding constants. To remove the polybiotinylated hybrids of the annealed plasmid DNA from genomic DNA samples, a streptavidin matrix was made (Warren, 1995). This matrix consists of streptavidin linked to oxirane acrylic beads via hexaglycine. It has been successfully used to remove the polybiotinylated hybrids of the annealed plasmid DNA. The removal of biotinylated hybrids and subsequent PCR amplification was carried out as follows:

15 uL matrix in phosphate buffered saline [PBS] was blocked with 1.5 uL 10um/mL tRNA, 1.5 uL 1 mg/mL BSA, 1.5 uL 0.1% Tween-20. After blocking, the matrix was washed three times with PBS containing 0.1% Tween-20. A 50 uL hybridization sample was added to the matrix, mixed and rolled very slowly on a tube roller at room temperature for 1 hr. After microcentrifugation for 2 minutes, the supernatant was collected and denatured at 95°C for 5 minutes. The denatured samples were immediately put on ice and a 50 uL ice cold 2 x PCR mixture was added. The final 100 uL reaction mixture included : 250 ng purified genomic DNA; 200 uM dATP, dTTP, dGTP and 50 uM dCTP; 0.2 uL ^{32}P-dCTP (2 Ci); 0.1 mg/mL BSA,; 2 mM MgCl2; 50 pmoles of each primer, 1.5 units of Taq DNA polymerase, in the buffer [20 mM$(NH4)_2SO_4$ 75 mM Tris-HCl pH 9.0, 0.01% Tween]. PCR was carried out for 35 cycles at 92°C for 15 seconds, 63°C for 30 seconds, 72°C for 60 seconds. The products were then analyzed by electrophoresis on a 10% denaturing polyacrylamide gel.

2.3 Results and Discussion

2.3.1 Results

This technique was used in PCR screening for the GUS and glutenin genes in kanamycin resistant plants, which were produced either via *Agrobacterium* mediated transformation or via TAXI mediated transformation.

The results from titration of plasmid removal (Figure 2.2) demonstrates that the plasmid can be completely removed at a suitable hybridization ratio under the conditions described in section 2.2. After removing contaminating plasmids from genomic DNA, the negative control of untransformed rye plants gave no false positive signals in the PCR products (Figure 2.3). Thus specific PCR amplification can be used to demonstrate conclusively the presence of the transgene in plants. However, Southern blots will still be required to determine integration into the plant genome.

2.3.2 Efficiency of PCR detection

Rye has a large genome of 7.6 x 10^9 bp. The T-DNA regions of the plasmid vectors used in this investigation were about 5.1 x 10^3 bp. In Southern blot analyses, 10 ug crude genomic DNA extract of each sample was loaded on agarose gels. After transferring onto a nylon membrane, perhaps only about 5 ug genomic DNA containing approximately 3.3 pg single copy transgenes was on the membrane. For detecting such small amounts of genes on a solid membrane, a probe with very high radioactivity and a long exposure time are required. In contrast, although only 0.17 pg single copy transgenes are present in 250 ng genomic DNA template used in PCR, after 35 cycles the gene would be amplified multi million times. The products can be easily observed on an ethidium bromide stained gel.

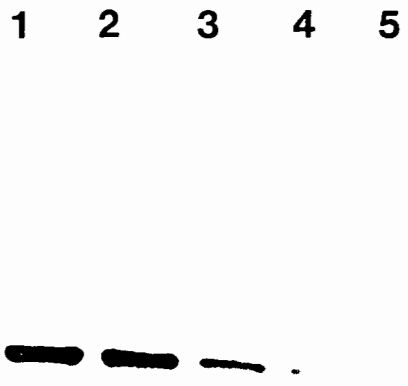


Figure 2.2 Titration of Plasmid Removal

Polybiotinylated ssDNA hybridized with 250 ng genomic DNA containing 0.5 pg plasmid pMKG at different ratios. After removal of hybrids, the elimination of plasmid from these genomic DNA samples were analyzed by PCR amplification of a transgene sequence, from -129 of the CaMV 35S promoter to + 257 of the 5' end of the GUS gene. The hybridization ratios of plasmid : polybiotinylated ssDNA in genomic DNA samples which were used as PCR templates in each lane are listed as below :

Lane 1	1 : 0	Lane 2	1 : 100
Lane 3	1 : 500	Lane 4	1 : 1000
Lane 5	1 : 2000		

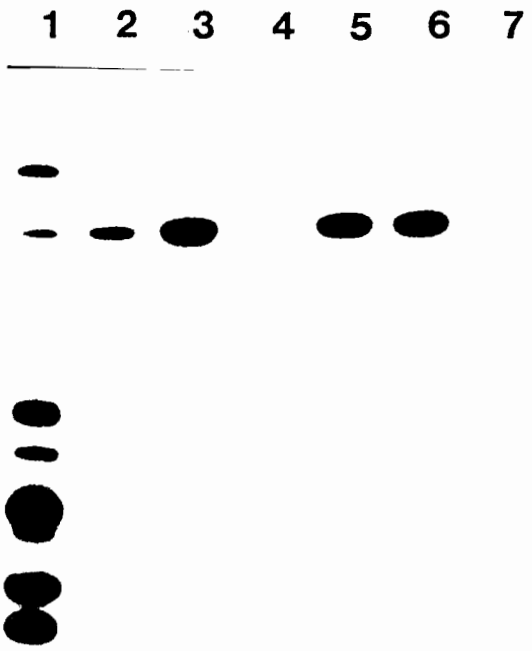


Figure 2.3 Detection of the Removal of Plasmid Contamination in Genomic DNA by PCR

Denaturing polyacrylamide gel electrophoresis analyses of PCR products amplified from the mixture of plasmid pKG43 and the genomic DNA of untransformed plants, with or without removal of plasmid contamination.

Lane 1: pBR322 HpaII digested molecular weight markers.
Lane 2: 0.1 pg pMKG43
Lane 3: 0.1 pg pMKG43 plus 250 ng genomic DNA from untransformed plants
Lane 4: DNA sample as in lane 3 but after removal of plasmid
Lanes 5&6: 200 & 250 ng unpurified genomic DNA from untransformed plants
Lane 7: DNA sample as in lane 6, but after removal of plasmid contamination

There are several factors effecting transgene amplification by PCR :

(1) The quality of genomic DNA samples is important for efficient amplification. Many plant DNA preparations contain varying amounts of polysaccharides which will inhibit the activity of *Taq* DNA polymerase during PCR amplification. Thus DNA-preparations free of polysaccharides are required.

(2) The concentration of genomic DNA in the PCR mixture can also effect the amplification efficiency. Too much genomic DNA template in the reaction solution will result in greater difficulty for the primers to anneal to their target sequences under the conditions used. Titration results indicated that 2.5 ng genomic DNA per uL, prepared as described, is the optimum concentration for subsequent PCR amplification.

(3) Complete heat denaturation is necessary before starting amplification cycles, Genomic DNA randomly coils in the solution. Completely denatured template will make it easier for the primers to find and anneal to a rare target sequence within the unordered genomic DNA. Therefore, before being added to the ice cold 2 x PCR mixture, purified genomic DNA samples were heat denatured at 95°C for 5 minutes.

2.3.3 Production of sufficient amounts of probe

The preparation of an efficient trap in sufficient amounts is one of the most important steps, which will determine whether or not plasmid contamination can be removed completely.
Nick translation to produce a biotin labelled probe would appear the simplest method to prepare the probe. However, I found that the nick translation labelled biotinylated probe easily reanneals to itself during hybridization, reducing the chance that the probe anneals to contaminating plasmid.

Single stranded biotinylated probes produced by PCR were also tested as a trap. However, producing ssDNA by PCR is less efficient than producing dsDNA. In addition, a certain amount of template is required for the amplification. This unlabelled template may also anneal to contaminating plasmid during hybridization, and these hybrids cannot be removed by streptavidin matrix. Furthermore, the purification of PCR products by gel electrophoresis would yield only very small amounts of the probe. When using the streptavidin matrix to capture the biotinylated PCR products, and then using these matrix-fixed probes to hybridize with genomic DNA, the high hybridization temperature would destabilize the linkage between biotin and streptavidin, resulting in hybrids released from matrix. Therefore, the particular procedure (Figure 2.2) was developed for producing a suitable ssDNA probe in sufficient amounts to remove the contaminating plasmid(s).

2.3.4 Streptavidin beads

Streptavidin oxirane acrylic beads were made using a procedure developed in our laboratory (Warren, 1995). Compared to the commercially available streptavidin agarose matrix, this matrix has some advantages :

(1) In commercial streptavidin agarose, the internal surface is larger than the outer surface. Therefore, most streptavidin is linked to the internal surface. However, in oxirane substituted nonporous microspheres streptavidin links only to the outer surface. This positioning of streptavidin will make it easier to react with the biotinylated macromolecular hybrids and capture them.

(2) The long arm of hexaglycine allows streptavidin to be more mobile. This, therefore, improves the interaction possibilities between streptavidin and hybrids.

(3) The density of oxirane acrylic beads allows streptavidin-hybrid complexes to be easily separated from the hybridization mixture by centrifugation.

Commercially available streptavidin magnetic beads can also be used for this removal. However, using the "home made" beads is more economical. The preparation protocol is described in Appendix B.

The principles elaborated in this technique should be useful in many experimental situations where it is essential to remove contaminating plasmids carrying the target gene(s), prior to PCR amplification.

Chapter 3

TAXI, a New Vehicle for the Transfer of Genes to Plants

Summary

A novel macromolecular complex, imitating the T-DNA complex in the *Agrobacterium* system, was constructed as a vehicle for the transfer of genes to rye plants. This complex consists of a linear single stranded DNA, containing a selectable marker gene and a reporter gene, bound to histone H1. This complex is referred to as the TAXI. Transgenic rye was recovered by transformation with the TAXI. Integration into the rye genome and expression of transgenes in the R1 and R2 generations of transformed rye demonstrated that the TAXI is a promising tool for the direct transfer of genes into cereals, bypassing the complications posed by genome type specificity in the *A.tumefaciens* system and the tissue culture process in direct gene transfer systems.

3.1 Introduction

Gene transfer into cereals via *Agrobacterium tumefaciens* mediated transformation has been attempted in a number of laboratories, although it is well known that cereals are insensitive to the infection of *A.tumefaciens* under natural conditions. To date, the production of transgenic rice has been the most successful (Hiei et al. 1994). The high frequency of transformation (12-28 %) of scutellum callus was similar to that obtained from *A.tumefaciens* mediated transformation in some dicot species. This success suggests that there may still be a possibility to achieve *A.tumefaciens* mediated transformation in cereals. Therefore, in the first stage of this study, the investigation focused on whether transgenic rye could be produced by the use of acetosyringone and glucose as inducers of *vir* genes in *A.tumefaciens* to improve the T-DNA transfer. The plasmid pBI121 (Figure 3.1) carrying the *nptII* and *uidA* genes was used as a vector. Although some positive results were obtained from PCR amplification, Western dot blot and histochemical assays, proof of the integration of transgenes was not achieved. The frequency of kanamycin resistance in progenies exhibited no increase in R1, R2 and R3 generation. This indicates that the positive results may have come not from true transformation, but rather from the transformation of endophyte.

To achieve true transformation in rye, an artificial T-DNA transfer system was designed, which was hoped to overcome the host limitation of T-DNA transfer, and achieve the integration of foreign genes into cereals. It has been noted that the T-DNA in its specific form of macromolecular complex in the *Agrobacterium* system may be a very important factor for the successful transfer and single locus integration of transgenes in the recipient plant. Therefore, the structure of the natural T-DNA complex was imitated in the design and construction of an artificial macromolecular complex. This novel vehicle for the transfer of genes to cereals has been termed the TAXI.

3.2 TAXI design

3.2.1 Increasing T-DNA copy number *in vitro*

(A) The optimal form of T-DNA : single strand or double strand ?

During *A.tumefaciens* mediated transformation in dicots, three types of T-DNA derivatives are produced: circular double stranded DNA (Zambryski et al., 1988); linear double stranded DNA (Steck et al., 1989); and single stranded DNA (Stachel et al.,1986). The circular T-DNA is produced in low quantities. In contrast, the single and linear double stranded T-DNA are produced at much higher levels and at similar rates. Tinland et al. (1994) reported results in favor of ssT-DNA being the form entering the plant cell nucleus. Their results strongly support Stachel's finding (Stachel et al., 1986). Rondenburg et al.(1989) found that ssDNA leads to a 3-10 fold higher frequency of stable transformation than dsDNA in *Nicotiana* protoplasts.

It is not yet clearly understood why a plant cell prefers a ssDNA rather than a dsDNA molecule for transformation. Two possible reasons have been suggested : (1) The DNA, in the physical form of single strandedness, may enter a plant cell and its nucleus more easily (Rodenburg et al., 1989). (2) ssDNA may integrate into a plant genome more efficiently, in analogy to the events promoted by the RecA protein that was found to match single stranded DNA to a complementary sequence of genome in *E.coli* (Mayer et al., 1985). For these reasons the single stranded form of T-DNA was chosen for the construction of the TAXI.

(B) Production of single stranded T-DNA fragment *in vitro*

In order to obtain a large number of T-DNA copies for the construction of the TAXI, the T-DNA fragment can be produced either *in vivo* or *in vitro* :

a) Isolating ssT-DNA from *A.tumefaciens* induced by dicot wound compounds. Because this T-DNA is naturally produced, it might be integrated into the plant genome efficiently. However, the T-DNA copy number in an induced *A.tumefaciens* is very low. It has been estimated that only less than 10 copies of T-DNA may be produced by each *A.tumefaciens* during dicot transformation (Binns, 1990). Such a low copy number and the DNA in the form of T-complex will make the isolation more complicated and less efficient.

(b) Isolating a dsT-DNA fragment from a high copy number cloning vector by restriction enzyme digestion. Linear ssT-DNA could be produced by heat denaturation of dsDNA. This method is the simplest way to obtain a large number of T-DNA fragments for the construction of the TAXI. However, the two complementary strands separated by heating will easily anneal to each other under suitable conditions. To solve this problem, an appropriate protein could be used to bind the ssDNA immediately after heat denaturation. This DNA binding protein not only prevents two complementary single strands from annealing, but also protects ssT-DNA from nuclease attack.

3.2.2 The T-DNA binding protein

In choosing a ssT-DNA binding protein for the TAXI construction, the protein should have the functions of both VirD2 and VirE. As a chromosomal DNA binding protein, histone H1 was chosen as the artificial ssT-DNA binding protein because it possesses a number of important characteristics.

a) It is well known that basic polypeptides, e.g. poly-ornithine, poly-arginine and poly-lysine, stimulate cell pinocytosis, increase the permeability of the cell membrane and cause lesions in cell membranes (Hugues et al., 1965, Burgess et al., 1973, Laroche et al., 1988). Of the five histones, the H1 type has the highest positive charge density. Unlike the core

histones the binding between DNA and H1 is not very strong. In solution histone H1 is envisaged to extend both a basic N-terminal and C-terminal region from a more globular central core (Shannon and Wells, 1987). Two terminal tails of histone H1 exhibit structural analogies to these basic polypeptides, which may cause a functional similarity to basic polypeptides. Hugues et al. (1965) found that histones and basic polypeptides were taken up by mammalian cells at rates up to 3000 times greater than serum albumin. When serum albumin was mixed with histone, the uptake of albumin increased 10 - 50 fold. Furthermore, Drew et al. (1970) reported that barley roots exposed to a histone solution (0.5 mg/ml) take up the protein in amounts as much as 0.4 % of the root's fresh weight in less than 30 minutes. It therefore appears that histone H1 may facilitate the entry of biological macromolecules into eukaroytic cells.

b) Histone H1 is a chromosomal DNA binding protein. As such it contains amino acid targeting sequences for entry into cell nucleus. This nuclear targeting sequence is present in the C-terminal tail of histone H1 (Dingwall et al., 1984). It has been found that histone H1 accumulates in the nucleus after injection into the cytoplasm of *Xenopus oocytes*. Thus the use of histone H1 as ssT-DNA binding protein will probably also facilitate the entry of T-DNA into the cell nucleus.

c) Histone H1 may have the function of recombinase which catalyzes ATP-independent DNA strand transfer and promotes integration of ssDNA into a chromosome. Kawasaki et al.(1989) found that calf thymus histone H1 catalyses the incorporation of a linear double stranded tet^r DNA into M13 mp8-tet^r single stranded viral DNA. Sobczak et al.(1988) reported that histone H1 catalyzed the intermolecular ligation of both the cohesive and blunt ends of DNA fragments. These findings suggested that histone H1 may possibly have the potential to have some function in DNA recombination. Thus histone H1 derived from sea urchin sperm was chosen as the substitute of viral protein in the TAXI construction.

3.3 Materials and Methods

3.3.1 Construction of T-DNA vectors

Two vectors have been constructed for producing artificial T-DNA molecules. A modified pBluescript SK, in which the *Sma*I and *Bam*HI sites were deleted, was used as the T-DNA cloning vector. It has been found that the border sequences of T-DNA are partially integrated together with the T-DNA in tobacco transformants (Yadaw et al., 1982, Zambryski et al., 1982) and rice transformants (Hiei et al., 1994). At this stage it is unresolved whether the border sequences aid in the integration of T-DNA, analogous to the role of repeat sequences in other eukaryotic mobile DNA elements. The investigations establishing the essential role of the right border in the initial T-DNA mobilization in *A.tumefaciens* (Wang, 1984) do not exclude the possibility of a further function with respect to integration. Thus a synthetic 30 bp T-border element, corresponding to the right border sequence of the nopaline Ti plasmid and an *Eco*RI recognition sequence, was inserted into the pBluescript SK between the *Eco*RI and *Eco*RV sites. The GUS gene fragment driven by CaMV 35S promoter was excised from pBI121 and inserted into the pBluescript between *Hind*III and *Cla*I sites. This T-DNA vector was termed pMBG.

For the construction of a selectable GUS T-DNA vector, a neomycin phosphotransferase gene (*nptII*), driven by the nopaline synthase promoter, was inserted into the pMBG *Hind*III site between the right border sequence and the GUS gene. This vector was termed pMKG (Figure 3.2a).

Another T-DNA amplification vector used in this investigation was pKG43, which carried the glutenin gene of the cDNA clone number 43 isolated from immature wheat embryos in

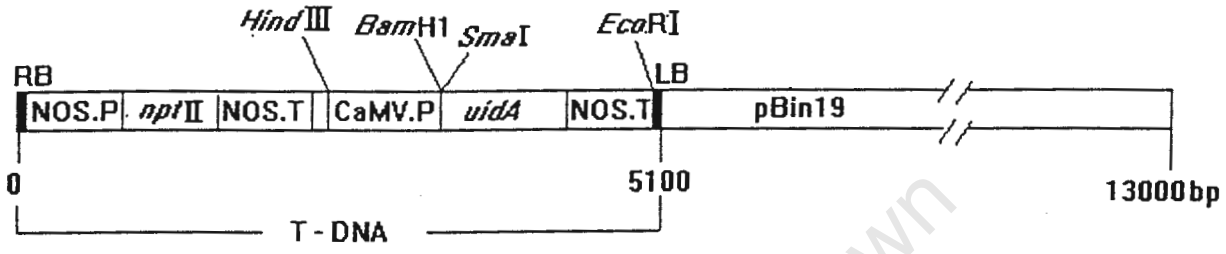


Figure 3.1 The Map of pBI121

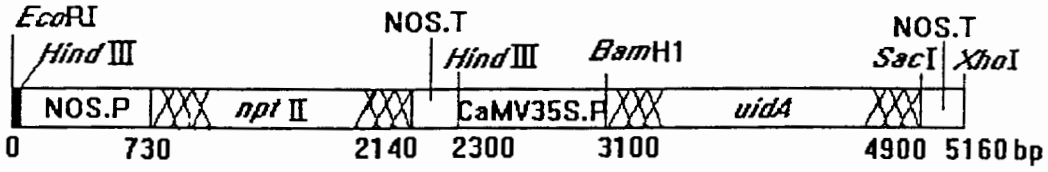
pBI121, a vector in the GUS fusion system, was constructed by Jefferson (1987). The T-DNA region contains a marker gene (*nptII*) and a reporter gene (*uidA*) in between the direct repeat border sequences.

RB & LB : right and left border sequences of nopaline Ti plasmid.

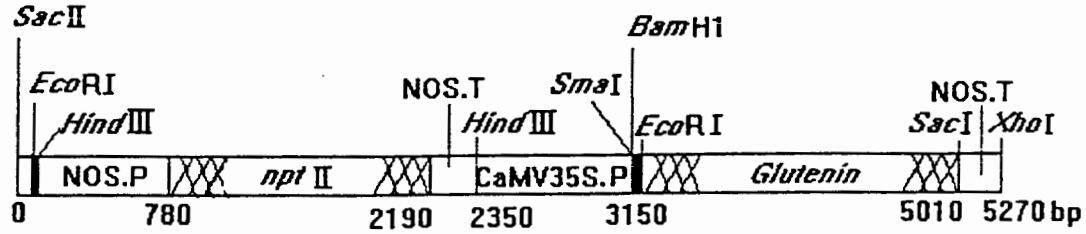
Nos.P & Nos.T : neomycin phosphotransferase promoter and 3' terminator sequence.

CaMV.P : cauliflower mosaic virus 35S promoter

E : *Eco*RI H : *Hind*III S : *Sma*I B : *Bam*HI



a) The *nptII - uidA* fragment of transfer DNA from pMKG



b) The *nptII - Glutenin* fragment of transfer DNA from pKG43

Figure 3.2 Maps of Transfer DNA

The transfer DNA fragments are cloned in pBluescript SK between *Eco*RI and *Xho*I sites (for *nptII-uidA* T-DNA) or *Sca*II and *Xho*I sites (for *nptII*-glutenin gene T-DNA).

RB	black box, ar the left end of the T-DNA
NOS.P	Nopaline synthe-ase promoter
NOS.T	Nopaline synthease 3' terminal sequence
nptII	Neomycin phosphotransferase II gene
uidA	Glucuronidase gene
CaMV.P	Cauliflower mosaic virus 35S promoter
Glutenin	Glutenin cDNA clone number 43

this laboratory. After deleting the GUS gene from pMKG, the glutenin cDNA was inserted into between the *Sma*I and *Sal*I sites (Figure 3.2b).

These T-DNA vectors were transferred to and amplified in the XL-blue strain of *E.coli*. The T-DNA fragments were obtained by digestion with suitable restriction enzymes followed by agarose electrophoresis.

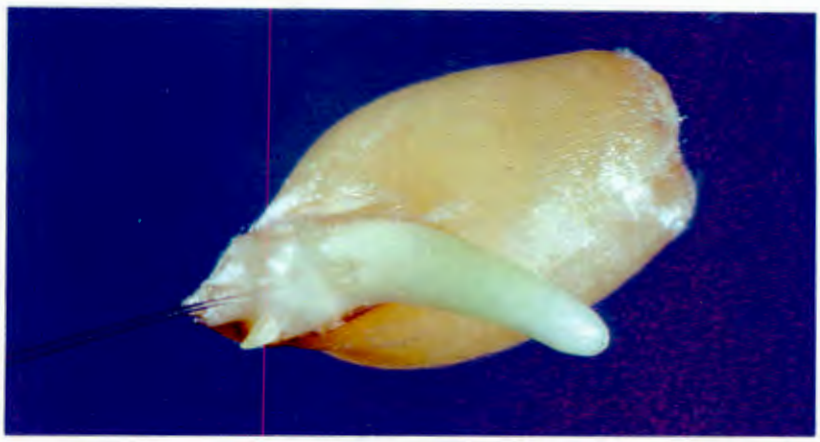
3.3.2 The assembly of ssT-DNA-histone H1 complex

Histone H1 isolated from sea urchin sperm (von Holt et al., 1989) was purified and used as the binding protein for the ssT-DNA construct. T-DNA fragments, isolated from agarose gels, were heat denatured and immediately put on ice. An ice cold histone H1 solution was then mixed with the ssT-DNA and left on ice to allow protein-DNA binding to occur and result in a soluble ssT-DNA-histone H1 complex. The optimal mass ratio of histone H1 (MW = 2.1 x 10^4) to ssT-DNA (5.1 kb, MW = 1.78 x 10^6) was established to be 2.5 : 1 ; giving a molecular ratio of approximately 200 : 1. At this binding ratio, one histone molecule can bind to about 25 bases of the ssT-DNA forming a soluble complex. Although the precise number of histone H1 molecules attached to ssT-DNA has not been determined, it is presumed that histone H1, through interaction of its basic N- and /or C-terminal arm(s), covers the whole ssDNA strand to protect the latter against nuclease attack.

3.3.3 Transformation

TAXI carrying the *nptII* gene together with either the GUS gene (pMKG) or the glutenin gene (pKG43) were individually transferred into rye plants by injection of germinated embryos and 2 day-old seedlings into the meristem of the growth point (Figure 3.3). In

A



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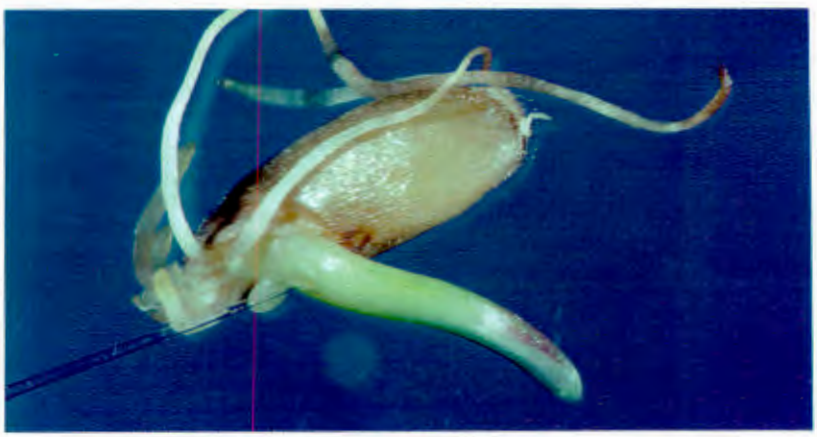


Figure 3.3 Seedling Injection

TAXI-pMKG and TAXI-pKG43 were transferred to rye seedlings by injection into :
A) germinating one day old embryos
B) 2 day-old seedlings
The glass needle of the injector was very gently pierced into the embryos and seedlings at the growth point. 5 uL TAXI solutions were injected into each embryo or seedling. The injected embryos and seedlings were kept in moist Petri dishes overnight before being transferred to vermiculite.

both transformations, 5ul solution containing 0.2 ug DNA per uL or 1 ug DNA per uL were injected per seedling. 50 embryos or seedlings each were injected with the TAXI per transformation experiment. Five such transformation experiments were done with the TAXI-pMKG and four with the TAXI-pKG43.

Injected embryos or seedlings were incubated overnight in a moist Petri dish, followed by transfer to moist vermiculite, and incubated for further 3 - 5 days at room temperature. These presumptively transformed seedlings were then planted into soil. After about three months in a plant chamber under illumination and humidity control, mature seeds were collected. Compared with the seeds of untransformed rye, the seeds from the putatively transformed plants were fewer in number and appeared shrivelled. From 25 putatively transformed rye plants on average 400 to 600 seeds were collected. These seeds were tested for kanamycin resistance.

3.4 Results

3.4.1 Kanamycin selection

pMKG and pKG43 carry the *nptII* gene, which encodes neomycin phosphotransferase, as a selectable marker. This enzyme specifically phosphorylates aminoglycoside antibiotics of the neomycin family, such as kanamycin and G418 (Klee et al., 1987). Kanamycin binds to 70S ribosomes of bacteria and chloroplasts, causing misreading of messenger RNA. However, modified kanamycin can no longer interact with ribosomes. When plant seeds germinate in a solution containing kanamycin the seedlings will become etiolated. These plants, white or pink in colour, can only survive until the two leaf stage due to the lake of chlorophyll with ensuing absence of photosynthesis. As a result of chloroplast ribosome inhibition by kanamycin, any one of the enzymes or proteins synthesized by the chloroplast

and needed in the photosystem I and II may become defective due to misreading of their respective mRNA. This would finally result in etiolation.

Seeds derived from plants transformed with both the TAXI-pMKG and the TAXI-pKG43 were selected by germination in the presence of kanamycin (800 ug / mL). Fully kanamycin resistant plants exhibit green leaves throughout their seedling development stages; partially kanamycin resistant plants have partially etiolated leaves or some green and some white leaves; kanamycin sensitive plants have white or pink leaves only (Figure 3.4). Approximately 90 % of seeds from untransformed rye did not germinate under selection conditions, and only 0.2 - 0.5 % of them survived to become mature plants.

The data indicated that low numbers of R1 and R2 plants were resistant to kanamycin (Table 3.1). However, when the plants transformed with the TAXI-pMKG were tested for NPT II activity *in vitro*, according to the method described by Peng et al.(1993), a much higher transfer rate of the gene was obtained (Figure 3.5). In the R1 and R2 generation plants, 13 out of 70 (18 %) and 49 out of 70 (70 %) were positive.

3.4.2 Analysis of the TAXI-pMKG transgenic rye

To identify transgenic rye from kanamycin resistant progenies, PCR screening and Southern blot analyses were used. Genomic DNA was isolated from leaves of kanamycin resistant plants (see Appendix B2.2.2). To eliminate the plasmid contamination, genomic DNA was purified as described in Chapter 2 prior to molecular analysis.

For PCR amplification of the GUS gene, primers were chosen which amplified a 386-bp fragment containing a part of the CaMV 35S RNA promoter and the 5' end of the GUS gene sequence. To determine whether the PCR product was the GUS specific amplicon, the

1 2 3 4 5

Figure 3.4 Kanamycin Selection of Transformed Plants

Dry seeds collected from putatively transformed rye plants (R0 generation) were soaked in kanamycin solution (800 ug / mL for the Boehringer product or 1 mg / mL for the Sigma product) for 2 days. Subsequently the seeds were germinated in vermiculite and watered with the same kanamycin solution. 3-5 days after germination the seedlings were planted into soil. The same kanamycin solutions were used to water the plants until their third leaf appeared.

1 : Control plants either could not germinate or exhibited white or pink leaves. The large majority did not survive under these selective conditions. Only 0.2 -0.5 % of control plants grew to maturity.

2-4: Some putatively transformed plants exhibited partial kanamycin resistance with partially white and partially green leaves.

5 : Fully kanamycin resistant plants exhibited green leaves throughout their seedling develoment and in the mature state.

Table 3.1 The Frequency of Kanamycin Resistance in the Progeny of Transformed Rye

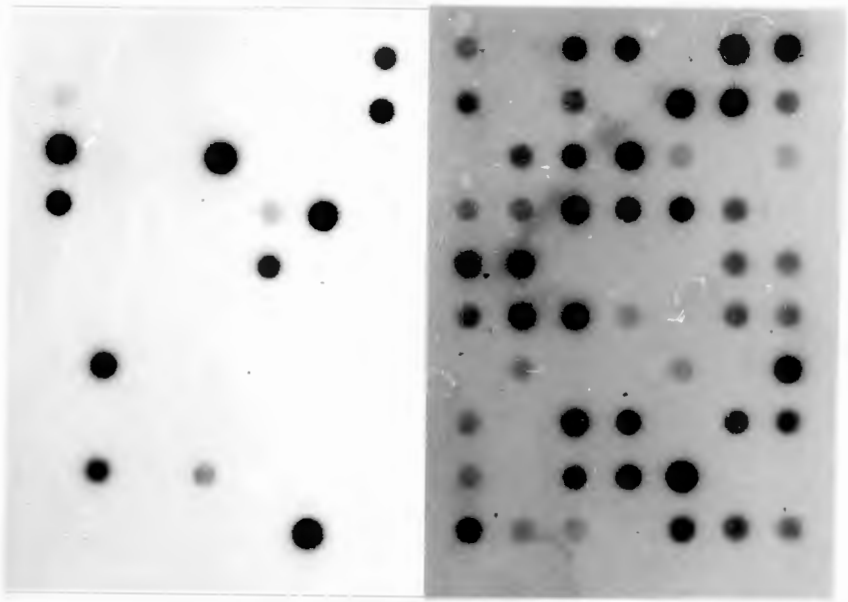
(a) The frequency of kanamycin resistance in progeny of the TAXI-pMKG transformants

Plant progeny	No. of seeds tested	No. of seeds germinated on kanamycin	Plants grown to maturity on kanamycin	Kanamycin resistance (%)
R1	1800	389	133	7.4
R2	800	283	93	11.6
control	1200	77	6	0.5

(b) The frequency of kanamycin resistance in progeny of the TAXI-pKG43 transformants

Plant progeny	No. of seeds tested	No. of seeds germinated on kanamycin	Plants grown to maturity on kanamycin	Kanamycin resistance (%)
R1	1400	169	52	3.7
R2	400	158	26	6.5
control	1000	68	2	0.2

* 200 seeds of each transformed and control plants were tested in every individual selection experiments. The numbers listed above are sums of all experiments.



A B

Figure 3.5 **Expression of the *nptII* Gene in R1 and R2 Plants Transformed with the TAXI-pMKG**

Leaves from individual seedlings derived from seeds of the R0 plants and R1 kanamycin resistant plants were cut into small pieces, put into a microtiter plate and incubated with γ-^{32}P-ATP and kanamycin. Incubation was carried out for 30 minutes at room temperature. The panels A (R1) and B (R2) represent the autoradiographs of the supernatants.

amplified DNA fragments were transferred from 10 % polyacrylamide gels to Hybond-N^+ membranes. The blots were then probed with a ^{32}P-labelled *Bam*HI / *Eco*RI *uidA* fragment from pBI121. Positive signals were observed in both R1 and R2 transformants (Figure 3.6).

To investigate integration events, Southern blot analysis was carried out. Genomic DNA from putatively transformed and untransformed rye was probed with a ^{32}P-labelled *uidA* fragment or a *Hind*III *nptII* fragment from pKG43 prior to and after digestion with a suitable restriction enzyme. Figures 3.7 and 3.8 demonstrate the analysis of the *uidA* gene in R1 and R2 plants. Figure 3.9 demonstrates the analysis of the *nptII* gene in R1 and R2 plants.

The expression of the GUS gene was determined by immunological and histochemical assays. Proteins isolated from second generation immature seeds reacted with a specific anti-GUS antibodies (Figure 3.10), demonstrating the expression of the GUS gene. Histochemical assays also showed this in the embryos (Figure 3.11). Further investigations were carried out on a large number of R1 and R2 seedlings. Figure 3.12 demonstrates the results of GUS activity fluorescence assays. Expression of the *nptII* and *uidA* genes in R1 and R2 seedlings are summarized in Table 3.2. Co-expression of both genes was not always observed.

4.4.3 Analysis of the TAXI-pKG43 transgenic rye

In the case of glutenin transformants, Southern blot analysis of putatively transformed rye cannot be used because wheat glutenin genes and the rye HMW secalin gene share considerable amounts of homology (Shewry et al., 1994). Various probes were designed from fragments of glutenin clone 43 in order to find a specific one. However, all of them hybridized with genomic DNA from untransformed rye. There is not sufficient sequence

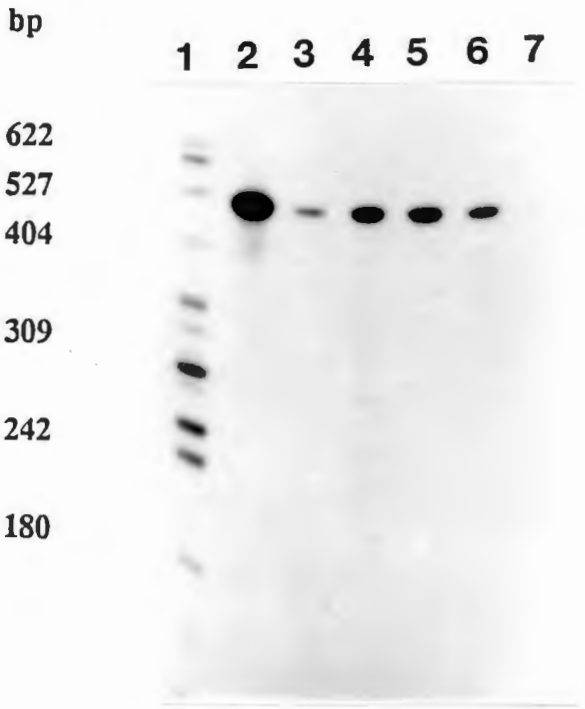


Figure 3.6 **PCR Amplification of The GUS Gene Fragment from Genomic DNA of Kanamyin Resistant Plants Transformed with the TAXI-pMKG**

250 ng plasmid-free genomic DNA of each plant was used as the template for PCR amplification. The forward primer anneals to -129 to -108 of the CaMV 35S promoter region and the reverse primer aneals to +157 to +236 of the coding region of the GUS gene. The 386-bp PCR product was analyzed by PAGE on a 10 % denaturing gel and hybridized with a ^{32}P-labelled *Bam*HI / *Eco*RI *uidA* fragment from pBI121. The PCR template of each lane is listed below.

Lane 1: pBR322 *Hpa*II molecular wight markers
Lane 2: 0.1 pg pBI121
Lanes 3&5: genomic DNA of R1 plants
Lane 4&6: genomic DNA of R2 plants
Lane 7: genomic DNA of an untransformed plant

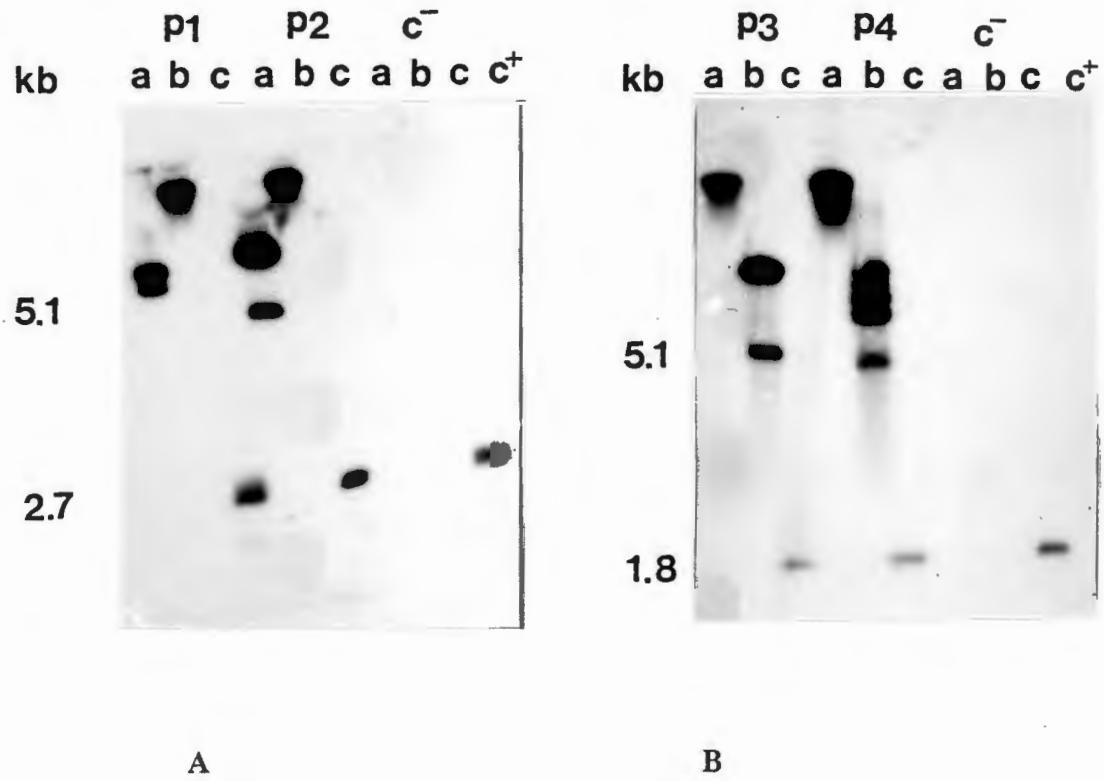


Figure 3.7 Southern Blot Analysis of the GUS Gene in R1 Plants Transformed with the TAXI-pMKG

Genomic DNA was isolated from leaves of kanamycin resistant R1 plants. 15 ug of each sample was electrophoresed on a 0.8 % agarose gel and the blots probed with a ^{32}P-labelled *Bam*HI / *Eco*RI *uidA* fragment from pBI121. The plants in A and B were produced from two separated transformation experiments. C^+ is a positive control of the *Hind*III / *Sac*I fragment of pMKG. C^- is a negative control of untransformed rye.

(A) In each set of three lanes, lane a: genomic DNA digested with *Eco*RI, lane b: undigested genomic DNA, lane c: genomic DNA digested with *Hind*III and *Sac*I.

(B) In each set of three lanes, lane a: undigested genomic DNA, lane b: genomic DNA digested with *Eco*RI, lane c: genomic DNA digested with *Bam*HI and *Sac*I.

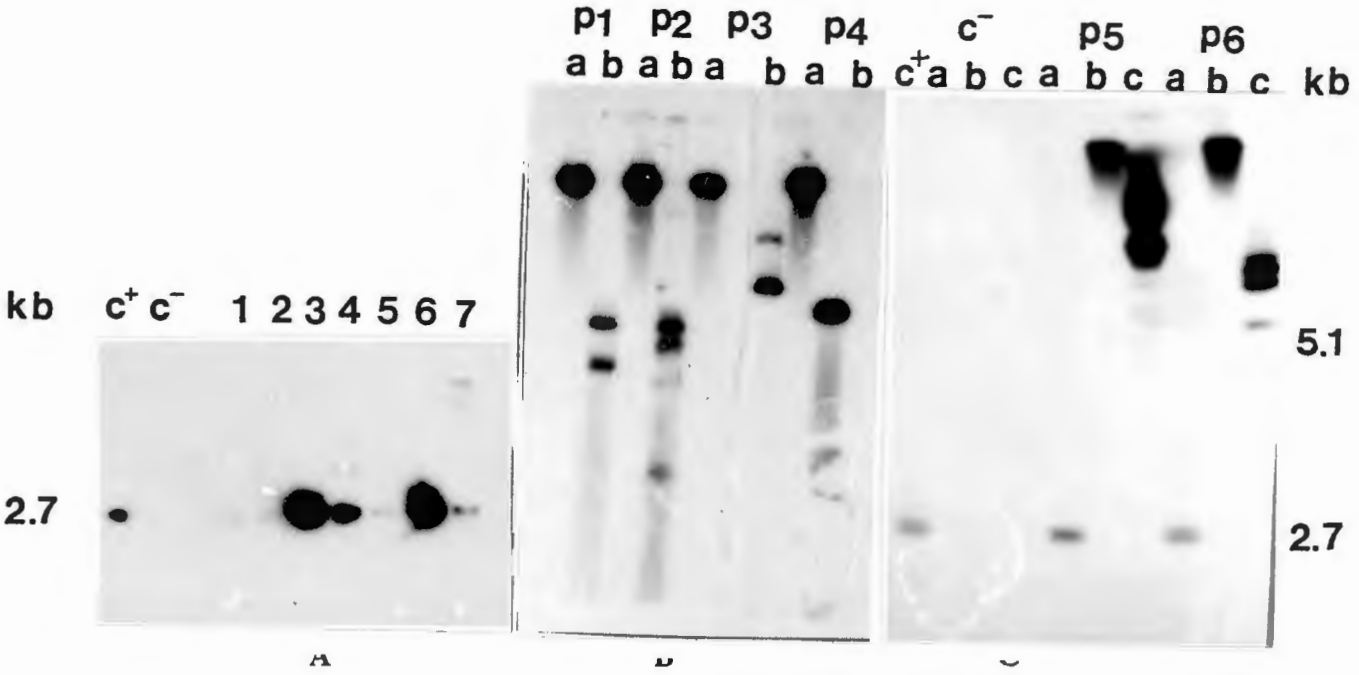


Figure 3.8 Southern Blot Analysis of the GUS Gene in R2 Plants Transformed with the TAXI-pMKG

Genomic DNA was isolated from leaves of kanamycin resistant R2 plants. 15 ug of each sample was electrophoresed on a 0.8% agarose gel and the blots probed with a ^{32}P-labelled *Bam*HI / *Eco*RI *uidA* fragment of pBI121. A: analysis of the intact GUS gene in R2 plants. B and C : analysis of the integration of the GUS gene in R2 plants produced from two separated transformation experiments. C^+ is a positive control of *Hind*III / *Eco*RI GUS fragment of pBI121. C^- is a negative control of untransformed rye.

(A) 1-7: genomic DNA digested with *Hind*III and *Sac*I.

(B) In each set of two lanes, a: undigested genomic DNA, b: genomic DNA digested with *Eco*RI.

(C) In each set of three lanes, a: genomic DNA digested with *Hind*III and *Sac*I, b: undigested genomic DNA, c: genomic DNA digested with *Eco*RI.

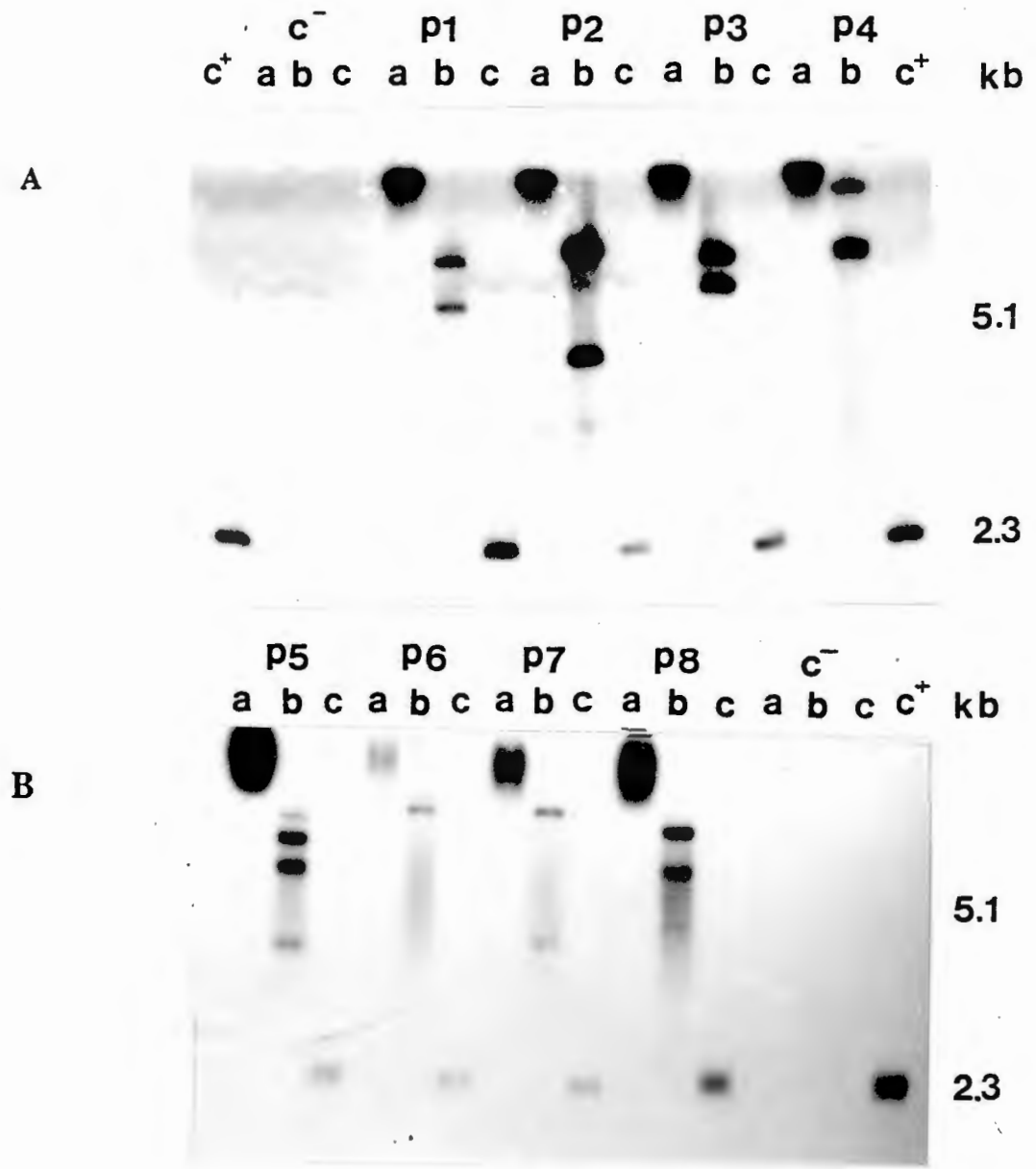


Figure 3.9 Southern Blot Analysis of the *nptII* Gene in R1 and R2 Plants Transformed with the TAXI-pMKG

Genomic DNA was isolated from leaves of kanamycin resistant R1, R2 plants and untransformed rye. 15 ug of each sample was electrophoresed on a 0.8 % agarose gel and the blots was probed with a ^{32}P-labelled *Hind*III *nptII* fragment of pMKG.

A and B are the blots of the R1 and R2 generations respectively. In each set of three lanes, a: undigested genomic DNA; b: genomic DNA digetsed with *Xho*I: c: genomic DNA digested with *Hind*III. C^+ is a positive control of the *Hind*III *npt*II fragment of pMKG. C^- is a negative control of of untransformed rye.

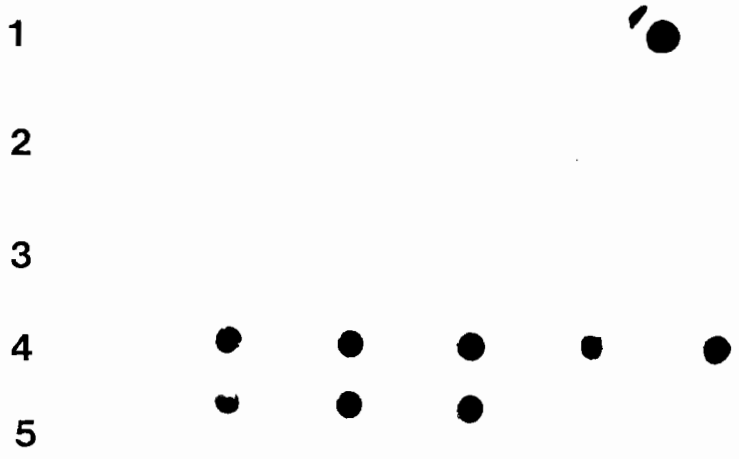


Figure 3.10 **Western Dot Blot Analysis of the GUS Protein in R2 Seeds from Plants Transformed with the TAXI-pMKG**

Proteins were extracted from the immature endosperm of R2 seeds and seeds from untransformed plants. The dot blot was probed with anti-GUS antibody linked to a chemiluminescence assay.

Row 1: 10 units glucuronidase (one sample only)

Rows 2 & 3: proteins from 10 untransformed seeds

Rows 4 & 5: proteins from 10 R2 seeds

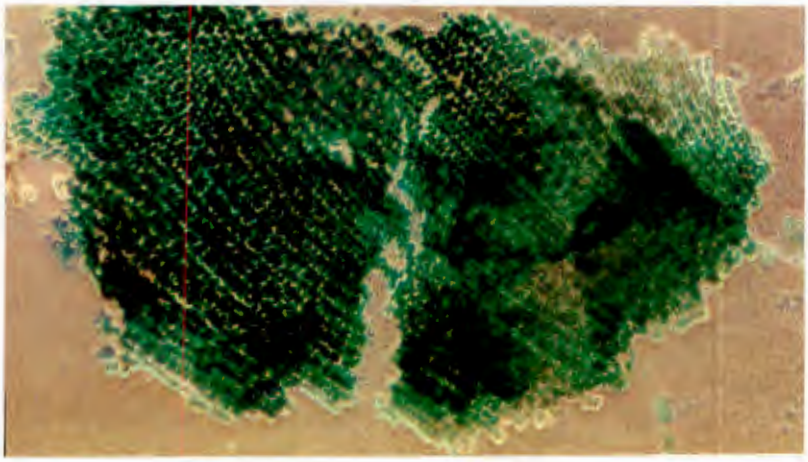
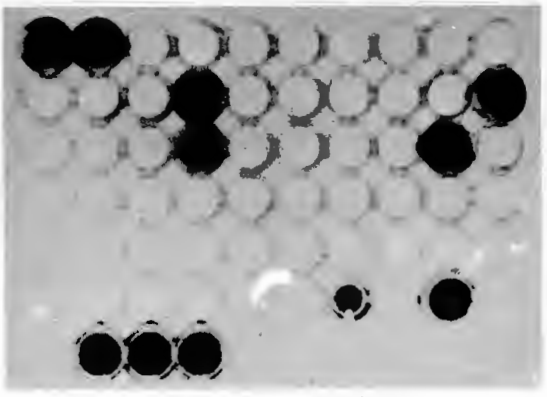


Figure 3.11 Histochemical Assay of GUS Activity in the Embryo of an R2 Plant Transformed with the TAXI-pMKG

A section of an embryo of the R2 generation was incubated with X-gluc. Pigment shows GUS activity.

A



B

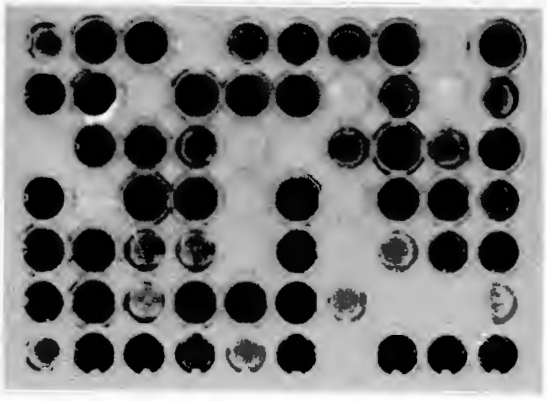


Figure 3.12 Expression of the GUS Gene in R1 and R2 Plants Transformed with the TAXI-pMKG

70 leaf samples of each R1 and R2 seedlings were used to analyze the GUS activity by fluorescence assay according to the method described by Peng et al. (1993).

A: R1 seedlings

B: R2 seedlings

Table 3.2 Expression of Transgenes in R1 and R2 Plants Transformed with the TAXI-pMKG [a]

Gene	Generation	Seedlings tested	Positive / Negative	positive (%)
nptII	R1	70	13 / 57	18
	R2	70	49 / 21	70
uidA	R1	70	10 / 60	14
	R2	70	54 / 16	77

[a] Because these seedlings were geminated from seeds collected from a group of R0 plants or R1 kanamycin resistant plants, the data cannot be used to test for Mendelian segregation.

information on rye HMW secalin available to design a specific probe for glutenin. PCR amplification of a fragment containing part of the CaMV 35S RNA promoter and the 5' end of the glutenin gene seemed, therefore, to be the only possible method that could be used to detect the glutenin gene in rye, because the CaMV promoter sequence should not exist in normal rye plants.

A forward primer corresponding to -129 to -108 of the CaMV 35S promoter region and a reverse primer corresponding to +187 to +168 of the 5' end region of the glutenin gene were made (Appendix B5.3). The gap between the promoter and the glutenin gene start codon is 63 bp. The length of the amplified fragment, therefore, should be 379 bp. Figure 3.13 demonstrates the result of PCR amplification. A 379-bp glutenin fragment was obtained from genomic DNA samples, free of plasmid, from some of the R1 and R2 kanamycin resistant plants. However, the result of PCR amplification cannot prove the integration of the glutenin gene into the plant genome.

In order to prove this, Southern blot analysis was done using a *nptII* fragment as a probe, because the *nptII* gene is 5' to the glutenin gene in pKG43 (Figure 3.2b). AP^{32}labelled *Hind*III *nptII* fragment from pMKG was used as the probe to hybridize with genomic DNA of R2 plants transformed with the TAXI-pKG43. Figure 3.14 demonstrates the hybridization results.

Because of the presence of homologous sequences, analyzing the expression of the glutenin gene in rye poses a similar problem to Southern blots. Prior to determining whether the glutenin gene was being transcribed in immature seeds of putatively transformed rye, it was necessary to identify the differences between secalin-mRNA and glutenin-mRNA. Both secalin and glutenins exhibit non-repetitive N-terminal and C-terminal domains which flank a large domain of a highly repetitive oligopeptides. Both the unique terminal domains and repetitive regions are highly homologous in these two protein families (Forde et al.,

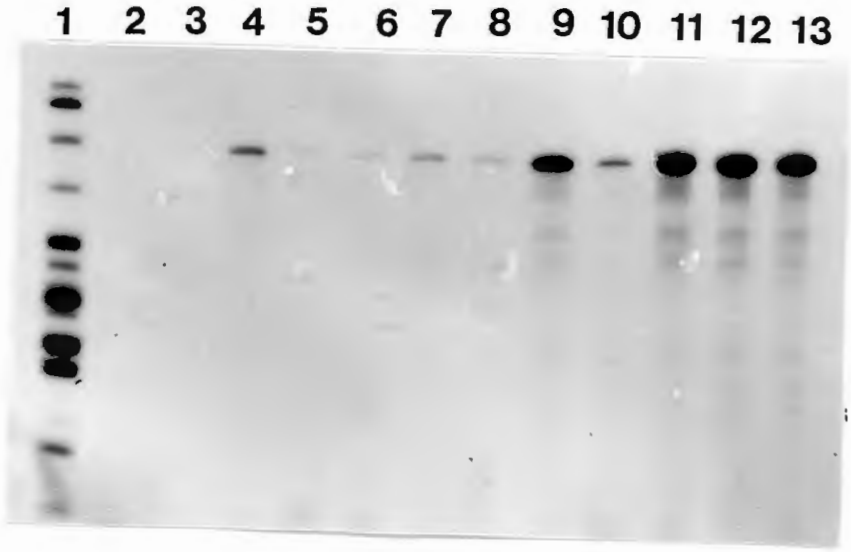


Figure 3.13 **PCR Amplification of a Glutenin Gene Fragment from Genomic DNA of R1 and R2 Plants Transformed with the TAXI-pKG43**

The 379-bp PCR products containing the 63-bp pBluescript polylinker between the CaMV 35S promoter and the glutenin gene 5' end were analyzed by PAGE (10 % denaturing gel). The blot was probed with a ^{32}P-labelled clone number 43 of wheat glutenin cDNA.

1 : pBR322 *Hpa*II molecular weight markers

2&3 : untransformed rye

4&5 : 0.1 pg, 0.01 pg glutenin clone 43 plasmid

6,7&10,11 : R1 plants produced from two individual transformation experiments

8,9&12,13 : R2 plants produced from two individual transformation experiments

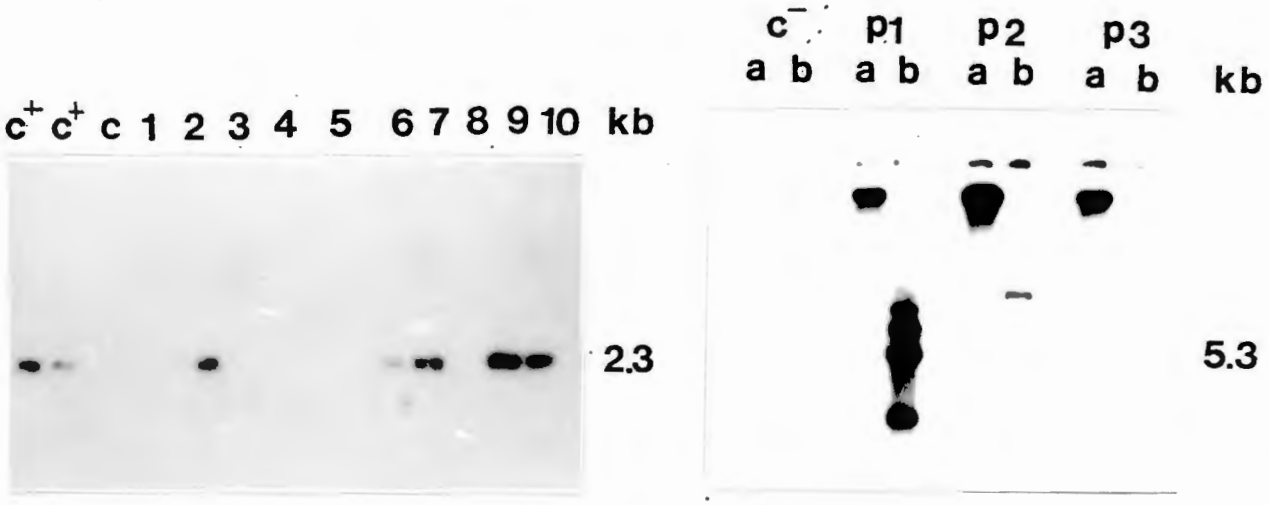


Figure 3.14 Southern Blot Analysis of the *nptII* Gene in R2 Plants Transformed with the TAXI-pKG43

Genomic DNA was isolated from leaves of kanamycin resistant R2 plants and untransformed rye. 15 ug of each sample were electrophoresed on a 0.8 % agarose gel and the blots probed with a ^{32}P-labelled *Hind*III *npt*II fragment of pMKG. C^+ is a positive control of the *Hind*III *nptII* fragment of pMKG. C^- is a negative control of untransformed rye.

(A) Analysis of the intact *nptII* gene in R2 plants. 1-10: genomic DNA of R2 plants digested with *Hind*III.

(B) Analysis of the integration of the *nptII* gene in R2 plants. In each set of two lanes, a: undigested genomic DNA. b: genomic DNA digested with *Xho*I.

1983). It is highly likely that HMW secalin contains these homologous regions at its two termini as well. In glutenin, the C-terminal homologous region contains approximately 26 amino acid residues, followed by a further 14 amino acid residues till the C-terminus is reached. In terms of the DNA sequence, the total length of this 3' terminal region would be about 120 bp. The difference in the 3' terminal region of the two mRNA species should become obvious by comparing the distances of the ultimate repetitive oligonucleotide sequences in the central homologous domains from the unique but also homologous 3' terminals in the two mRNA species. A forward primer corresponding to a glutenin repeat sequence, and a reverse primer corresponding to the 3' end of the glutenin codon region, were used for PCR amplification (Appendix B5.3). This reaction should produce more than one amplified fragment because the forward primer can anneal to every repeat region.

mRNA was isolated from immature seeds of rye as described in Appendix B2.3. RT-PCR was performed as in Appendix B5.2. The results (Figure 3.15) indicated that some of the RT-PCR products from untransformed rye mRNA and glutenin cDNA differ in size being 358 and 409 bp respectively. This difference could be used as positive and negative controls in the transcriptional analysis of the glutenin gene in putatively transgenic rye (Figure 3.16).

The analysis was next carried out at the protein expression level. The codon region of the glutenin clone 43 is approximately 1800 bp, corresponding to 600 amino acid residues. Discounting the first 10 - 20 residues which, because of the leader sequence, will be removed on storage in the seed, the expected molecular weight of the glutenin will be in the region of 64 - 65 kDa. Figure 3.17 shows a Western blot of seed storage proteins reacting with anti-glutenin antibody.

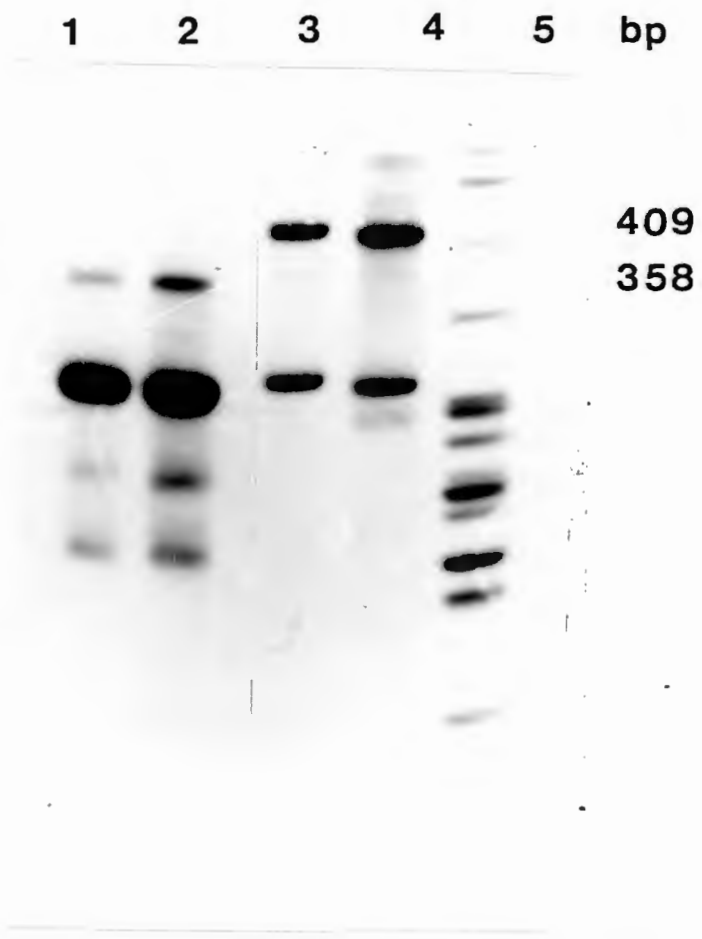


Figure 3.15 **PCR Analysis of cDNA 3' End Regions of HMW Secalin and Glutenin**

Total RNA was isolated from immature seeds of wheat and rye. mRNA was then separated from total seed RNA by oligo-dT magnetic beads. cDNA was obtained by reverse transcription. The 3' region of the HMW secalin and glutenin cDNA were amplified by PCR using a forward primer corresponding to a glutenin gene nonapeptide repeat region and a reverse primer corresponding to the 3' end of the glutenin coding region. Multiple PCR products were analysed on a 10 % denaturing polyacrylamide gel.

Lanes 1&2: PCR products of the HMW secalin mRNA 3' region from rye

Lanes 3&4: PCR products of the glutenin mRNA 3' region from wheat

Lane 5: pBR322 *Hpa*II molecular weight markers

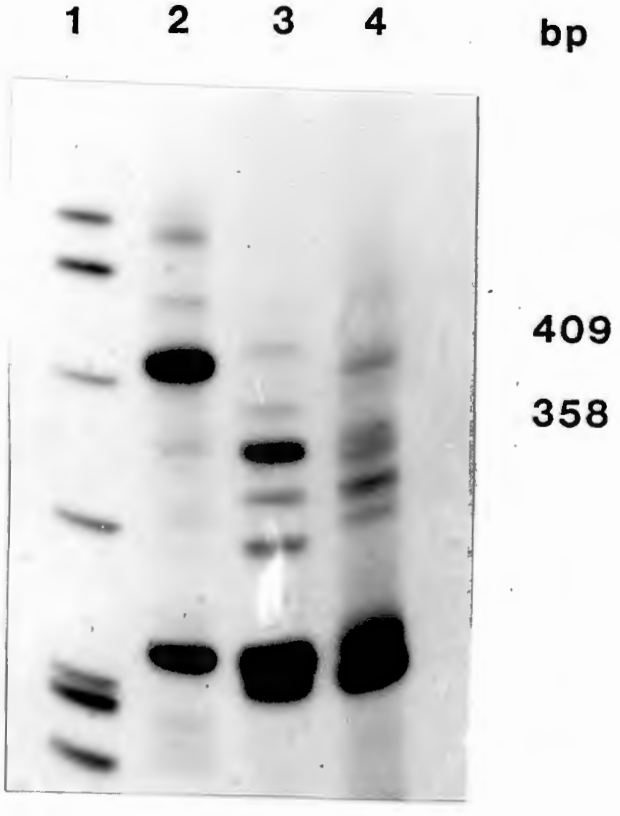


Figure 3.16 **RT-PCR Analysis of the cDNA 3' End Region of Seed Storage Proteins of an R2 Plant Transformed with the TAXI-pKG43**

mRNA was isolated from a kanamycin resistant R2 plant transformed with the TAXI pKG43. The 3' end of mRNA was amplified by RT-PCR. PCR products were analysed by PAGE (10 % denaturing gel).

Lane 1 : pBR322 *Hpa*II molecular weight markers.

Lane 2 : glutenin cDNA

Lane 3 : cDNA from untransformed rye

Lane 4 : cDNA from an R2 plant

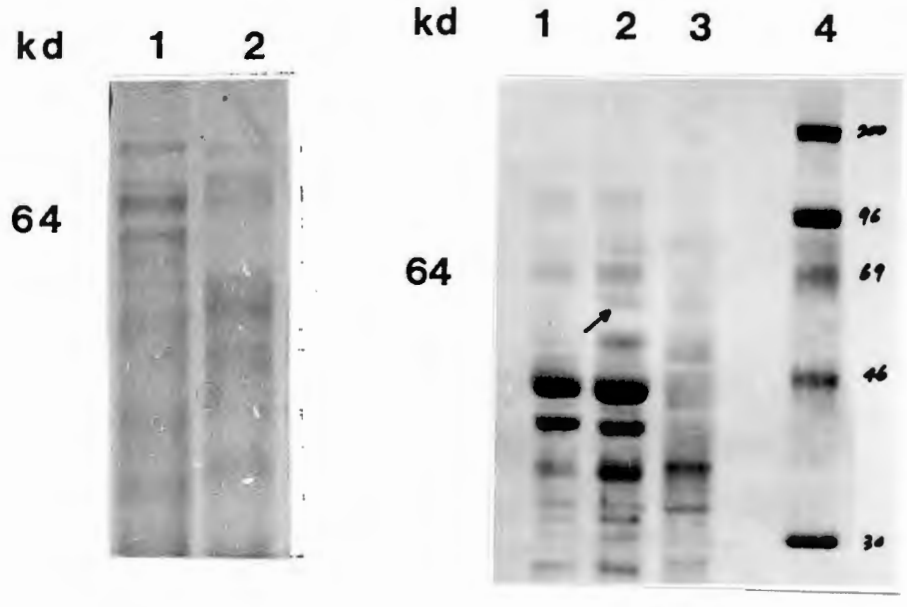


Figure 3.17 **Western Blot Chemiluminescence Assay of an R2 Plant Transformed with the TAXI-pKG43**

Proteins were separated by SDS gradient PAGE and the electroblot probed with anti-glutenin antibody linked to a chemiluminescence assay.

(A) Western blot of seed storage proteins

lane 1: seed storage proteins from an R2 plant

lane 2: seed storage proteins from an untransformed rye

(B) PAGE of seed storage proteins

lane 1: seed starage proteins from an untransformed rye

lane 2: seed storage proteins from an R2 plant

lane 3: seed storage proteins from wheat

lane 4: molecular weight markers

3.5 Discussion

3.5.1 Integration and inheritance of transgenes

On the blots of both R1 and R2 transformants of the TAXI-pMKG, *uidA* and *nptII* positive signals were found in the HMW region of unrestricted genomic DNA, and one to three positive signals were observed of different molecular weights in *Eco*RI (for *uidA*) and *Xho*I (for *nptII*) digested genomic DNA in individual transformants. Most of them appeared equal to or larger than the expected minimum size, indicating the integration and inheritance of one to three copies of pMKG. The smaller fragments may be the result of rearrangements or deletions during integration. On the blots of *Hind*III and *Sac*I (for *uidA*) and *Hind*III (for *nptII*) restriction digests, fragments having the size expected for intact *uidA* or *nptII* genes were demonstrated in both R1 and R2 plants.

Hybridization results of the TAXI-pKG43 transformants showed positive signals when the blots from R2 plants were probed with a *nptII* fragment. These signals were absent from untransformed rye. One R2 plants had a single band larger than pKG43, while another showed three larger and one smaller, similar to the integration and inheritance of pMKG.

These results have, therefore, proved that gene transfer by the TAXI can result in stably transformed rye plants with one to three copies of transgenes being inserted in different chromosomal loci in individual plants.

4.5.2 Expression of transgenes

Out of 4400 seedlings in the various experimental groups grown under kanamycin selection, a total of 304 plants grew to maturity. Between 3.7% and 11.6% of R1 and R2 progenies of putative transformants produced via either the TAXI-pMKG or TAXI-pKG43

were kanamycin resistant. In contrast, out of 2200 uninjected control seedlings only 8 naturally resistant rye survived (0.36 %). However, due to the fact seeds were pooled, the increase in kanamycin resistance from the R1 to the R2 generation cannot be used to show Mendelian segregation. In addition, expression of the integrated *nptII* gene may not be very high in rye due to the use of the *nos* promoter, which is not strong in cereals, and the use of very stringent selection conditions in these experiments.

This is supported by the *in vitro* analysis of NPT II activity which revealed a much higher gene expression rate, being 18 % for R1 and 70 % for R2 progeny respectively. This difference between the expression of NPT II activity tested *in vivo* (via kanamycin selection) and *in vitro* could be explained by the very stringent selection conditions used and the high sensitivity of the *in vitro* test.

Expression of the uidA gene also revealed from the R1 to the R2 generation (14% to 77%).

Expression of the glutenin gene in rye was demonstrated at both mRNA and protein levels. In the RT-PCR results, a 409-bp fragment only appeared in glutenin cDNA samples but not in untransformed rye samples. A 358-bp fragment was amplified from the latter which was absent in the glutenin cDNA samples. Both the 409-bp and 358-bp bands were amplified from the mRNA of R2 plants transformed with the TAXI-pKG43. Western blot analysis of glutenin showed an extra band in the expected molecular weight region (64 kDa) from R2 plants. These results indicate that the glutenin gene was expressed in the R2 progeny. Thus three genes have been successfully transferred into rye via the TAXI.

3.5.3 The mechanism of TAXI transfer of genes into plant cells

DNA transfer with the TAXI may be through the interaction between histone H1 and the plant cell membrane. Electron microscopic investigations have demonstrated that basic

polypeptides cause lesions in cell membranes and stimulate pinocytosis (Burgess et al., 1973). Laroche et al. (1988) found that only short polylysine molecules (MW about 4000; 35-38 amino acid residues) destabilize the phosphatidylcholine / phosphatidic acid bilayer. When the short polylysine molecules bind to the bilayer, they induce a decrease in the liquid-crystalline transition temperature, resulting in the membrane becoming more fluid and unstable. Histone H1 is a very basic protein. It contains a long tail in its C-terminus consisting largely of lysine intermixed with mainly alanine residues. There are no acidic amino acids in that region to balance the charge (Strickland et al., 1980a, 1980b). It is generally assumed that the C-terminal tail of histone H1 may move freely, in the absence of counter balancing acidic charges, similar to polylysine. When this tail comes into contact with a cell membrane, a very basic local charge may be produced. The membrane becomes more fluid and unstable. This interaction, in an analogy to basic polypeptides, may cause swelling of the cell and the production of lesions on the membrane. Such lesions, caused through a toxic effect of the histone H1, could constitute the port of entry for the TAXI.

The DNA within the complexes is presumably very well protected against nuclease attack because of the basic surface of the complex due to the presence of histone H1. This basic surface will inhibit any enzymatic action of nuclease(s) which may be present in the central vacuole of the plant cell. In this organelle, the enzymes operate in the region of pH5. This pH is maintained only inside the central vacuole by a proton pump (Boller et al., 1986).

Once the histone-DNA complex has entered a cell and subsequently the nucleus, facilitated through the NLS sequence in the C-terminus of histone H1, the ssDNA may become integrated into the plant genome during DNA replication again aided by histone H1 because of its recombinase and ligase activity. Whether the integrated transgenes can be clonally transmitted will very much depend on the transformed cell entering the cell cycle before it is lignified. Clonal proliferation of transformed meristem cells, into which the

foreign genes have been integrated, may have a developmental connection to the germ cell line. Transgenes may thus be propagated in subsequent plant generations.

In conclusion, TAXI mediated gene transfer constitutes an efficient tool for the genetic engineering of rye. It may well be applicable generally for bypassing genome type specificity and the complexity of plantlet regeneration. However, there were several oversights in the design and performance of the experiments reported here. These include the choice of the selectable marker gene, the absence of an intron sequence upstream of the GUS gene which could lead to GUS positive results due to bacterial contamination, uncontrolled pollination and the pooling of seeds from individual plants. In order to improve this new methodology for the genetic engineering of cereals, and also to remedy these oversights, the investigation was carried on to a third stage where a combination of TAXI mediated transformation and microprojectile bombardment was investigated.

Chapter 4

Establishment of a Rapid Regenerable Callus Line of a Grass Species (*Digitaria sanguinalis*)

Summary

A rapidly regenerable callus line was required for the investigation of the TAXI-biolistic process. A grass species (*Digitaria sanguinalis*) was chosen as the candidate. Embryogenic callus was initiated from young inflorescences on MS medium containing 2.5 mg/L 2,4-D. Plantlets were produced on the same nutrient medium without 2,4-D. Fertile regenerated mature plants were successfully obtained 8 - 10 weeks after callus initiation.

4.1 Introduction

In order to investigate the events of gene transfer, integration and inheritance in cereals using a new approach, the TAXI microprojectile bombardment, a rapid regenerable callus line would be desirable. Therefore, the establishment of a tissue culture procedure of somatic embryogenesis and plant regeneration was viewed as an essential component of this investigation into the TAXI biolistic technique.

Cereal and other grass species have generally been considered to be rather recalcitrant to plant tissue culture techniques and have also been among the most difficult to manipulate *in vitro* (Vasil, 1988). The earliest use of plant cell culture techniques in grass species was in 1944 (Brink et al.). However, in early work most plant regeneration was unreliable (Vasil and Vasil, 1986). Explants used as starting material in these studies were from differentiated and mature tissues or organs, resulting in root meristem proliferation which produced masses of nondividing terminally differentiated cells (Cure and Mott, 1977, King et al. 1978, O'Hara and Street, 1978). Improvements in the ability to obtain long-term, high frequency regeneration from tissue culture of cereals and other grass species occurred during the 1980s (for reviews see Vasil, 1986, 1987). This was because of the use of tissue / organ explants containing undifferentiated meristematic cells, high concentrations of 2,4-D, and plant regeneration by somatic embryogenesis (Vasil, 1982, 1983, 1984, 1985).

A Guinea grass species, *Panicum maximum* Jacq, was shown to undergo somatic embryogenesis and plant regeneration from suspension cells, leaf tissue, immature embryos, mature embryos and young inflorescences (Lu and Vasil, 1981a,b, 1982). These results provided detailed information useful for the production of a rapid regenerable callus line from the same species. A grass species, *Digitaria sanguinalis*, was therefore chosen as the test plant for the transfer of genes to cereals by TAXI microprojectile bombardment.

D.sanguinalis possesses similarities to cereal crops in some characteristics, e.g. there is some homology in their genomes and in the nature of their differentiation and development. An additional reason for this choice is that *D.sanguinalis* is sensitive to maize streak virus (MSV). It will therefore be a suitable test plant for another project, that of MSV resistance in transgenic maize. In this Chapter the induction of somatic embryogenesis and recovery of fertile plants from the young inflorescence of *D.sanguinalis* will be described.

4.2 Materials and Methods

4.2.1 Callus induction

Young unemerged inflorescences of *D.sanguinalis* were collected from field grown plants. Surface sterilization was carried out by washing with 70 % ethanol for 1 minute followed by 3.5 % sodium hypochlorite for 20 minutes, and then washing four times with sterile distilled water. Inflorescences were cut into 5 mm segments and transferred to agar medium. Murashige and Skoog's (1962) medium containing 2,4-D at concentrations of 2.5 mg/L was used for callus induction. The medium was adjusted to a pH of 5.8 with 0.1 N KOH before autoclaving and solidified with 0.8 % agar. Cultures were maintained in the dark at 26°C for the initiation of callus.

4.2.2 Plant regeneration

Embryogenic callus was transferred onto MS medium containing 0.1 mg/L NAA and 10 mg/L BA at 26°C with 16 hours of diffuse light for the initiation of plant regeneration. The callus having small plantlets was then transferred onto MS medium without hormones or plant growth regulators. Plant regeneration was carried out in agar in bottles, in a 33 : 33 : 33 mix of sand, compost and palm peat, in plant growth chambers and in potting soil.

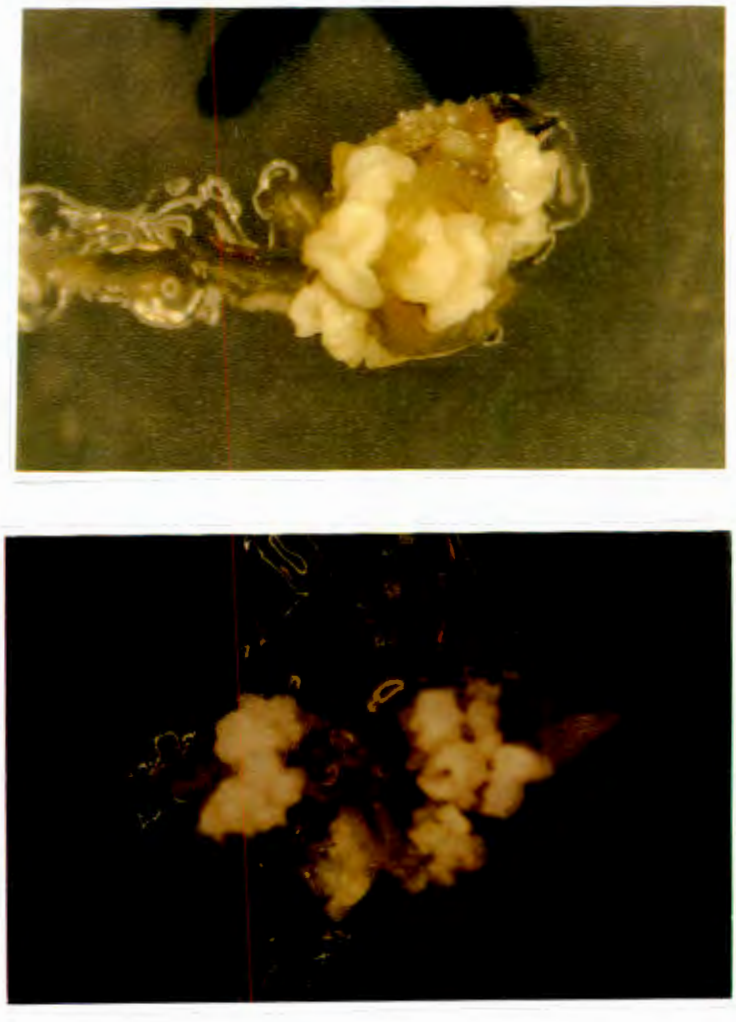
4.2.3 Germination of seeds collected from regenerated plants.

Seeds collected from regenerated plants were dried at 37°C in the dark for three days and then incubated at 4°C for ten days. In order to increase the frequency of germination, GA and a smoke-treated solution (provided by Dr. N.Brown, Kirstenbosch National Botanical Gardens) was used. Seeds were soaked in 0.05 % GA at room temperature for three hr and then transferred and incubated in a smoke-treated solution at room temperature for 24 hr. Seeds were soaked in 70 % ethanol for one minute and washed three times with sterile distilled water, before being transferred to the agar medium containing MS salts only. Germinated seedlings grew to maturity in a plant growth room.

4.3 Results and discussion

4.3.1 Callus initiation

The frequency of embryogenic callus production and plant regeneration depends very much on the nature of the initiating material and its developmental stage. In general most mature and differentiated tissues fail to form callus or plants (Vasil and Vasil, 1986). Best results are obtained using immature organs or meristematic tissue, particularly immature embryos, young inflorescences and the basal parts of young leaves (Vasil, 1987). In this study young inflorescences, nodes of young stems and young leaves of *D.sanguinalis* were used as starting materials to initiate callus. Callus was only produced from young inflorescences which were in the proper developmental stage. These embryogenic callus are white in colour, compact and had an organized appearance (Figure 4.1). A significantly different nature and behavior of callus tissue was observed when the initiating tissue was at different developmental stages.



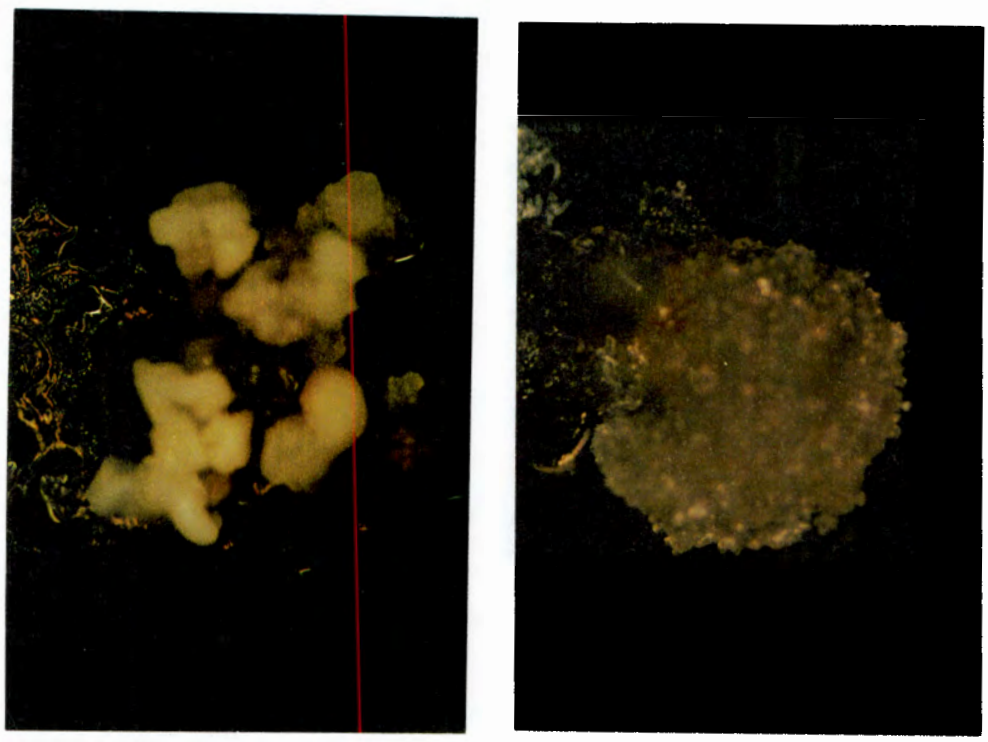
4.1 Callus Induction

Callus induced from young inflorescences of *Digitaria sanguinalis* 2 weeks after culturing on MS medium containging 2.5 mg/L 2,4-D.

It was found that when the stage was not right, either no callus was initiated or some soft, friable and translucent callus was produced (Figure 4.2). Such callus did not show any potential for embryogenesis. Similar findings have been reported by Lu and Vasil (1982). The use of inflorescences, in which the floral primordia were just being formed and had not fully differentiated, gave the best results.

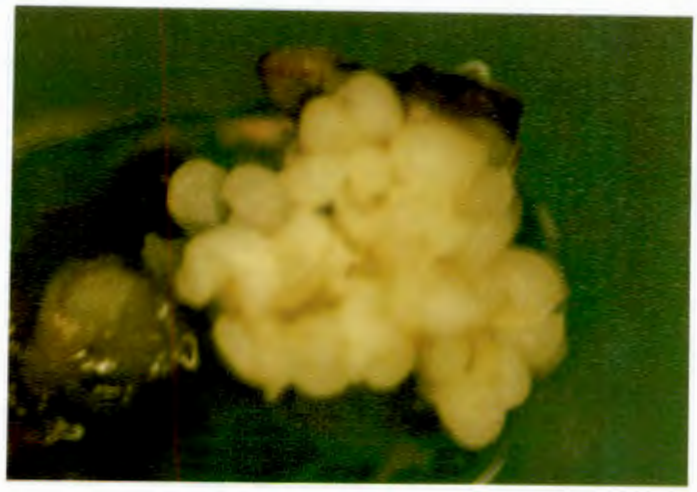
Callus tissue first became visible within 2 weeks on MS medium containing 2.5 mg/L 2,4-D. No significant differences were found in callus formation with the use of 2.5, 3.5 and 5 mg/L 2,4-D in the culture medium. Adequate levels of 2,4-D may play a role in perpetuating the embryogenic nature of cultures by continued divisions in embryogenic cells and the active meristematic zone of somatic tissues (Vasil, 1987). Reducing the concentration of 2,4-D in the culture medium, e.g. 0.2 mg/L, would result in organization of somatic embryos. In addition, it has been thought that some molecules, e.g. the plasma membrane arabinogalactan protein (AGP) epitope, may have a direct biological function in embryogenesis (Schmidt et al., 1994). Experiments reported by de Vries et al. (1988) and Kreuger et al. (1993) suggest that specific molecules, which are totally different from conventional plant growth regulators and clearly developmentally regulated, are able to direct the transition of somatic cells into embryogenic cells.

Embryogenic callus could be maintained for long periods of time without losing its embryogenic ability (Figure 4.3). In this work, *D.sanguinalis* calli have been maintained for seven months without their embryogenic ability being reduced.



4.2 Embryogenic and Non-embryogenic Calli

Two different types of calli were induced from tissues of *D.sanguinalis*. Embryogenic callus was white in colour, compact and organized in nature (left). Non-embryogenic callus was generally soft and friable (right).



4.3 Somatic Embryos

The appearance of somatic embryos of *D.sanguinalis* on the initial young inflorescence tissue within 3 weeks of culture.

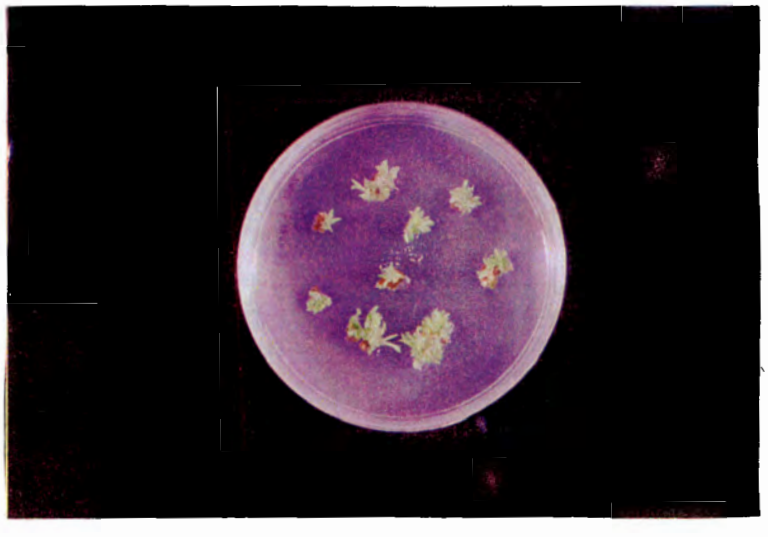
4.3.2 Plant regeneration

The compact and white callus continued to proliferate on 2,4-D medium. When the callus was transferred to a medium containing 0.1 mg/L NAA and 10 mg/L BA, the typically organized grass embryos were formed on the surface of the callus within 10 days. These embryos germinated to form plantlets on MS medium without hormones or plant growth regulators (Figure 4.4). Regenerated mature plants were successfully obtained 3 weeks after plantlets were transferred to soil (Figure 4.5). Generally 20 - 30 mature plants from each inflorescence segment were produced in about 8 - 10 weeks (Figure 4.6).

Formation of somatic embryos of *D.sanguinalis* took place rapidly in culture. Germination of these embryos was also rapid, but in some cases a few plantlets appeared white in color, and regenerated plants appeared precocious. It has been suggested that this precociousness can be partly controlled by the addition of abscisic acid to the nutrient medium (Vasil and Vasil, 1986).

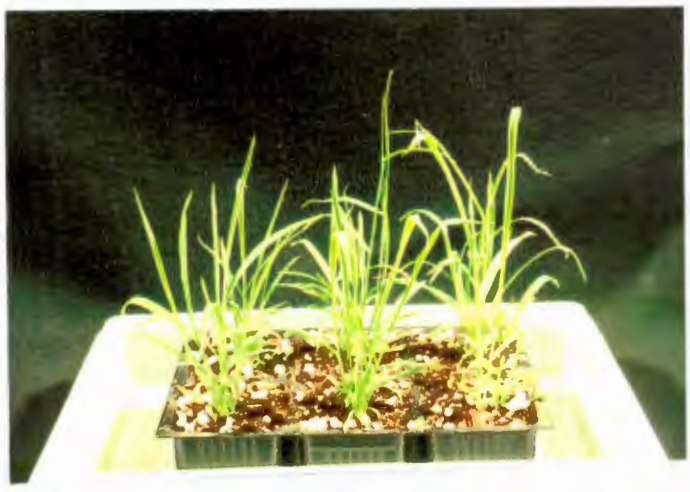
Seeds could be harvested from regenerated plants three months after starting callus initiation culture. A specific treatment is required for germinating seedlings (see Materials and Methods). Cold treatment and heat shock may function in imitating a winter- and spring-like process, by which the frequency of seed germination was greatly increased (data not shown).

The procedure described in this chapter contributed to the establishment of a rapid regenerable callus line of *D.sanguinalis*. This callus line can be a good candidate for investigating the transfer of foreign genes to cereals and other grasses.



4.4 Regeneration of Plantlets

Plantlets regenerated from somatic embryos 2 weeks after being transferred onto MS medium containing 10 mg/L BA and 0.1 mg / L NAA (Top). These plantlets were then transferred onto MS medium without hormone and plant growth regulators (bottom).



4.5 Growth of Regenerated Plants

Regenerated plants were transplanted from agar medium (top) into a solid mixture (bottom), and grown for about 10 days in a plant room before being transplanted into soil.

4.6 Fertile Regenerated *D. sanguinalis*

Regenerated plants flowering 3 weeks after being planted into soil. Seeds could be collected from regenerated *D. sanguinalis* 10 - 12 weeks after callus initiation.

Chapter 5

TAXI Bombardment, a New Approach for the Transfer of Genes to Plants

Summary

Histone H1-protected single stranded DNA, carrying a selectable marker gene (*bar*) and a reporter gene (*uidA*), was coated onto gold particles and delivered into embryogenic callus of *D.sanguinalis* by the biolistic process. Transformed calli were selected on a medium containing 3 mg/L bialaphos. R0 plants regenerated from bialaphos resistant embryogenic calli were analyzed by Southern blot hybridization. The results demonstrated independent transformation events and revealed single or low copies of the *bar* gene integrated into the plant genome. Inheritance of transgenes in R1 bialaphos resistant plants of five transgenic lines was analyzed by Southern blot hybridization and GUS activity assays. The presence of the *bar* gene in the progenies of all five transgenic lines demonstrated stable transformation events. Transgenic plants carried between one and three copies of the *bar* gene. Mendelian 3:1 segregation of transgenes was observed in four of the five R1 populations.

5.1 Introduction

The technique of TAXI mediated gene transfer to plants was developed in the production of transgenic rye (see Chapter 3). The major advantages of this technique is that a specific structural and functional macromolecular complex, imitating the structure of the natural T-DNA complex in the *Agrobacterium tumefaciens* transformation system, was used for the delivery of DNA into plant wound sites. This artificial T-DNA complex may improve the efficiency of the transfer and integration of genes into plant cells. However, the method of TAXI delivery was not ideal, as during its injection into seedling meristems using a fine needle most cells in the wound sites were destroyed. Only a few intact cells have the chance of being transformed. Integration of genes carried by the TAXI is dependant on the number of intact cells transformed, whether these cells would carry on division to form colonal growth, and whether these transformed clones would develop correctly. Any strategy to increase the frequency of the TAXI mediated transformation should, therefore, focus on the method of DNA delivery and the choice of the target tissue.

Microprojectile bombardment is the only available technique for direct gene transfer where the cell wall need not be considered as an obstacle (Sanford, 1993). This technique does not suffer from many of the restrictions characteristic of others, notably, it is not restricted to certain specific genome, tissue and cell types. For these reasons this technique has been successfully used to produce transgenic cereals which appear to be difficult to obtain by other methods. On the one hand, the biolistic process is extremely efficient in the delivery of DNA into intact tissues and cells. On the other hand, however, because it has no direct effect on chromosomal integration mechanisms as the *Agrobacterium* system apparently does, lower frequencies of stable transformation (Birch and Franks, 1991) and multiple copies of inserted transgenes which may result in transgene inactivation (Finnegan and McElroy, 1994), render this powerful method somewhat suboptimal.

Considering all the advantages and disadvantages inherent in both the TAXI transformation and the biolistic process, a combination of these two systems to develop a new approach for the transfer of genes into plants led to the investigation of a third stage. The TAXI-biolistic process has, therefore, been tested and developed in the production of stable transgenic *D.sanguinalis*.

5.2 Materials and Methods

5.2.1 Vector construction

The plasmid used in this study was pUGB35 (Figure 5.1), derived from pBluescript SK. The bialaphos resistance gene (*bar*) and the GUS gene (*uidA*) were used as selectable marker genes. The *bar* gene, fused between the CaMV 35S promoter, the intron 1 sequence of rice alcohol dehydrogenase (*Adh1*) and the *nos* terminator sequence, was taken from pMFBAR (supplied by Mr. K. Palmer, Department of Microbiology, University of Cape Town). The *uidA* gene, under the control of the maize ubiquitin promoter and intron 1 sequences, was taken from pAHC25 (Taylor et al., 1993). These two genes were joined in the same orientation and inserted into the *Hind*III and *Cla*I sites of pBluescript SK. A synthetic oligonucleotide, supplying the right border sequence of the nopaline Ti plasmid, was inserted between the *Eco*RI and *Eco*RV sites of pBluescript SK.

5.2.2 Coating gold particles

The *Eco*RI site in the maize ubiquitin intron 1 sequence of pUGB35 appears insensitive to restricted digestion. The plasmid could, therefore, be linearized by *Eco*RI digestion at the end of the right border sequence without the breakage of transgene regions. Single stranded

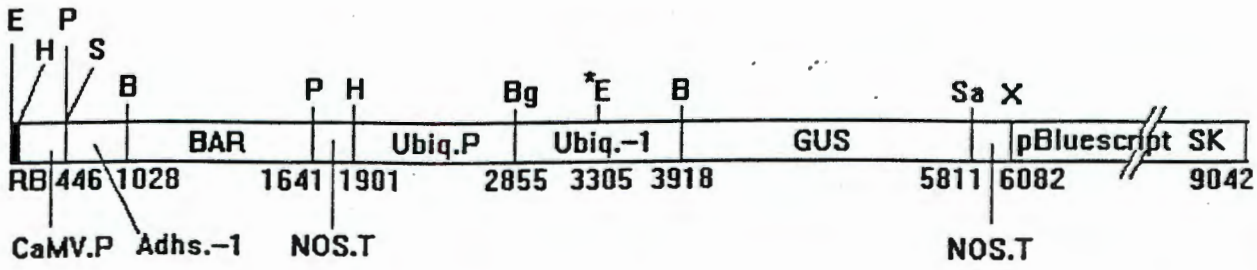


Figure 5.1 The Map of pUGB35 Used For TAXI Bombardment

Vector pUGB35 was derived from pBluescript SK. The *bar* gene, driven by the CaMV 35S promoter plus Adhs intron-1, and the GUS gene, driven by the maize Ubiquitin promoter plus intron-1, were inserted into the polylinker region of pBluescript SK.

RB : black box, right border sequence of nopaline Ti plasmid.
CaMV 35S.P : Clauliflower mosaic virus 35S RNA promoter
Adhs.-1 : Rice alcohol dehydrogenase gene intron-1
Ubiq.P. : Maize ubiquitin promoter
Ubiq.-1 : Maize ubiquitin gene intron-1

E : *Eco*RI H : *Hind*III P : *Pst*I S : *Sal*I
B : *Bam*HI Bg : *Bgl*I Sa : *Sac*I X : *Xho*I

DNA was produced by heat denaturation and bound to histone H1 at the mass ratio of 1 : 2.5 (see Chapter 3). This DNA-protein mixture was kept on ice for 30 minutes to allow stable binding to occur.

Gold particles (about 1 um) in 50 % glycerol, prepared as described by Dunder and Pace (1993), were precipitated by spinning in a microcentrifuge for five seconds and resuspended in 10% TE buffer. For one shot, 20 uL gold particles (0.5 mg/uL) mixed with an equal volume of ssDNA-protein complex (50 ng/uL of ssDNA). The coating was accomplished by a 30 min vortex. The gold particles were collected by centrifugation, washed with 10 % TE. resuspended in 10 uL 10 % TE and the coated gold particles were loaded onto a disk, and dried in a desiccator under vacuum. It is important to use these particles for bombardment as soon as possible. Gold particles were coated with plasmid alone according to the method described by Dunder and Pace (1993).

5.2.3 DNA delivery into callus

The addition of an osmoticum to the bombardment medium can dramatically increase the rate of transformation (Sanford et al., 1993). Embryogenic calli of *D.sanguinalis* were transferred onto MS medium containing 2.5 mg/L 2,4-D, 100mg/L myositol and 0.2 M mannitol overnight before being used as target materials for bombardment.

Embryogenic calli were bombarded with gold particles coated with plasmid or the TAXI using the Dupont PDS- 1000 / He (helium) system. The amount of gold used for one shot was 480 ug, two shots per dish, with rupture discs that burst at 1100 and 1550 psi at a distance of 6cm between stopping screen and the target. Bombarded calli were incubated for 24 hr before being transferred onto the same medium without manitol. Embryogenic callus bombarded without DNA was used as a control in all experiments.

5.2.4 Selection of bialaphos resistant callus and plant regeneration

Calli were transferred to selection medium containing 2.5 mg/L 2,4-D and 3 mg/L bialaphos seven days after bombardment. Subculturing was carried out every second week. Calli growing on selection medium for four weeks were transferred onto regeneration medium (see Chapter 4) with 3 mg/L bialaphos for another four weeks. Regenerated bialaphos resistant plantlets continued growing on MS medium without hormones and plant growth regulators, with 3 mg/L bialaphos before being transplanted into a solid mixture. Further selection was carried out at the mature stage of regenerated plants. A herbicide solution, IGNET (supplied by Institute of Fruit Technology), containing 0.0066% PPT was used to spray regenerated plants growing in soil. Seeds could be collected from PPT resistant *D.sanguinalis* four months after bombardment.

5.3 Results

5.3.1 Microprojectile bombardment and selection of transformed callus lines

Callus bombarded with different acceleration pressures, 1100 or 1550 psi, had slightly different effects in the TAXI mediated transformation. Using 1100 psi gave rise to the greatest number of bialaphos resistant callus. Control calli were placed on MS medium containing 2.5 mg/L 2,4-D and 1 mg/L, 2mg/L, 3 mg/L or 4 mg/L bialaphos in order to determine a concentration suitable for selection. About 30%, 10%, few and no control calli could survive in the presence of 1, 2, 3, and 4 mg/L bialaphos after two subcultures (four weeks) of selection. Plants were not obtained from the surviving control calli when transferred onto a regeneration medium containing 3 mg/L bialaphos. The calli became

brown in 15 - 20 days on this medium. Therefore, 3 mg/L bialaphos was used in both selection and regeneration precedures.

Selection of transformed callus was initiated in a medium containing 3 mg/L bialaphos one week after bombardment. After four weeks selection, resistant calli were transferred onto a regeneration medium containing 3 mg/L bialaphos for another four to six weeks. Regenerated bialaphos resistant plantlets were then grown to mature in a solid mixture and soil in a plant room. Table 5.1 summarizes three transformation experiments and Figure 5.2 demonstrates callus selection and plantlet regeneration.

5.3.2 Analysis of R0 plants

Transgene integration and expression was analyzed in the R0 plants. The enzyme activity of phosphinothricin acetyltransferase (PAT) encoded by the *bar* gene was determined by inactivation of phosphinothricin (PPT). A series of concentrations of a herbicide solution, IGNET, was used in titration experiments by spraying control plants. No plants survived three to four days after spraying 5% IGNET solution containing 66 mg/L PPT. When the same solution was used for spraying plants regenerated from 16 transformed lines, the plants from 14 lines remained healthy and green (Figure 5.3). Leaves of the plants from two lines showed some necrotic areas.

GUS activity was analyzed histochemically and by fluorescent means, according to the methods described by Jefferson (1987) and Peng et al (1993). Blue spots were observed in the leaf tip of some R0 plants (Figure 5.4a), however, most gave negative histochemical results. GUS activity in transformed plants was therefore analyzed using a fluorescence assay. Instead of MUG, a very sensitive substrate, ß-trifluoromethyl umbelliferyl ß-

Table 5.1 The Results of Three Independent Transformation Experiments

Experiment number	Velocity (psi)	Callus samples bombarded	Regenerated plants/callus line
I	T-1550x2	63	8 / 3
	T-1100x2	60	8 / 5
	P-1550x2	59	0 / 0
II	T-1550x2	27	2 / 2
	T-1100x2	29	3 / 2
	P-1550x2	31	0 / 0
III	T-1550x2	32	1 / 1
	T-1100x2	34	5 / 3
	P-1550x2	30	2 / 1
Total	T-1550x2	122	11 / 6
	T-1100x2	123	16 / 10
	P-1550x2	120	2 / 1

* T-1550x2 : bombarded twice with TAXI using 1550 psi.
T-1100x2 : bombarded twice with TAXI using 1100 psi
P-1550x2 : bombarded twice with plasmid using 1550 psi

A B

Figure 5.2 Selection and Regeneration of R0 Plants Transformed with the TAXI-pUGB35

Bombarded embryogenic calli of *D.sanguinalis* were selected on MS medium containing 3 mg / L bialaphos and 2.5 mg / L 2,4-D for one month. Regeneration was carried out under the presence of 3 mg / L bialaphos.

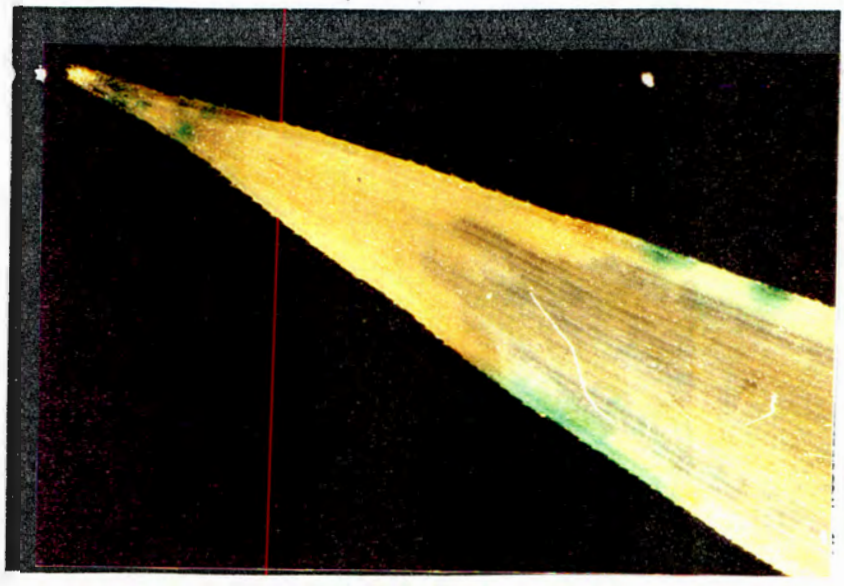
A: left: plantlets regenerating from callus bombarded with the TAXI-pUGB35.
right: unbombarded calli.

B: regenerated plantlets produced by TAXI bombardment.

5.3 Herbicide Resistant *D.sanguinalis*

Regenerated mature plants were tested for resistance to PPT. The IGNIT solution containing 66 ug / mL PPT was sprayed onto the plant surfaces. Four days after spraying, herbicide resistant plants were green (right) while sensitive plants (untransformed) became yellow and died (left).

A



B

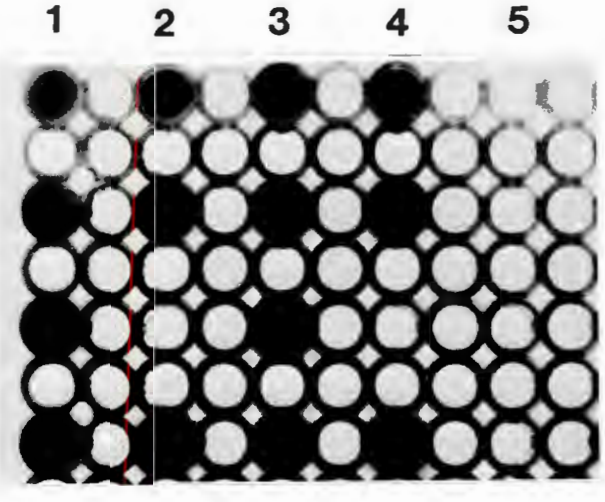


Figure 5.4 GUS Activity Assays of R0 Plants Transformed with the TAXI-pUGB35

Detection of GUS activity in R0 plants was performed either histochemically (Jefferson 1987) or by fluorescent means (Peng et al. 1993).

A: Histochemical assay. Pigment spots demonstrate GUS activity.

B: Fluorescent assay. Dark wells demonstrate GUS activity.

Lanes 1-4: bialaphos resistant R0 plants were inoculated in every second well in each row.

Lane 5 : untransformed plants

glucuronide (TFUG), was used in the reaction. 14 out of 16 bialaphos resistant plants were GUS positive (Figure 5.4b).

Transgene integration was analyzed by Southern blot hybridization. 16 TAXI-pUGB35 transformed and two plasmid pUGB35 transformed R0 plants were analyzed. Non-restricted and *Eco*RI digested genomic DNA were probed with a ^{32}P-labeled 0.6-kb *Sma*I *bar* fragment from pDPG165 (Figure 5.5) or the 1.8-kb *uidA* fragment from pBI121.

On the blots probed with the *uidA* fragment, the results appeared complicated (Figure 5.6). Hybridization occurred with high molecular weight DNA of these transformed plants. However, positive signals were also observed in one untransformed plant. This phenomenon has also been observed in other transformation experiments reported by Castillo et al. (1994).

On the blots probed with the *bar* fragment, positive signals were observed in the high molecular weight region of R0 plants, but absent in untransformed plants (Figure 5,7A). Figure 5.7B demonstrates the hybridization of *Eco*RI restricted genomic DNA from R0 and untransformed plants.

5.3.3 Analysis of R1 plants

Germination of seeds collected from 14 transgenic lines following self pollination was attempted as described in 6.2.4, but only five were successful. Tiny germinated seedlings were selected on a medium containing 3 mg/L bialaphos for four weeks. Herbicide sensitive seedlings became brown and died after 10 - 15 days. Resistant seedlings remained green and continued to grow (Figure 5.8). After four weeks of selection, resistant plants

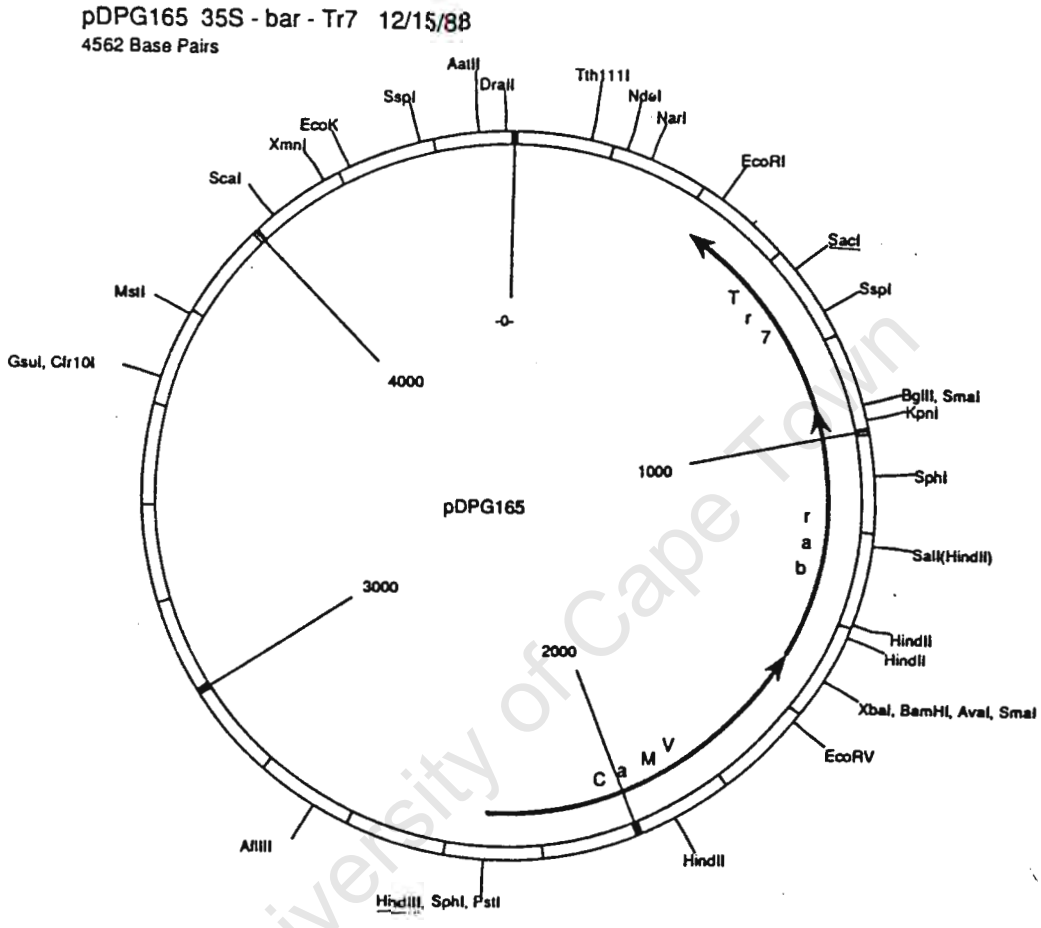


Figure 5.5 The Map of pDPG165

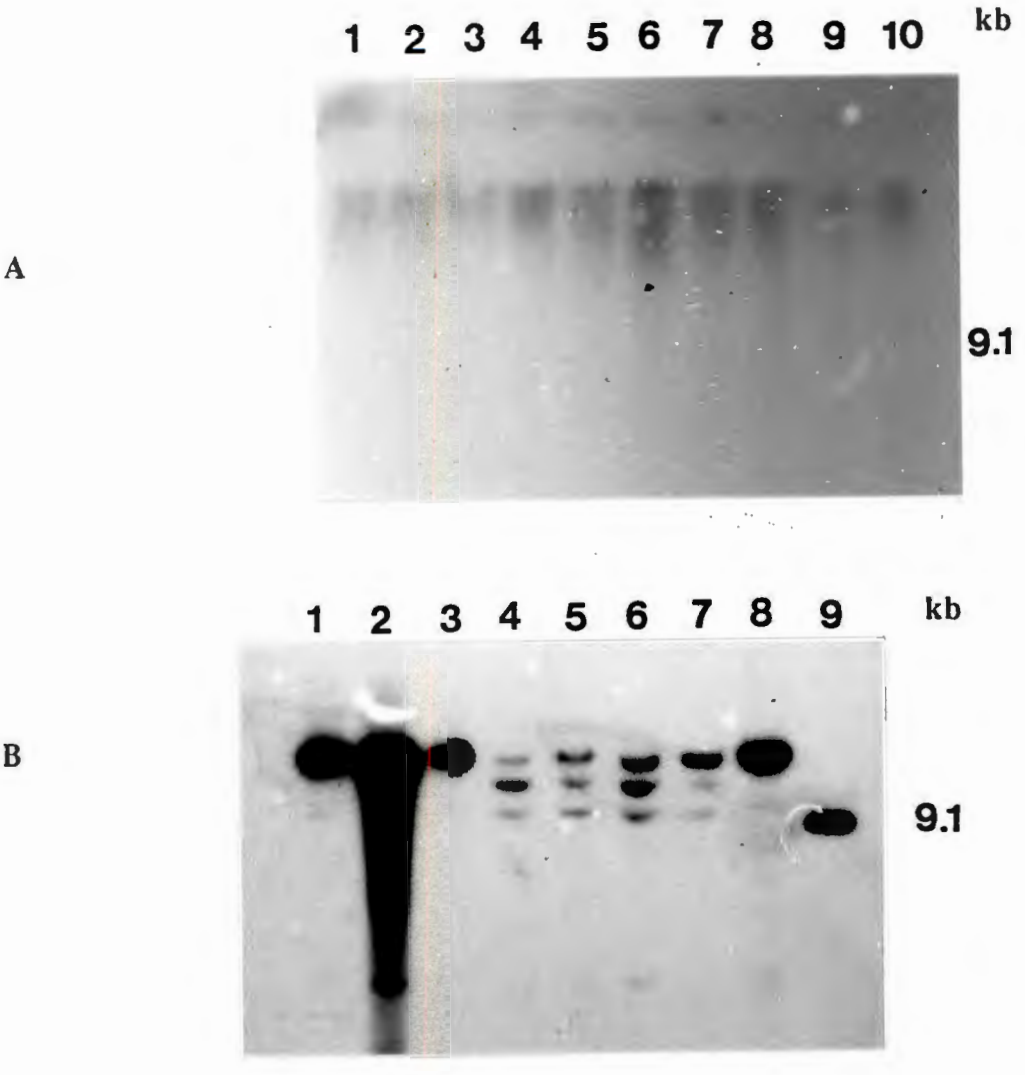


Figure 5.6 **Southern Blot Analysis of the GUS Gene in the R0 Bialaphos Resistant Plants Transformed with the TAXI-pUGB35**

Genomic DNA isolated from bialaphos resistant R0 plants hybridized with a ^{32}P-labelled 1.8-kb *Bam*HI/*Eco*RI *uidA* fragment from pBI121.

A: The blot of unrestricted genomic DNA.
- Lanes 1&2 untransformed plants
- Lanes 3-10: R0 plants

B: The blot of *Eco*RI restricted genomic DNA
- Lanes 1-7: R0 plants
- Lane 8: untransformed plant
- Lane 9: linear pUGB35

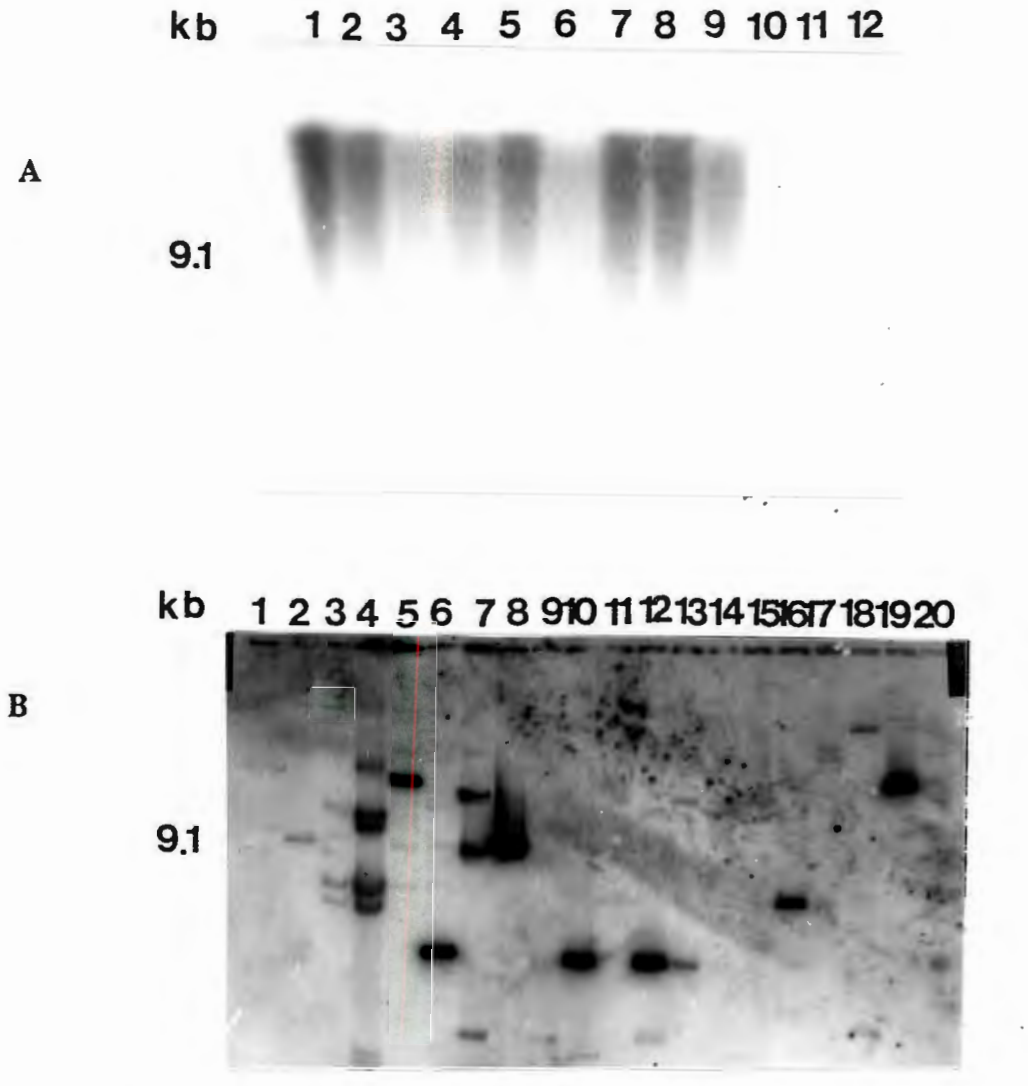


Figure 5.7 Southern Blot Analysis of the *bar* Gene in Bialaphos Resistant R0 Plants Transformed with the TAXI-pUGB35

Genomic DNA was isolated from bialaphos resistant R0 plants and hybridized with a ^{32}P-labelled 0.6-kb *Sma*I *bar* fragment from pDPG165.

A: Hybridization of unrestricted genomic DNA.
- lanes 1-10: R0 bialaphos resistant plants.
- lanes 11&12: untransformed plants.

B: Hybridization of *Eco*RI restricted genomic DNA.
- lane 1: untransformed plant
- lane 2: linear pUGB35
- lanes 3&4: plants transformed by naked plasmid pUGB35
- lanes 5-20: 16 R0 plants individually transformed with the TAXI-pUGB35

A

B

Figure 5.8 Bialaphos Selection of R1 Seedlings

Seeds collected from regenerated *D.sanguinalis* were germinated on agar containing MS salt only. Germinated seedlings were then transferred onto a medium containing MS salt and 3 mg/L bialaphos for 4 weeks.

A: R1 seedlings of *D.sanguinalis* transformed with the TAXI-pUGB35 under the selection conditions.

B: Bialaphos resistant selection was carried out for 4 weeks.

were transplanted and grown in a solid mixture and soil. These plants were then analyzed for the inheritance and expression of transgenes at the molecular level.

Inheritance of transgenes in the R1 plants was confirmed by Southern blot analysis. Blots containing *Eco*RI digested genomic DNA from leaves of R1 and untransformed plants were hybridized with a ^{32}P-labeled 0.6-kb *Sma*I *bar* fragment from pDPG165. Four R1 plants from each transgenic line were analyzed (Figure 5.9).

Expression of the transgenes was analyzed by herbicide resistance and GUS activity assays. Figure 5.10 demonstrates the results of the lattter. Table 5.2 summarizes the expression of transgenes in R1 progeny of five transgenic lines. Mendelian segregation ratios of 3 : 1, were shown in four out of five lines ($P > 0.1$).

5.4 Discussion

Microprojectile bombardment is an effective technique for the transfer of genes into intact plant tissues and cells. Evidence of transient expression of a reporter gene, *uidA*, in plant cells after bombardment indicated that more than 90% of GUS expressing cells directly received the foreign gene in their nuclei (Yamashita et al., 1991, Hunold et al., 1994). It was reported (Yamashita et al., 1991) that approximately 3 % of bombarded cells were observed to contain gold particles and in most cases these were located in the vacuole or cytoplasm of the cells. Only in a few cases, did cells have a gold particle in their nuclei. This low frequency appears to be one of the main disadvantages in gene transfer by the biolistic process.

In order to increase the frequency of transformation, the use of histone H1 as a ssDNA binding protein was hoped not only to protect single stranded DNA from nuclease attack,

Table 5.2 Expression of Transgenes in R1 Plants of Five Transgenic Lines

Lines	No. of plants tested	Bialophos resistance (R / S)	(%)	P[a]	GUS activity in resistant plants[b]
TAXI-1	68	47 / 21	68	0.3-0.5	24 / 3
TAXI-3	45	32 / 13	71	0.5-0.7	21 / 0
TAXI-6	34	18 / 17	53	< 0.01	10 / 0
TAXI-8	16	11 / 5	69	0.5-0.7	7 / 0
TAXI-9	23	19 / 4	83	0.5-0.7	10 / 0

[a] P: probability that bialaphos resistance in the R1 plants conforms to the Mendelian inheritance ratio of 3 : 1.

[b] Not all bialaphos resistant plants were tested.

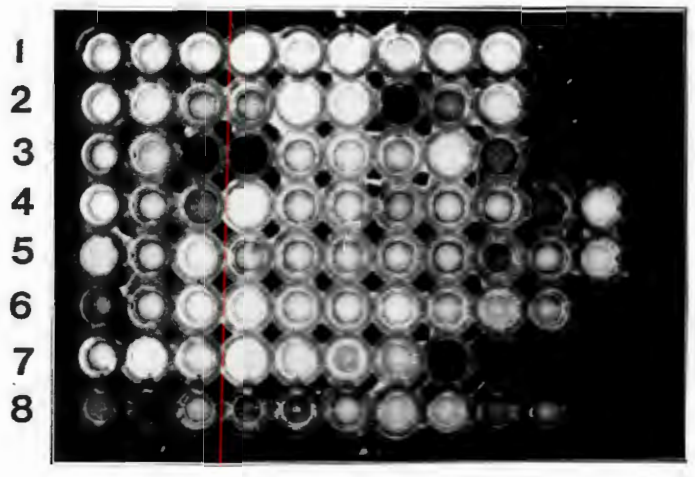


Figure 5.10 Fluorescence Assays of the GUS Activity in R1 Bialaphos Resistant Plants

Fluorescence assays of GUS activity in R1 bialaphos resistant progenies of five transgenic lines were carried out as described by Peng et al. (1993).

Rows 1 - 3: TAXI-1 R1 plants
Rows 4 - 5: TAXI-3 R1 plants
Row 6: TAXI-6 R1 plants
Row 7: TAXI-8 R1 plants
Row 8: TAXI-9 R1 plants

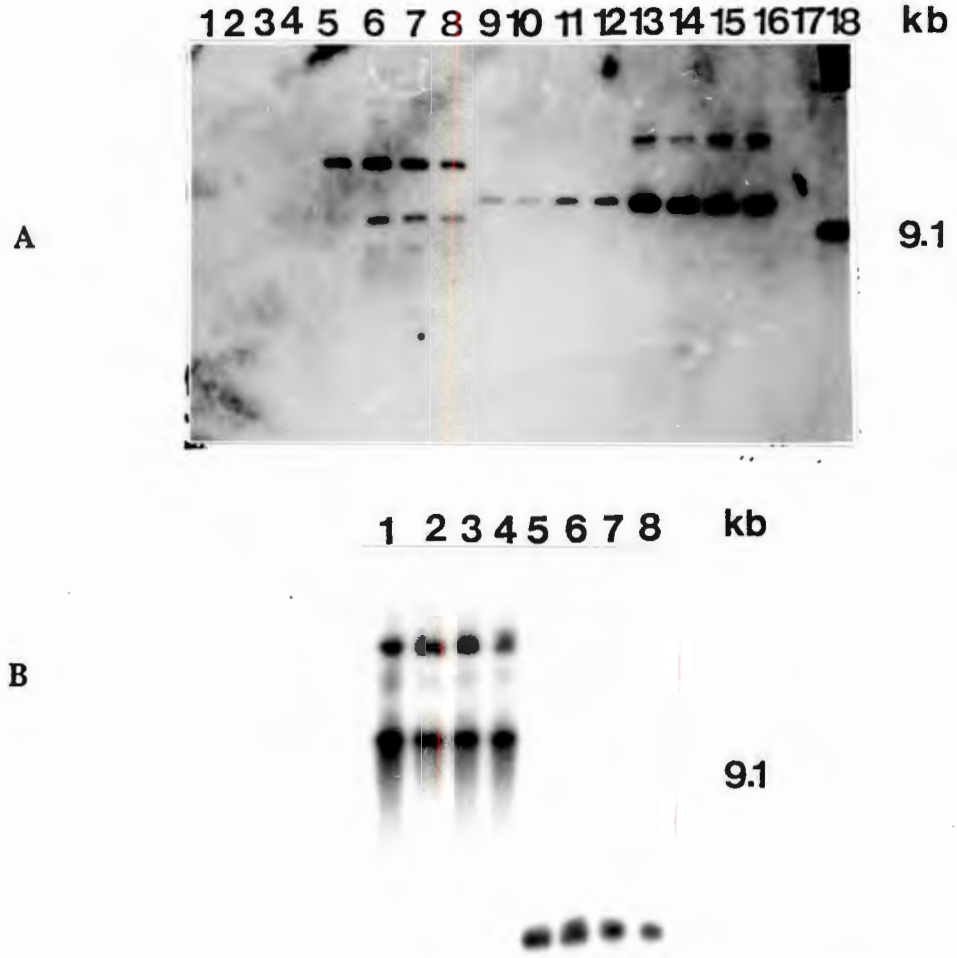


Figure 5.9 Southern Blot Analysis of the *bar* Gene in Bialaphos Resistant R1 Plants Transformed with the TAXI-pUGB35

Genomic DNA was isolated from R1 bialophos resistant progenies of five transgenic lines, digested with *Eco*RI and hybridized with the ^{32}P-labelled 0.6-kb *Sma*I *bar* fragment from pDPG165.

A:	Lanes 1-4:	untransformed plants
	Lanes 5-8:	4 R1 plants from TAXI-3
	Lanes 9-12:	4 R1 plants from TAXI-9
	Lanes 13-16:	4 R1 plants from TAXI-1
	Lane 17:	no DNA sample
	Lane 18:	linear pUGB35
B:	Lanes 1-4:	4 R1 plants from TAXI-8
	Lanes 5-8:	4 R1 plants from TAXI-6

but also to pilot DNA into the cell nucleus. The investigation of the TAXI transformation described in Chapter 4 demonstrated that histone H1 may play an important role in nuclear targeting of ssT-DNA, because of its nuclear location sequences (NLS). Therefore, using a histone H1-ssDNA complex to deliver foreign genes into plant cells by particle bombardment should theoretically increase the frequency of transformation. In addition, the use of a precisely linearized single stranded DNA, instead of a cyclic double stranded plasmid, may also improve the integration of transferred DNA (discussed in Chapter 3).

5.4.1 The evidence for stable transformation

The experiments reported here demonstrated that stably transformed *D.sanguinalis* plants were produced by the TAXI biolistic process. The evidence for this is as follows :

1) Selection and regeneration of transformed calli occurred in the presence of bialaphos in the culture medium.

2) The expression of the transgenes in R0 plants was detected by GUS activity assays and resistance to the direct application of PPT.

3) Transgene integration in R0 and R1 plants was demonstrated by Southern blot analysis.

4) Mendelian segregation of the *bar* gene in R1 plants demonstrated inheritance of the transgene in the progeny of four transformed plants.

A more detailed discussion of these four lines of evidence is given below.

1) Selection and regeneration of transformed calli in the presence of bialaphos in culture medium.

In an early report on transgenic wheat, many false positive and non-transformed but Basta resistant lines were obtained (Vasil et al., 1992). In this study, to prevent non-transformed calli escaping from selection, bialaphos was used to substitute for Basta not only in the

selection medium but also in regeneration media. The processes of selection and regeneration were carried on for eight to ten weeks. Only the callus which could survive and regenerate to a plantlet under these conditions was considered as a putatively transformed line. Later evidence by the direct application of PPT to plants and molecular analysis showed that only two out of 16 resistant lines were "escapes". It is therefore critical to regenerate plants under selection. This observation is in agreement with the results reported in barley (Wan and Lemaux, 1994) and rye (Castillo et al., 1994).

2) The expression of the transgenes in R0 plants was detected by GUS activity assays and direct application of PPT

The apical portions of leaves of the 16 bialaphos resistant plants were stained with X-Gluc to screen for GUS activity. However, only two of these showed blue spots at the leaf tips. A fluorescence assay, using TFUG as the substrate instead of MUG, made the detection more sensitive. 14 out of the 16 plants were GUS positive. However, it is difficult to understand why the histochemical assay was not sensitive enough to detect the expression of the GUS gene, because the maize ubiquitin promoter is considered to be very strong.

A 0.0066 % PPT solution, IGNIT, was used to spray untransformed and regenerated plants. 14 out of 16 plants showed full resistance to PPT. The other two plants showed necrotic areas on some leaves. Untransformed plants, however, could not survive and wilted one week after spraying. These results corresponded well with Southern blot analysis, as the DNA of the two GUS negative R0 plants (No.5 and No.7) showed no hybridization with the *bar* probe. The reason for these two plants regenerating under selection may be because the original calli consisted of both transformed and non-transformed cells. The possible chimeric nature of transgenic calli is not surprising given the multicellular target initially bombarded. Non-transgenic cells may be protected by the transgenic cells in the callus, allowing the former to proliferate while under selection. The use of bialaphos selection

during the regeneration process may prevent non-transgenic plants regenerating from chimeric transgenic calli (Fromm et al., 1990, Castillo et al., 1994, Becker et al., 1994). However, the escape of a few chimeric plantlets from selection may still occur because of their partial resistance.

3) Transgene integration in R0 and R1 plants was demonstrated by Southern blot analysis

Southern blot analysis showed that all R0 plants surviving from direct application of PPT contained one to three insertions of the *bar* gene in their genome. Different patterns of integration were seen in plants from different lines. These results indicate that the *bar* gene had been inserted in different chromosomal loci in the individual transformation events. Southern blot analysis of R1 plants from five transgenic lines further proved that the *bar* gene had been stably integrated and inherited in R1 progenies. Four plants each of four transgenic lines showed one to three insertions of the *bar* gene in their genome. Compared with R0 plants the DNA bands of the R1 plants of line TAXI-1, TAXI-3 and TAXI-9 are constant. However, that of line TAXI-8 were moved to a higher molecular weight region. It is suspected that modifications such as methylation in one or two *Eco*RI restriction sites could be responsible for the altered DNA migration pattern, because these plants showed the same range of herbicide resistance as the parent. Both R0 and R1 plants of line TAXI-6 showed a low molecular weight band, about 3.5 kb, hybridizing with the *bar* probe. These plants are bialaphos resistant and showed GUS activity in their leave (Figure 5.10), which demonstrated the expression of both transgenes. The 3.5-kb band on the blot of *Eco*RI restricted genomic DNA may have resulted from the activation of the *Eco*RI site in the ubiquitin intron-1 region after the T-DNA had been integrated into the plant genome. None of the untransformed plants showed hybridization.

A major achievement of the TAXI biolistic transformation compared with standard plasmid transformation is the single or low numbers of integrations in the transformed plant genome. It has been noted that microprojectile bombardment commonly results in multiple copies of the gene being inserted and complex integration patterns (Birch and Franks, 1991). The integration of only one copy is desirable for practical genetic engineering to avoid potential problems of suppression of expression caused by multiple gene integration (Napoli et al., 1990). The role of the T-DNA complex in the *Agrobacterium* transformation systems may be not only in the transfer of T-DNA from the bacterium to the plant cell nucleus, but also in the T-DNA integration process which results in single or low numbers of insertions of transgenes in the plant genome. The reason for single or low number insertions achieved in the TAXI transformation system are not yet understood. It may result from the specific artificial form of the DNA imitating the T-DNA complex.

4) Mendelian segregation of the *bar* gene in R1 plants demonstrated the inheritance of the transgene in the progeny of transformed plants.

Mendelian and non-Mendelian segregation of transgenes have been observed in previous studies on transgenic rice (Goto et al., 1993, Peng et al., 1992, Rathore et al., 1993) and maize (Spencer et al., 1992). In this investigation the phenotypic segregation ratio of BAR in R1 plants of four transgenic lines was approximately 3 : 1 ($P > 0.3 < 0.7$), demonstrating Mendelian inheritance. Co-segregation of the *bar* and *uidA* genes indicated their genetic linkage in all of the five R1 populations.

5.4.2 The efficiency of transformation

Summarizing the results of three independent transformation experiments, there is a significant difference between bombarding with the TAXI-pUGB35 and the naked plasmid pUGB35 (Table 5.1). Using 1550 psi, six transgenic callus lines were produced from a total of 122 callus samples bombarded with the TAXI-pUGB35. In contrast, only one transgenic callus line was recovered from 120 callus samples in the case of plasmid bombardment. TAXI-biolistic transformation appears to be more efficient. However, the efficiency of microprojectile mediated gene transfer is not consistent because a number of factors interact to affect it (Birch and Franks, 1991, Sanford, 1993). In this particular situation, at least two key factors influence the frequency of transformation :

1) The coating procedure of gold particles

The efficiency of naked plasmids or the TAXI complexes attaching to gold particles is not comparable, as they are two completely different procedures. Although the same amount of DNA was used for coating gold particles in both procedures, it is not possible to determine the exact amount of DNA coated on particles in independent experiments. Any slight variation in coating may cause changes in the recovery of transgenic plants.

2) Impact velocity of the microprojectile / DNA complex

Ethanol cannot be used for the precipitation of the DNA-histone H1 complex because it will change the configuration of histone H1, resulting in the loss of its biological function. Therefore, the gold-TAXI solution loaded on the disk had to be dried under vacuum before being used for bombardment. There is a significant difference between bombarding with gold-plasmid particles and gold-TAXI particles using the same velocity. In the latter case particles adhere to the disk more tightly. The transformation frequencies, therefore, are not comparable, even when the same velocity was used to deliver gold-plasmid and gold-TAXI particles to target cells. In this study, only 1550 and 1100 psi were tested. 650, 900 and

1300 psi have since been tested and the results showed that 900 psi may be close to the optimal velocity for the TAXI biolistic process (data not shown).

In conclusion, the TAXI-biolistic process is a new approach for the direct transfer of genes to plants. It combines advantages of the *Agrobacterium* transformation system and the biolistic process. The method makes it possible not only to deliver DNA into intact plant tissues or cells, but also results in nuclear targeting of transferred DNA, and single or low numbers of insertions of transgenes in the plant genome. However, the technique is still in its early developmental stage and further investigations are necessary for the technique to be perfected.

Abbreviations

CTAB	hexadecyltrimethyl ammonium bromide
DCC	dicyclohexylcarbodiimide
DEPC	diethylpyrocarbonate
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
NHS	N-Hydroxysuccinimide
PEG	polyethylene glycol
PVP-40	polyvinylpyrrolidone (MW: 40,000)
SDS	sodium dodecyl sulphate
X-gluc	5-bromo-4-chloro-3-indolyl ß-D-glucuronic acid
TFUG	ß-trifluoromethylumbelliferyl ß-glucuronide
2,4-D	2,4-dichlorophenoxyacetic acid
NAA	naphthaleneacetic acid
PPT	phosphinothricin

Appendix A

Media, Buffers and Solutions

A1. Media

A1.1 Luria-Bertani medium (for 1 litre)

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g

Adjust pH to 7.5 with Sodium Hydroxide

A1.2 YMA medium (for 1 litre)

Yeast extract	1.0 g
Mannitol	10.0 g
K_2HPO_43HQ	0.05 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g

A1.3 MSSP medium (for 1 litre)

MS medium salt (supplied by Flow Laboratories)

Glucose	30.0 g
Na_2HPO_4	2.225 g

Adjust pH to 5.5

A1.4 High osmotic medium (for 1 liter)

MS medium salt

Sucrose	30 g
Mannitol	0.2 M
Myositol	100 mg
2,4-D	2.5 mg
Agar	8.5 g

A2. Buffers

A2.1 TE

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

A2.2 STE

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)
100 mM NaCl

A2.3 10 x TBE

0.89 M Tris-HCl
0.89 M boric acid
10 mM EDTA (pH 8.0)
10 mM EDTA
Adjust pH to 8.3

A2.4 50 x TAE

2 M Tris-ace
0.1 M EDTA (pH 8.0)

A2.5 PBS

137 mM NaCl

2.7 mM KCl

4.3 mM $Na_2HPO_4.7H_2O$

1.4 mM KH_2PO_4

A2.6 20 x SSC

3 M NaCl

0.3 M Na-citrate.2H O

Adjust pH to 7.0 with 1 M HCl

A3. Solutions

A3.1 TSB

LB medium pH 6.1

10 % PEG (6000)

5 % DMSO

20 mM $MgCl_2/MgSO_3$

A3.2 Guanidinium solution (extract bufferA)

4 M Guanidinium thiocyanate

25 mM sodium citrate (pH 7.0)

0.1 M ß-Mercapto-ethanol

0.5 % N-laurylsarcosine (Sarkosyl)

A3.3 CTAB solution (extract bufferB)

100 mM Tris-HCl (pH 8.0)

20 mM EDTA (pH 8.0)

1.42 M NaCl

5 M Ascorbic Acid

2 % CTAB

2 % PVP-40

A3.4 Extract solution for genomic DNA

200 mM Tris pH 7.5

250 mM NaCl

10 mM EDTA

0.5 % SDS

A3.5 Oligonucleotide probe hybridization solution:

A3.5.1 Prehybridization solution (pH 7.2) :

125 mM NaCl

17 mM $Na_2 HPO_4$

8 mM $NaH_2 PO_4.2H_2O$

173 mM SDS

12.5 mM PEG (6000)

A3.5.2 Hybridization solution (pH 7.2) :

125 mM NaCl

17 mM NaH_2PO_4

8 mM $NaHPO_4 \cdot 2H_2O$

173 mM SDS

A3.5.3 Washing solution

the same as hybridization solution except that SDS is at a concentration of 17.3 mM.

A3.6 SOC solution

0.5 % Bacto yeast extract

2 % Bacto tryptone

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM glucose

Appendix B

General Techniques

B1 Microbial techniques

B1.1 Preparation of competent cells

B1.2 Transformation

B2 Nucleic acid isolation

B2.1 Plasmid preparation

B2.1.1 Miniprep

B2.1.2 Maxiprep

B2.2 Isolation of genomic DNA

B2.2.1 For Southern blot analysis

B2.2.2 For PCR amplification

B2.3 Isolation of RNA

B2.3.1 Isolation of total RNA

B2.3.2 mRNA preparation

B3 DNA manipulation

B3.1 Techniques for vector construction

B3.1.1 Restriction enzyme digestion

B3.1.2 Recovery of DNA fragments from gel slices

B3.1.3 Production of blunt end DNA by Klenow fragment and Nuclease P1

B3.1.4 Dephosphorylation

B3.1.5 Ligation

B3.2 Probe labelling techniques

B3.2.1 Nick translation

B3.2.2 End labelling by terminal transferase

B3.3 DNA sequencing

B4 Nucleic acid transfer and hybridization

B4.1 Southern blot

B4.2 Northern dot blot

B5 PCR techniques

B5.1 DNA amplification by PCR

B5.2 RNA RT-PCR

B5.3 Sequences of primers and other oligonucleotides

B6 Isolation and analysis of proteins

B6.1 Isolation of seed storage proteins

B6.2 Separation by gradient SDS PAGE

B6.3 Electroblotting

B6.4 Chemiluminescence assay

B2 Nucleic acid preparation

B2.1 Plasmid preparations

B2.1.1 Small scale (miniprep)

Plasmid was isolated using the alkaline lysis miniprep method in 1.6.1 "Current Protocols in Molecular Biology" (1991).

B2.1.2 Large scale (maxiprep)

Alkaline lysis method was used in large scale plasmid preparation and followed by CsCl equilibrium centrifugation, according to the method in "Current Protocols in Molecular Biology" (1991) 1.7.1.

2.2 Isolation of genomic DNA from rye leaves and immature seeds

B2.2.1 Isolation of genomic DNA for Southern blot analysis

Approximately 50 mg fresh tissue was frozen in liquid nitrogen, and ground by using a mini-homogenizer. 500 uL extract buffer A (see Appendix A) was added to the tissue powder. The mixture was homogenized for 10 seconds, and then heated at 60°C for 10 minutes. The extract was centrifuged for 5 minutes. The supernatant was extracted with 400 uL phenol:chloroform (1:1), and the aqueous phase was further extracted with 400 uL chloroform. The aqueous phase was precipitated with 150 uL 5M NaCl and 1 mL 96% Ethanol at -20°C for 2 hr. After centrifugation for 15 minutes at 4°C, the genomic DNA pellet was washed with 75% ethanol and dissolved in TE.

B2.2.2 Isolation of genomic DNA for PCR amplification

Approximately 50 mg fresh tissue was ground in liquid nitrogen by using a mini-homogenizer. 600 uL extract buffer B (see Appendix A) was added in the tissue powder and homogenized. 60 uL 2M sodium acetate and 600 uL phenol were added and mixed well. 120 uL chloroform:isoamyl-alcohol (49:1) was added and vortexed for 15 seconds, then kept on ice for 15 minutes. The extract was centrifuged for 10 minutes and the aqueous phase was precipitated with an equal volume of iso-propanol at -20°C overnight. The precipitate was collected by centrifugation at 15000

rpm for 30 minutes at -10°C. The pellet was further extracted by 600 uL CTAB extract buffer (see Appendix A)and 500 uL chloroform:isoamyl-alcohol (24:1), then precipitated with an equal volume of iso-propanol at room temperature for 2 hr. The precipitate was collected by centrifugation at 20000 rpm for 20 minutes at 22°C. The pellet was washed with 75% ethanol and dissolved in TE.

B2.3 Isolation of RNA

B2.3.1 Isolation of total RNA

Approximately 50 mg fresh tissue was quickly frozen in liquid nitrogen and ground by means of a mini-homogenizer. 1 mL RNA extraction reagent (supplied by Advance Biotechnologies LTD) was added to the tissue powder and homogenized. The homogenate was kept at 4°C for 5 minutes. 200 uL chloroform was added, mixed well and kept on ice for 10 minutes. The extract was centrifuged at 12000g at 4°C for 5 minutes. The aqueous phase from the repeated chloroform extraction was then precipitated by adding an equal volume of isopropanol at 4°C for 2 hr. The precipitate was collected by centrifugation at

20000 rpm at 4°C for 20 minutes. The RNA pellet was washed and dissolved in DEPC treated sterile distilled water.

B2.3.2 mRNA preparation

mRNA preparation was carried out by using "Dynabeads mRNA DIRECT kit" [supplied by DYNAL (UK) LTD]. 500 uL of lysis/binding buffer was added to the sample of total RNA and mixed well.

200 uL of conditioned Dynabeads with Oligo $(dT)_{25}$ were mixed with the RNA sample and annealed for 5-10 minutes at room temperature. The mixture was then placed in the Dynal MPC (a magnetic Eppendorf tube rack) for 2 minutes and the supernatant was removed. The mRNA annealed beads were washed once with "Washing buffer with Lids" and three times with "Washing buffer". 20 uL elution buffer was added and kept at 65°C for 2 minutes. The sample was placed in the Dynal MPC magnetic rack and the supernatant containing mRNA was transferred to an RNase-free Eppendorf tube.

B3 DNA manipulation

B3.1 Techniques used in vector construction

B3.1.1 Restriction enzyme digestion

The conditions for plasmid DNA digestion was at final DNA concentrations of 0.5 to 1 ug/uL and an enzyme concentration of 0.5 units/ug DNA.

For double digestion either a buffer compatible with both enzymes was chosen or the sample was digested first with the enzyme requiring the lower salt concentration and the salt concentration was then adjusted to the requirements of the second enzyme.

B3.1.2 Recovery of DNA fragments

a) From agarose gel slices

Isolation and purification of DNA fragments from agarose gel slices were carried out using "Gene clean II kit" (supplied by BIO 101 Inc).

b) From polyacrylamide gel slices

DNA was recovered in an ammonium acetate elution buffer (2.7.3 "Current Protocols in Molecular Biology" 1991). An additional precipitation was usually necessary to get a pure DNA fragment which is suitable for ligation.

B3.1.3 Production of blunt end DNA by Klenow fragment and Nuclease P1

Certain ligation procedures require DNA fragments with blunt ends. To produce blunt ends from 5' overhang DNA fragments generated by restriction enzymes, the Klenow fragment of *E.coli* DNA polymerase was usually used to fill in the overhang.

A typical reaction consists of 1 ug DNA in 20 uL reaction buffer, 0.5 pmol of each deoxyribonucleotide and 2.5 units of Klenow. The reaction is performed for 15 minutes at 30°C and stopped by heating to 75°C for 10 minutes or by adding 1 uL 0.5 M EDTA.

To produce blunt ends from 3' overhang DNA fragments, Nuclease P1 is normally chosen. Nuclease P1 has phosphodiesterase activity which catalyses the hydrolysis of phosphodiester linkages in ssDNA and RNA. A typical reaction consists of 1 ug linearized 3 kb DNA fragment with 3' overhangs and 0.05 units nuclease P1 in 20 uL buffer with incubation at 37°C for 15 minutes. The reaction is stopped by heating for 10 minutes at 80°C.

B3.1.4 Dephosphorylation

Calf intestinal phosphatase (Boehringer Mannheim) was used as in "Current Protocols in Molecular Biology"(1991) 3.10.1.

B3.1.5 Ligation

A typical reaction includes :

1 uL 10x buffer (200 mM Tris-HCl pH7.5, 50 mM $MgCl_2$ 50 mM DTT)

1 uL 5 mM ATP

1 uL 0.5 mg/mL BSA

100 pg vector DNA (dephosphorylated)

at least an equimolar amount of insert

(optimum ratio of vector:insert at 1:3 to 1:5)

1 unit T4 ligase for sticky ends ligation or

5 units T4 ligase for blunt ends ligation

The mixture is incubated at 12°C overnight and used directly for transformation.

B3.2 Probe labelling

B3.2.1 Nick translation

^{32}P-dCTP labelled DNA fragment is produced by nick translation. The labelling method used is described in "Current Protocols in Molecular Biology"(1991) 3.5.3. A "Nick Translation kit" (supplied by Promega) was used for the reaction.

Unincorporated nucleotides are removed by using a spin column (Maniatis 1982). 1 uL of the probe shouLd be counted to determine the specific activity.

B3.2.2 End labelling by terminal transferase

To produce ^{32}P-dCTP labelled oligonucleotide probes, end labelling is catalysed by terminal transferase. The reaction can be done according to "Current Protocols in Molecular Biology"(1991) 3.6.1. Purification and determination of specific activity are performed as above.

B3.3 DNA sequencing

A routine dideoxy method in "Current Protocols in Molecular Biology" 7.4 was used in DNA sequence experiments. After the reaction, 4 uL of each heat denatured sample is loaded onto a gel that consists of 6% acrylamide (19:1 acryl:bisacrylamide), 8 M Urea and 1x TBE. Samples are electrophoresed at 1500 volts at 55°C for 2 to 6 hr. Gels are dried under vacuum in a gel-drier for 20 minutes at 60°C and autoradiographed onto a Cronex-4 X-ray film at -70°C overnight.

B4 Nucleic acid transfer and hybridization

B4.1 Southern blot

When the interesting DNA fragments are longer than 600 bp and run on an agarose gel, Southern blot and hybridization are performed as described by Maniatis ("Molecular Cloning" 1989).

When the interesting DNA fragments are shorter than 600 bp, the sample is separated on a denaturing polyacrylamide gel (i.e DNA sequence gel). After completion of gel electrophoresis, the gel is transferred onto an Immobilon-S membrane or a nylon membrane

(Hybond-N^+; Amersham) and transferred overnight in buffer (0.5xTBE, pH 8.7). After the membrane is air dried, the DNA is crosslinked by UV-irradiation for 6 minutes.

Prehybridization is carried out at 55°C in a sealed plastic bag containing prehybridization solution (See Appendix A). After 1 hr incubation with gentle shaking in a water bath, the contents are squeezed out and replaced with the hybridization solution (See Appendix A). Oligonucleotide probes must be used in this hybridization. Hybridization is carried out with gentle shaking in a water bath at 55°C for 3 hr. The blot is washed in a washing solution (See Appendix A) for 5 minutes at room temperature. The washing step is repeated 4 times. The membrane is then covered in 'gladwrap' and autoradiographed at -70°C.

B4.2 Northern dot blot

For Northern blots and Northern dot blots, RNA samples are glyoxal denatured. Glyoxal (7M) is deionized by repeated passage through a mixed bed resin (Unilab Amberlite MB-1) until the pH reaches 6. The glyoxal is then stored at -70°C in small aliquots.
Two glyoxal mixtures are prepared: with and without DMSO. The mixture without DMSO is used when it is essential to keep the volume to a minimum. DMSO is not essential for denaturation (Thomas 1983).

DMSO glyoxal mix:	15 uL	7 M glyoxal
	50 uL	DMSO
	2 uL	0.5 M phosphate pH 7.0

Two volumes of this mixture are added to one volume of RNA solution (3.5 ug/ uL).

Glyoxal mix : 15 uL 7 M glyoxal

9 uL H_2O

1 uL 0.5 M phosphate pH 7.0

One volume of this mixture is added to three volumes of RNA solution (3.5 ug/ uL).

This solution is incubated for 1 hr at 50°C and kept on ice until used.

Denatured RNA samples are spotted onto a nylon membrane pretreated with 10x SSC, using a vacuum-driven slot- and dot-blotting apparatus. After the membrane is dried, it is UV-irradiated. The glyoxal adduct is removed by soaking the membrane in 20 mM Tris-HCl, pH 8.0 at 100°C for 10 minutes.

Hybridization is performed as in "Current Protocols in Molecular Biology"(1991) 4.9.1.

B5 PCR techniques

B5.1 DNA amplification by PCR

250 ng (50 uL) of purified genomic DNA (see Chapter 4) was heat denatured at 95°C for 5 minutes and kept on ice. 50 uL of ice cold 2 x PCR mixture, containing 10 uL 10x buffer, 4 mM $MgCl_2$, 400 M of each dNTP, 0.02% BSA, 50 pmol of each primer and 2.5 units Taq DNA polymerase were added to genomic DNA samples and mixed.

PCR was carried out : 92°C 15 seconds

primer T_m -5°C 30 seconds

72°C 60 seconds

for 30 - 35 cycles, and then 72°C for 5 minutes. The annealing temperature of primer and template depends on primer T_m. Usually a temperature which is 3 - 5°C lower than the primer T_m is chosen as the annealing temperature. An extension time of 60 seconds is

usually sufficient for products up to 1500 bp. The sizes of the products are examined by polyacrylamide gels or agarose gels.

B5.2 RNA RT-PCR

Enzymatic amplification of RNA by PCR has been used for detecting rare RNA. In order to maximize the efficiency of annealing, poly(A)$^+$RNA and cDNA primer were heated to 90°C and cooled slowly to 67°C. The sample was centrifuged for 1 second and the condensate was collected. In this investigation, because the poly(A)$^+$RNA was annealed to the oligo dT magnetic beads, cDNA primer was not required. The first cDNA strand extended directly from oligo dT. This reaction of reverse transcription produced a cDNA library on the solid magnetic beads. The reaction mixture of reverse transcription contained :

15 uL poly(A)$^+$RNA annealed to oligo dT beads
4.0 uL 5 x buffer
1.5 uL 10 mM dNTP
0.2 uL (24 units) RNase inhibitor
1.0 uL (200 units) M-MLV reverse transcriptase

The reaction mixture was incubated and shaken very gently at 42°C for 1 hr. Denaturing at 95°C for 1 minute allowed poly(A)RNA to separate from the cDNA strand. Ice cold denatured samples were placed on an ice cold Dynal MPC magnetic rack to remove aqueous solution from the beads.

100 uL 1 x PCR mixture as mentioned earler was added to the cDNA beads immediately. Synthesis of the second strand of cDNA was performed for 2 cycles at 92°C for 20 seconds, 60°C for 1 minute, 72°C for 3 minutes. The solution was denatured by heating at 92°C for

40 seconds in order to release the second strand of cDNA from the beads. Consequently the beads were removed as described above.

An additional 1 unit of Taq DNA polymerase was added to the reaction. PCR amplification was done as described in B5.1.

B5.3 Sequence of primers and oligonucleotides

B5.3.1 PCR primers

(optimised according to computer "primer" programme)

The fragment of **CaMV 35S promoter-GUS gene**:

forward primer	reverse primer
-129 to -108	+257 to +236
5' CGCACAATCCCACTATCCTTCG 3'	5' CTGACACCGCGATCAAAAAACC 3'

The fragment of **CaMV 35S promoter-glutenin gene**:

forward primer	reverse primer
-129 to -108	+187 to +168
5' CGCACAATCCCACTATCCTTCG 3'	5' TCCGCGACTTGGCTGACGGC 3'

The fragment of **glutenin gene 3' end**:

forward primer	reverse primers
repeat region	3' end
5' ACTACCCAACTTCTCCACAGCA 3'	5' AGTTCTATCACTGGCTGGCC 3'

B5.3.2 sequence of **right border of T-DNA**

5' AATTCGTTTACCCGCCAATATATCCTGTCA 3'TS

B6 Isolation and analysis of proteins

B6.1 Isolation of seed storage proteins

Immature seeds or water soaked mature seeds were weighed to obtain an average weight of the seed. A grinding buffer containing 100 mM Tris-HCl pH 7.4, 5 mM DTT and 0.1 % SDS was used (10 uL/ mg seeds). After grinding and mixing, the extracts were heated at 100°C for 5 minutes, and then centrifuged at 12000g for 10 minutes. 9 times volume of ice cold acetone was added to the supernatants, and proteins precipitated at -20°C for 1-2 hr. After collecting the precipitates by centrifugation at 12000g 10 minutes, the pellets were extracted with 96% ethanol at 4°C overnight. The storage proteins were obtained by centrifugation as above, and were then dissolved in 1% SDS solution.

B6.2 Separation on a gradient SDS polyacrylamide gel

The seed storage proteins were separated by electrophoresis on a 5%- 10% gradient SDS polycrylamide gel. The gel preparation and electrophoresis were done as described in "Current Protocols in Molecular Biology" (1991) 10.2.10 .
After completion of electrophoresis, half of the gel was transferred by electroblotting onto a nitrocelluLose membrane (Hybond C). The duplicate samples on the other half of the gel were stained by Coomassie Brilliant Blue, destained and photographed.

B6.3 Electroblotting

The process of electroblotting was performed as described in "Current Protocols in Molecular Biology" (1991) 10.8.1-8.

B6.4 Chemiluminescence assay

Horseradish peroxidase, coupled to the secondary antibody, in the presence of hydrogen peroxide catalyses the oxidation of cyclic diacylhydrazides like luminol. A reaction product in an excited state is thus formed, which decays to the ground state by emitting light. 4-iodophenol acts as a radical transmittor between the oxigen radical and luminol, and strongly enhances the light emission.

Chemiluminescence Western Blotting Reagents were supplied by Boehringer Mannheim. The process of immunoreaction and detection consists of 8 steps :

(1) Membrane blocking

Non-specific antibody binding was blocked by incubating the membrane for 2 hr in blocking solution (1 x PBS, 0.1% Tween-20, 3% BSA) and shaking at room temperature.

(2) Primary antibody reaction

The membrane was incubated for 1 hr with primary antibody diluted 1 in 500-1000 in blocking solution containing 1% BSA.

(3) Washing

The membrane was washed 4 times in a washing buffer (1 x PBS, 0.1% Tween-20) for 15 minutes.

(4) Horse Radish peroxidase conjugated protein A

The membrane was incubated for 1 hr with horse radish peroxidase conjugated protein A diluted 1 in 1000 in washing buffer.

(5) Washing Repeat step (3)

(6) Enzyme reaction

The excess buffer was removed from the membrane. The detection reagent was added and incubated with the membrane for 2 minutes.

(7) Exposure

The membrane was inserted in a plastic bag and put into a film cassette and exposed to X-ray film for 30-300 seconds.

(8) Film development

B7 Other techniques

B7.1 GUS histochemical assay

5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) is a widely used substrate for histochemical localization of GUS activity in tissues and cells. The product of glucuronidase action on X-gluc is not colored. It must undergo an oxidative dimerization to form the insoluble and highly colored indigo dye. This oxidation reaction is catalyzed by a ferricyanide / ferrocyanide mixture and gives a blue precipitate at the site of enzyme activity.

GUS activity histochemical assay was carried out according to the protocol described by Jefferson (1987).

B7.2 Purification of Histone H1

Sea urchin sperm histone H1 was isolated and purified as described in "Methods of Enzymology" (von Holt et al. 1987). After being freeze-dried, 500 mg histone H1 was dissolved in 25 mL 0.25 M HCl and transferred into a dialysis tube. The sample was dialysed overnight against 2 litres of 0.025 M HCl at 4°C and then against 2 litres of H_2O at 4°C. The sample was freeze-dried.

B7.3 Purification of antibodies

Anti-GUS and anti-BMV antibodies were purified by immunoaffinity chromatography.

(1) Virus dissociation

BMV particles were dissociated before being coupled onto a solid support. BMV (in 0.5 M phosphate buffer pH 6.0) was concentrated by centrifugation at 30000rpm at 4°C for 2 hr. The pellet was resuspended in 1.2 mL of the same buffer. The virus was then dissociated by overnight dialysis against 1 M NaCl, 100 mM sodium borate pH 9.5 at 4°C. This dissociated BMV was used to prepare an affinity column.

(2) Preparation of affinity column

a. Preparing Cyanogen Bromide-activated beads

3g cyanogen Bromide (CNBr) was dissolved in 5 mL dioxane and mixed with 10g CL-4B sepharose beads pre-wetted with water. The mixture was stirred and incubated at 20°C in a water bath for 15 minutes. 2 M NaOH was slowly added to maintain a pH of 11 during the reaction. The reaction was stopped by quickly adding ice cold water. The mixture was transferred to a glass filter, vacuum filtrated and the beads washed with a large volume of sodium borate buffer (pH 9.5).

b. The coupling of virus and activated beads

After washing, the dissociated BMV was added to the beads which were resuspended in 10 mL sodium borate buffer. The coupling reaction was done by rolling the beads overnight at

4°C. 5 mL of 1 M ethanolamine was added to stop the reaction. The beads were again rolled overnight at 4°C.

The BMV coupled beads were packed into a 10 mL column.

(3) Purification of antibody

The column was washed with 10 volumes of 10 mM Tris-HCl pH 7.5, 10 volumes of 100 mM glycine pH 2.5, 10 volumes of 100 mM triethylamine pH 11.5 and 10 mM Tris-HCl pH 7.5 , until the pH reached 7.5. The column was then ready for purifying anti-BMV antibody.

The affinity chromatography was performed as described in "Antibody" (Immunological Laboratory manual).

The anti-GUS antibody was coupled in an analogous fashion. Affinity chromatography with rye seed protein as a ligand was used to remove from the anti-glutenin immunoserum antibodies cross-reacting with secalin and other seed storage proteins present in rye seeds. The affinity column was prepared as desribed but using whole rye protein as ligand.

B7.4 Preparation of streptavidin-oxirane acrylic beads

The method of preparation of streptavidin-oxirane acrylic beads has been developed by R.Warren in our laboratory.

25 mg hexa-glycine is dissolved in 100 uL H_2O by adding 75 uL of 1 M NaOH. 1 mL of 0.5 M $NaHCO_3$ (pH 8.5) is then added, and the volume of the solution is adjusted to 2 mL with H_2O. 1 g oxirane acrylic beads are then added to the solution. The coupling reaction of hexa-glycine and the beads is carried out for 3 days at 37°C with stirring.

After coupling, the beads are dialysed against 4 x 20 litres of water at 4°C for 2 days to remove excess hexa-glycine. The matrix is washed twice by centrifugation with H_2O pH

2.0 at 8000 rpm for 10 minutes followed by washing twice with 10 mM HCl. The matrix is then dried by vacuum followed by drying at 37°C for 3 days in a dessicator over P_2O_5

The matrix is then resuspended in 5 mL distilled DMF containing 0.1 M DCC and 0.1 M NHS. The mixture is incubated for 5 hr with stirring at 37°C , in order to activate the hexaglycin carboxyl groups on the matrix.

The activated matrix is aliquotted into 10 Eppendorf tubes (500 uL each), and then washed with 4 x 1 mL dry DMF. The final washing is with 1 mL H_2O.

15 uL of a solution containing 5 ug streptavidin / mL and 100 - 200 uL 0.1 M $NaHCO_3$ pH 8.0 are quickly added into each sample and mixed well. The matrix is mixed by gentle vortexing overnight at room temperature. The uncoupled streptavidin is removed by washing with STE at least 3 times. The matrix is then stored at 4°C.

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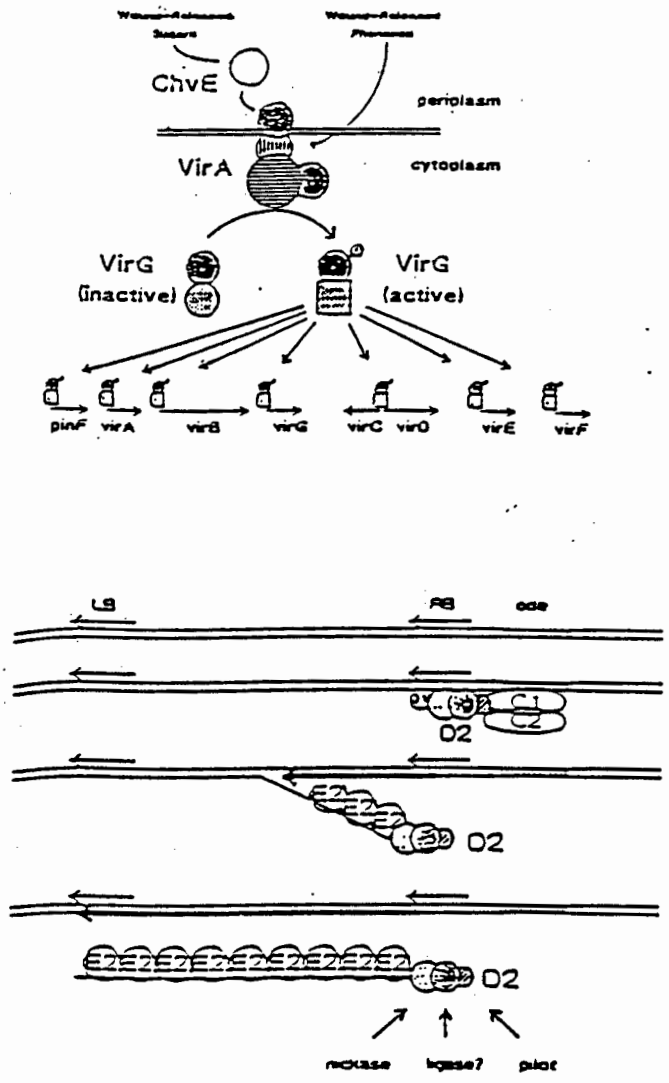

Figure 1.1 Proposed Mechanism of *vir* Gene Induction and T-DNA Formation

Top: The proposed function of the regulatory proteins VirA and VirG. VirA is a transmembrane protein kinase that may directly bind the phenolic inducer. VirG is a target of the VirA kinase and binds to *vir* promoters to activate their transcription.

Bottom: Proposed mechanism of synthesis of single stranded T-DNA. RB: right border; LB: left border; ode: overdrive; C1,C2,D1 and D2: the products of the *vir*C1, *vir*C2, *vir*D1, and *vir*D2 genes. Thick arrows indicate newly synthesized DNA which could displace the bottom strand of T-DNA.

Reprinted from Winans (1992)

Figure 1.2 The Proposed Secondary Structure of High Molecular Weight Glutenin Subunits

HMW glutenin subunits have molecular weights ranging from 60000 to 80000. The proteins consist of three domains: a repetitive central domain flanked by non-repetitive N- and C-terminal domains. The central domain contains two consensus repeat motifs, PGQGQQ and GYYPTSLQQ. The N- and C-terminal domains contain most or all of the cysteine residues : 3 or 5 in the N-terminal domain, and 1 in the C-terminal domain.Each amino acid residue is depicted as a sphere.

α-Helix region: blue, highly conserved sequences of N-terminal and C-terminal regions
Cysteine: yellow-green
ß-spiral region: domain of repeated sequences
concensus of hexapeptide: red
concensus of nonapeptide: orange

(Computer graphics : Dr. D. Maeder, based on model proposed by Miflin et al., 1976)