

OPTIMIZATION OF APEX-MEDIATED DNA TRANSFORMATION IN RICE

A Thesis

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Marsha Natalie Samuels
A.Sc., College of Agriculture, Jamaica, 1995
B.Sc., Louisiana State University, 1998
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ABSTRACT

Genetic transformation using the rice shoot apical meristem, derived from germinating seedlings, was established in this study for commercial varieties of *Oryza sativa*. To optimize apex-mediated DNA transformation in rice several parameters were tested to improve the efficiency and reliability of isolation of the shoot apices and regeneration of vegetative shoot apices. Results from these experiments indicated that certain factors were important in increasing the frequency of survival rate of the apex, co-cultivation and co-transformation. These factors included: the starting size of the apex had to range between 1.0 mm and 1.2 mm for maximum survival of the apex, concentration of the infecting bacteria (pCL3 and pCL4) at an OD of 0.6-0.8 at 600nm and pTOK233 at an OD of 1.5-1.8 at 600nm, the minimum lethal concentration of hygromycin B was 5 mg/L, and use of Rice Shoot Apex (RSA) medium. Furthermore, the presence of acetosyringone before and during co-cultivation at levels between 400 ppm and 1000 ppm increased the number of transformed apices by 40%. Vacuum infiltration at 20-21 Hg for 5 minutes did not have an effect on apex survival, however apex transformation rate was increased by 30–35%. Adhering to the development of the new protocol resulted in a 90% shoot apex culturing success rate and a 95% transformation rate of the apices.

Vectors containing the GUS gene with either the maize ubiquitin promoter or rice ubiquitin promoter resulted in > 85% transformation efficiency of the apices based on GUS assays. The vectors LBA4404 (pCL3) and LBA4404 (pCL4) proved to be more apex-specific than root-specific, an advantage over the super-binary vector LBA4404 (pTOK233). Preliminary vectors such as pRQGus, pRQHg and pSB41 were constructed for future use in experiments involving LBA4404 (pTOK233) and tissue culture experiments. Conducting experiments with both the results from tissue culture and vector construction could provide alternative routes to further enhance the efficiency of apex-mediated transformation.

CHAPTER I

INTRODUCTION TO STUDY

Rice is one the most important cash crops in the world and it plays a very significant role in the U. S. economy. As one the of the major food sources, rice is third to wheat and maize in the world total production of food grains (USDA 2000). More than 90% of the rice cultivated is consumed in Asia (Khush, 1997). Additionally the uniqueness of this grain has allowed it to adapt to different geographic, locations, soil types and environments, making it the world's most versatile crop (Ou, 1985). However, with the rapidly growing demand for rice worldwide, due to great increases in world's population that consume rice, the yield of rice must be increased through the development of improved farm practices and genetic improvement.

The cultivation of rice began in the United States over 200 years ago. It first began when colonists from Charleston, South Carolina began to grow rice. It was then introduced to Louisiana in 1718 by French explorers led by Bienville. For the following 150 years rice was grown on a limited basis in Louisiana, until after the Civil War which caused rapid increase in acreage particularly in the southwest part of the state (Linscombe, 1999).

Rice belongs to the Gramineae family and the genus *Oryza*. It is believed to have originated 130 million years ago. At present there are twenty-one wild species and two cultivated species in the genus *Oryza*, nine are tetraploid and the remainder are diploids. *O. sativa* is Asian rice, grown worldwide, while *O. glaberima* is grown only in limited areas of West Africa. These two cultivated species are diploid $2n=24$ and have the

genome AA. Some wild species have either $2n=24$ or $2n=48$ chromosomes representing AA, BB, CC, BBCC, CCDD, EE, FF, GG and HHJJ genomes (Khush, 1997). The wild species are important as they provide a gene pool from which useful resistance genes for disease and insects may be identified. However, due to the differences such as limited homology between the *O. sativa* genome and the wild *Oryza* species, gene transfer is difficult owing to limited recombination and low crossability of the chromosomes. With the assistance of biotechnology, the embryo rescue technique has helped in the possibility of gene transfer among distantly related species. An example is the transfer of genes of grassy stunt virus and bacterial blight resistance from an 'A' genome wild species into cultivated rice using embryo rescue techniques (Khush, 1997).

Rice became a model system for cereal genomic research because of its small genome size (4.2×10^8 bp) and its simple diploid character. This factor has paved the way for the development of detailed linkage maps and enabled large-scale sequencing of expressed sequences. (Arumuganathan and Earle, 1991). There are three subspecies of *Oryza sativa*: *japonica*, *javanica* and *indica*. Of the three, *indica* is the most widely cultivated and consumed in Asian countries. It is estimated to feed more than two billion people and accounts for 80% of cultivated rice worldwide. The *javanica* group is characterized as having a long grain and is commercially grown in the southern part of the U.S. *Japonica* differs because of its short or round grains and is grown primarily in the temperate climates of Japan, China, Taiwan and California.

Owing to the ever-increasing demand of rice world wide, new varieties with higher yields, multiple resistances to pests and diseases, and with tolerance to stresses such as acidity, salinity and/or flooding are greatly needed. In the last five years

remarkable and significant advances in plant molecular biology and gene delivery techniques have led to cellular and molecular approaches to crop improvement. These approaches show promise to increase the efficiency of traditional breeding methods and also provide new unconventional approaches to enhance and build the rice economy. The introduction of beneficial genes from other organisms such as those encoding disease and insect resistance via genetic transformation into the rice genome arose from developments in this area. Furthermore, the development of molecular maps, tagging important genes with RFLP markers and direct gene transfer were all made possible because of transgenic rice research. These important steps as dramatic genetic improvement were not possible through breeding and selection. (Gould 1996; Hiei et al 1997; Jiang, 1999; Wang, 2000).

The most commonly used methods for foreign gene transfer to plants are either mechanical or biological. The popular forms of mechanical methods are protoplast-mediated transformation, microprojectile bombardment of cells and tissues, electroporation, and polyethylene glycol (PEG). (Gould 1996; Jiang, 1999; Christou et al 1996; Toriyama et al., 1988; Kyojuka et al., 1991). The biological methods are evolved from *Agrobacterium*-mediated gene transfer. For this research the purpose was to develop and improve the practical application of *A. tumefaciens* for rice transformation since this was once theorized as not being possible for monocot transformation (Hiei et al 1997; Gould, 1996; Dong et al., 1996; Aldemita et al., 1996; Rashid et al., 1996). This recently, however, has become the method of choice because it had previously proven to be simple, offered more precise results in gene transfer, results in permanent genetic change, and is less likely to have a high occurrence of somaclonal variation (Jiang, 1999).

Use of the mechanical methods of gene transfer has not been as successful in producing transgenic rice as compared to biological methods owing to significant drawbacks. However, especially for the cereals these methods were soon found to have restrictions and limitations that were not promising favorable results. A more efficient, repeatable technique was being sought and this was found in the application of *Agrobacterium*-mediated transformation. In rice, this method offered several advantages including a high efficiency rate of transformation, the ability to transfer large pieces of DNA, it is inexpensive and it does not require the use of protoplasts. (Komari, et al., 1996; Park, 1996).

Even with the advent of this promising technique, it was evident that there were certain restrictions that would affect its widespread use and application. One such noted limitation was that the rapid, reliable production of regenerated competent rice explants from any genotype, was inconsistent and not properly developed for successful repeatability. This became an obstacle since the success of *Agrobacterium*-mediated transformation depended on the ability to readily regenerate *in vitro*. This was important, since the regeneration process had to be genotype independent. (Park, 1996). Other factors that heavily impacted the success of this technique were, types and age of the tissue being inoculated, kinds of vectors being used, strains of *Agrobacterium*, details of infection and various conditions that are conducive for proper tissue culture. (Hiei et al., 1997).

To alleviate these problems with *Agrobacterium* transformation in monocots, researchers sought ways to make substantial improvements. One such improvement was the use of the rice shoot apical meristem instead of immature embryos or other rice

explants. This decision soon proved to be a more successful avenue since the rice shoot apex succumbs to *Agrobacterium*-mediated infection (Gould, 1996; Hiei et al., 1997; Park et al., 1996; Rashid et al., 1996). Additionally, the apical meristem gave rise to plants that have both the identical genotypic and phenotypic make-up of the original plant intact.

Additionally, improvements also had to be made to improve the efficiency rate of rice transformation. Since a detailed repeatable method to increase the yield of transformed plants has not been fully established, constant changes to this technique are being consistently developed. This is owing to the fact that presently the reported *Agrobacterium*-mediated transformation rate stands between 10-30% (Gould, 1996). The focus of the research reported herein aimed at improving the efficiency rate of rice transformation not only by using the shoot apical meristem, but also by incorporating the use of an engineered vector that has the rice ubiquitin promoter and the selection gene hygromycin B. Making improvements using the rice ubiquitin promoter was essential since recent studies by Wang, (2000), showed that the ubiquitin promoter functioned very efficiently in the shoot apex. The decision to incorporate the use of the rice ubiquitin promoter and the ubiquitin promoter, came from the fact that these promoters are actively expressed in plants, because they are involved in the basic processes of cell functions, such as protein degradation, also they are very good promoters in monocot transformation systems and finally studies using the ubiquitin promoters have shown a 10-15 fold increase in successful transformation than CaMV 35S promoter (Wang, 2000, Viestra, 1996; Christensen et al., 1992; Christensen et al., 1996). This is in contrast to the conventional CaMV35S promoter that failed to show convincing results of its superiority

in the shoot apex of transgenic rice as stated by numerous studies that used this promoter for both dicot and monocot transformation (Christou et al., 1996; Odell et al., 1985; Peterhans et al., 1990; McElroy et al., 1994; Christensen et al., 1992). These results were clearly observed in transient GUS assays prepared from transgenic rice (Wang, 2000).

A few scientists showed that by incorporating the use of a super-binary vector such as pTOK233 (LBA4404) would eventually ensure a high efficiency rate for rice transformation (Komari et al., 1996; Hiei et al., 1996; Gould, 1996). Using LBA4404 is important since it carries a disarmed Ti-plasmid that with genetic manipulation, genes of interest can easily be introduced to tissue or plant of interest. Creating constructs of this nature would eventually lead to an increased rate of rice apex-mediated transformation. Therefore, using LBA4404 with hygromycin B as a means of achieving established and proven permanent gene expression versus expression attainable by GUS assays would be an area of interest for this research. An overall achievement of this kind would lead to an established and detailed, repeatable, method of optimized apex-mediated DNA transformation in rice.

The objectives of this study were to: (i) develop an efficient and reliable method for extracting and regenerating young vegetative shoot apices in rice for gene transfer, (ii) optimize various parameters for maximum gene transfer rates via co-cultivation of apices with *Agrobacterium*, (iii) develop Ti-plasmids with GUS and hygromycin genes driven by rice ubiquitin promoter, and (iv) evaluate the utility of engineered plasmids by transient GUS assays in the shoot apices, co-cultivated with *Agrobacterium*.

CHAPTER II

REVIEW OF LITERATURE

Introduction

An essential component of plant biotechnology is genetic transformation by mechanical or biological methods. It offers novel approaches to plant production, propagation, modification, improvement and preservation through direct transfer of genes to a variety of plant species (Bhojwani, 1990). Over the past decade major advances made in the laboratory has transformed this so-called 'art' into a rapidly developing industrial technology. The rising popularity of this technique weighs heavily on the increasing world population and global food production. Plants, especially the cereal grains wheat rice and corn, provide well over 80% of the total food consumed daily by humans (Chrispeels and Savada, 1994). To keep abreast with the growing population tactful management of plant growth and development is required, since the present situation unmonitored will severely place additional stress on the plant-growing capacity of the earth.

Among the cereals, rice (*Oryza sativa* L.) is the most important since it is the primary food source for over 40% of the world population, and based on population growth projections it has been estimated that rice yields must increase by over 5 million tons per year to maintain the current level of rice consumption per person (Hodges et al., 1990). Therefore, in order to accomplish this major task, extensive research efforts and progress have been made during the last three decades to improve yield. It was soon noted from these research efforts that high performance rice cultivars would be needed in

the future to make major improvements. Conventional breeding programs coupled with the current advances in *in vitro* cell culture of rice and genetic engineering using the tools of cellular and molecular biology will be most effective in the success of these types of research.

Genetic engineering of rice along with other cereals was not possible because of the fundamental problems associated with the introduction of genes into cells and in the regeneration of mature and fertile plants following genetic transformation (Hodges et al 1990), since monocotyledonous species exhibit natural resistance to *Agrobacterium* infection. However, the development of mechanical techniques such as, microprojectile bombardment, made it possible for foreign genes to be transferred to cereal grains. Furthermore, it has been stated that the ability to introduce specific genes for tolerance or resistance to salts, herbicide, insects, viruses, improved yields etc, will provide the next major development of new plant varieties. Even though these mechanical methods of gene transfer were being widely used, numerous difficulties with the techniques were becoming apparent. Therefore, most studies were turning to developing and improving biological transfer of genes in cereal grains by successfully identifying and isolating agronomically useful genes to be transferred between plant species (Christou, 1996)

Genetics of Rice

Rice belongs to the Gramineae family and the genus *Oryza* and is believed to have originated at least 130 million years ago. The genus *Oryza* has 21 wild species and 2 cultigens from *O. sativa* and *O. glaberrima*. Most of the *Oryza* species, including the cultigens, are diploid ($2n=24$) while nine are tetraploid ($2n=48$). Presently six genomes have been identified and are found to be either diploid or allotetraploid. The diploids

have the AA genome while the others have an AA, BB, CC, BBCC, CCDD, EE, FF, GG or HHJJ genome. The wild *Oryza* species are a very rich source for improvements to disease and pest resistance (Sitch 1990 and Ratnayaka, 1999). However, due to less homology between *O. sativa* genome and the wild *Oryza* related species transfer of genes between them via breeding is limited to low crossability and reduced recombination between the chromosomes. However, a technique known as embryo rescue has made it possible to transfer genes between distantly related species (Khush, 1997). The wild types and their useful traits are listed in Table 2.1.

Cultivated rice is the model system for cereal genomic research because of its small genome size (450 Mb – the smallest among the cereals), which makes it possible to develop detailed linkage maps and enables large-scale sequencing of expressed sequences. *Oryza sativa* has three subspecies, *indica*, *japonica* and *javanica*. *Indica* rice is grown in the humid regions of South and Southeast Asia, and China. It is naturally adapted to the monsoon climate, requiring little weed or water control. Plants of this subspecies are usually tall, leafy and respond to fertilizer by producing more vegetative parts rather than grain (Chrispeels and Savada, 1994; Ratnayaka, 1999). *Japonica* species are grown in the temperate regions such as Japan, Taiwan and the lower Yangtze valley of China and Korea. These require precise water, weed and pest control, since they are not well suited for the tropics. Their phenotypic characteristics include short stalks, with short or round grains and early maturation. *Javanica* subspecies are long grain plants commercially grown in the southern part of the U.S.

Table 2.1. The wild species of *Oryza* and related genera with their economic importance Adapted from “Gene Manipulation in Plant Improvement II” p.79. Showing the wild *Oryza* species presently known, along with the most useful economic trait.

Wild Species	Genome	Important trait
<i>O. glaberrima</i>	A ^g A ^g	Resistance to greenleaf hopper
<i>O. nivara</i>	AA	Resistance to grassy stunt virus and blast
<i>O. rufipogon</i>	AA	Tolerance of acid sulphate soils and stagnant flooding; source of cytoplasmic male sterility
<i>O. barthii</i>	A ^g A ^g	Resistance to bacterial blight
<i>O. longistaminata</i>	A ¹ A ¹	Floral characteristics for outcrossing
<i>O. eichingeri</i>	CC / BBCC	Resistance to brown plant hopper, green leaf hopper And white-backed plant hopper
<i>O. officinalis</i>	CC	Resistance to brown plant hopper, green leaf hopper, and white-backed plant hopper
<i>O. minuta</i>	BBCC	Resistance to brown plant hopper, green leaf hopper, white-backed plant hopper and blast
<i>O. australiensis</i>	EE	Resistance to brown planthopper and drought
<i>O. brachyanatha</i>	FF	Resistance to stemborer and rice whorl maggot
<i>O. ridleyi</i>	--	Resistance to rice whorl maggot, bacterial blight and blast
<i>O. longiglumis</i>	--	Resistance to bacterial blight and blast
<i>Porteresia coarctatum</i>	--	Tolerance of salinity

They were first considered to be an *indica* subspecies due to the morphological characteristics. It was later reclassified as a *japonica* subspecies based on the molecular characterization of DNA (Glaszmann, 1987).

In the past decade the need for the development of new varieties with higher yields, multiple resistances to diseases and pests, tolerances to flood, salinity is

recognized as the answer to solve the increasing demand for rice worldwide. Intensive research in the last ten years resulted in the development of two saturated molecular maps (> 1500 DNA markers) of the rice genome and localization of many important genes. Other accomplishments have seen the identification of quantitative trait loci (QTL's) associated with disease resistance, abiotic stress tolerance and yield potential in many ecosystems (McCough and Doerge, 1995).

Transfer of Genes in Rice

Until recently, the application of genetic engineering techniques were developed for dicotyledonous species only, since lack of reliable gene transfer was not properly regulated for monocotyledons (cereals). With constant genetic manipulation of the cereals, reliable and rapid transfer of genes is presently being developed. The most popular methods of gene transfer in cereal grains can be divided into (a) mechanical and/or chemical gene transfer, and (b) biological gene transfer. Mechanical and chemical methods of gene transfer currently include, microprojectile bombardment of cells and tissue, polyethylene glycol (PEG), electroporation and protoplast-mediated transformation. Biological methods of gene transfer are based on the evolved use of *Agrobacterium tumefaciens* a soil bacterium.

Mechanical Methods of Gene Transfer

Microprojectile Bombardment

This technique is also referred to as the biolistic or the particle gun or gene gun method. The technique briefly described involves plant tissues or cells being bombarded or shot with gold (3mg of 1 um-sized particles) or tungsten particles coated with plasmid DNA containing the genes to be transferred (Figure 2.1). The DNA coating on the

particles is incorporated into the cell's chromosome through a recombination or DNA repair process. The plant tissue treated is then subjected to a selection process using either kanamycin, hygromycin B or bialaphos. Subsequently, survivors are regenerated transformed from this tissue (Ratnayaka, 1999, Wilson et al., 1993).

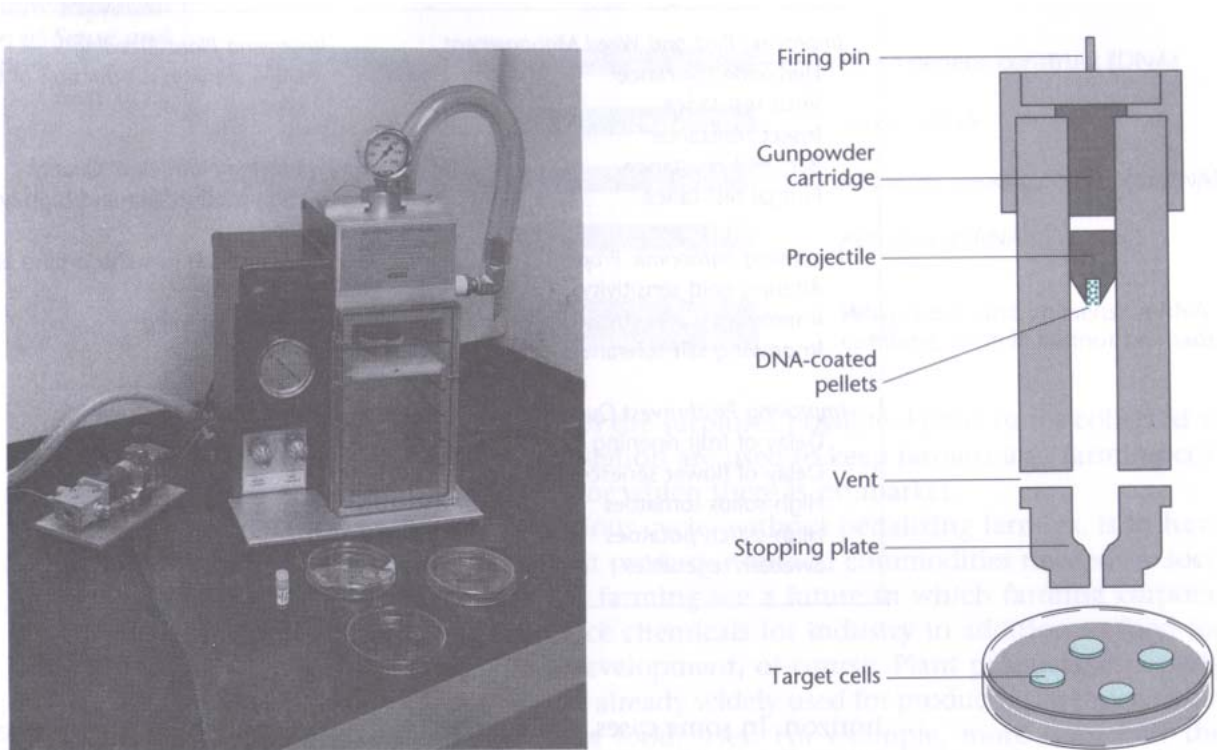


Figure 2.1. The DNA particle gun developed by John C. Sanford of Cornell University, fires tungsten pellets coated with DNA into plant cells. The pellets are held by a plastic microprojectile, which is accelerated by a gunpowder charge. The instrument shown is known as the Biolistic® system from BioRad. Source: Plant Genetic Engineering: New Genes Old Crops p. 405, 1994.

Advantages

Presently the only reported advantage of the gene gun is its efficiency over the protoplast-mediated transfer since any tissue (immature embryos, callus, immature panicles) can be used as an explant to facilitate regeneration.

Limitations

The limitations with this method are based on two reported observations, sterility and transgene inactivation (Christou, 1996; Tu et al., 1998; Wilson et al., 1993, Hiei 1996; Ratnayaka, 1999). The transgenic regenerants often show poor fertility and phenotypic abnormality. In addition the regenerated plants often appear stressed i.e. low seed production, premature senescence, poor reproductive development and stunted growth. This could be owing to the fact that even though a large amount of tissue is used, a great majority of the cells in the target tissue do not receive the coated DNA during bombardment (Wilson et al., 1993). The number of independent transgenic events is relatively low and transgene inactivation can be attributed to interactions between the inserted gene and its homologous DNA sequence (Tu et al., 1998, Ratnayaka, 1999). A study by Jiang 1999 proved that this technique was labor intensive owing to the large quantities of high quality immature embryos that had to be prepared and that it was difficult to adapt, while the transgenic plants were faced with somaclonal variation.

Polyethylene Glycol (PEG)

This method is basically used with protoplasts or osmotically sensitive cells. It involves the removal of the cell wall by using cell-wall-degrading enzyme, and then treating the exposed cells with a mixture of polyethylene glycol (PEG) and the transforming DNA. The exogenous DNA molecules are internalized in to the infecting tissue while a PEG-induced protoplast fusion takes place (Goldman et al., 1995). The PEG solution is importance since no transformation will occur without it.

Advantages

When coupled with protoplast technology it tends to increase the number of transformed plants from one in every eight to two in every eight plants regenerated (Tu et al., 1998; Ratyanaka, 1999; Wilson et al., 1993).

Limitations

The limitations are due to the types of cells being transformed, the type and concentration of PEG being used and the actually time of the cells being exposed (Goldman et al., 1995). The cells being used are usually very delicate and have to be handled with extreme care especially before the selection process. It is also very time consuming and application of the technique is not always successful as the efficiency rate of the protoplasts regeneration is relatively low.

Electroporation

This method was designed as an alternative to PEG transformation techniques. After the cell wall of the tissue is removed, the cell is exposed to an electric field that polarizes the membrane components and causes it to develop a potential difference across it. When the voltage exceeds the threshold level, the membrane breaks down in localized areas and the cells become permeable to exogenous DNA molecules (Goldman et al., 1995).

Advantages

Goldman and his colleagues reported that the popularity of this technique was due to its simplicity and reproducibility. However the limitations far surmounted the advantages especially if one was not familiar with the electric voltages used. Another

advantage was that transformation of the cells would still occur without the use of PEG, since it could be used on intact cells (Goldman et al., 1995).

Limitations

Electroporation could prove to be fatal to the cells being transformed if the electrical field exceeded the critical field. It became obvious that if this critical level was exceeded the damage to the cell would be irreversible thus rendered useless. Additionally, the transformation frequencies obtained are much lower than that of PEG, gene gun or other methods of gene transfer.

Protoplast-mediated Transformation

Using protoplasts for gene transfer arose from the fact that they are competent in both division and plant regeneration and also can be isolated in large quantities from embryogenic suspension cultures derived from mature seeds. An isolated protoplast has the ability to divide in 3-4 days and form colonies with an efficiency rate of 1-10% (Shimamoto, 1991). The isolated DNA is added to the protoplast suspension for uptake into the cell. Numerous studies have established this method as being successful because of its high efficiency in generating transgenic plants. The technique is further enhanced by using chemically treated liquid medium (PEG) or by applying an electrically pulse (electroporation) (Shimamoto, 1991; Kyojuka and Shimamoto, 1995; Ayres and Park 1994; Jiang, 1999; Ratanaka, 1999).

Advantages

Protoplast-mediated transformation is very well established. It is a popularly used method that has led to the development of fertile transgenic plants. The technique is relatively easy and straightforward and remains basically the same for dicots and

monocots plant species. It uses large quantities of dividing protoplasts that are able to form protoplast-derived colonies (Hiei et al., 1997; Park et al., 1996).

Limitations

The limitations are based on the plant material used. For example in rice, serious problems were associated with plants regenerated from *japonica* and *indica* varieties especially from protoplasts treated via electroporation. Somaclonal variations were most obvious in the transgenic lines produced, as there was significant variability in height, maturity, and seed production. Results also varied when different suspension culture media were used and environmental and seasonal problems also took a toll on the success of this technique. Overall, even when these limitations were best controlled, availability of suitable promoters, selectable markers and selective agents became significant problems (Jiang, 1999; Ratyanaka, 1999; Shimamoto, 1991; Christou, 1996; Kyozyuka and Shimamoto, 1995).

Biological Method of Gene Transfer

Agrobacterium Transformation

Agrobacterium transformation is made possible via the use of *Agrobacterium tumefaciens*, which is a soil bacterium that has evolved a unique permanent inter-kingdom gene transfer mechanism to infect, transform and parasitize plants. Its popularity can be attributed to the ease at which it can be used, since bacteria can penetrate cells at a wound site and actively integrate the transfer DNA stably into plant chromosomes (Ratnayaka, 1999; Mendel and Teeri, 1995). The pathogenic *Agrobacterium* also controls the regeneration process and subsequent proliferation, since the transferred DNA contains genes that are fully expressed in the transformed plant cell.

This will eventually lead to synthesis of plant hormones and further cell division, which will cause the transformed cells to develop into normally differentiated transgenic plants (Figure 2.2).

Prior to these studies it was thought that *Agrobacterium*-mediated transformation was virus-like and not able to result in permanent heritable genetic changes (Braun, 1962). In addition, the use *Agrobacterium* was restricted to dicotyledonous plants. This oversight was due to the fact that monocotyledonous plants were not natural hosts of *Agrobacterium tumefaciens* and lacked the ability to respond to wounding (callus formation), therefore rendering the regeneration of transgenic plants impossible (Ratyanaka, 1999; Hiei et al., 1996; Gould, 1991; Mendel and Teeri, 1995; Park et al., 1996).

Advantages

Scientists have been busily making several attempts to develop the use of *Agrobacterium* in cereals, since its discovery in 1982 (Zambriski et al., 1982), that reported important advantages of it compared to the direct non-biological gene transfer methods. *Agrobacterium* transformation technique is simple and relatively inexpensive to use worldwide. Compared to the mechanical methods of transformation, the gene integration is often low or a single copy; it also has the ability to incorporate itself into the plant genome without rearrangement (Ratyanaka, 1999; Gould, 1996, Doug et al., 1996). Its transformation rate is relatively higher than the mechanical methods. Ishida and colleagues reported a 10-50% transformation rate in tobacco (Ishida et al., 1996). Large segments of DNA (> 200 kb) including BAC fragments can be transferred. Compared to other methods of transfer, it is genotype independent.

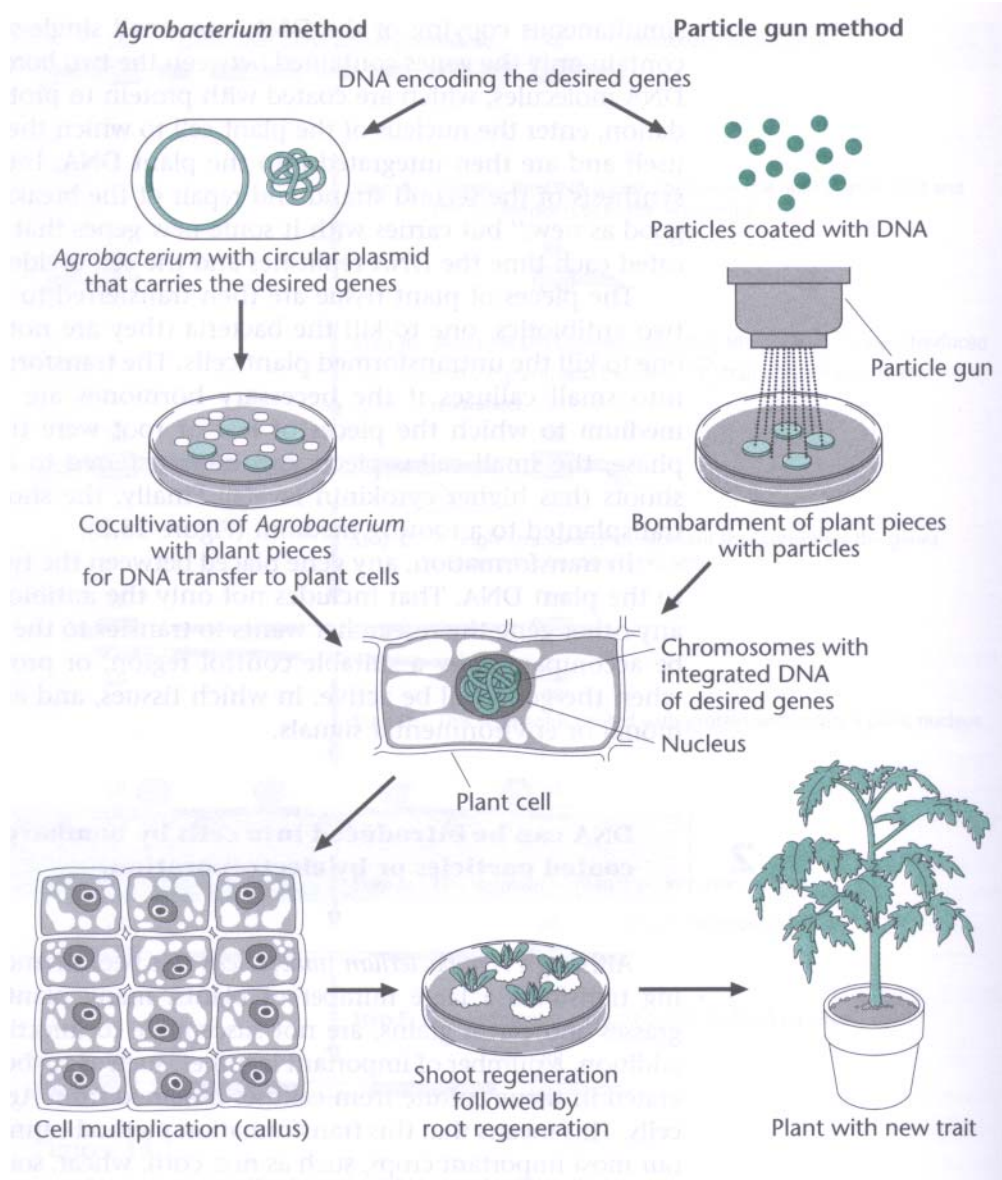


Figure 2.2. Schematic representation showing the *Agrobacterium* method of gene transformation versus the particle gun method. Source: Plant Genetic Engineering: New Genes, Old Crops, Chrispeels and Sadava, p. 405, 1994.

Limitations

The most evident restriction with this technique was its poor establishment. It had inconsistencies with the ability to repeatedly result in rapid and reliable production of transgenic plants. This posed a threat since for *Agrobacterium*-mediated transformation to be successful in rice (or cereals), it had to be readily regenerated *in vitro* and the regeneration had to be genotype-independent (Gould, 1996; Park et al., 1996). Other factors that heavily affected the success of this technique are (i) types and age of the tissue being inoculated, (ii) kinds of vectors being used, (iii) strains of *Agrobacterium*, (iv) details of infection, (v) use of acetosyringone, and (vi) concentration of selective agent (Hiei, et al., 1997; Gould 1996; Park et al., 1996; Komari et al., 1996; Khanna and Raina, 1999).

Alleviation of the Limitations

To alleviate these problems stated previously came in the form of the shoot apical meristem. Researchers saw this as a substantial improvement, since the shoot apex can succumb to *Agrobacterium*-mediated transformation (Gould, 1991; Gould 1996; Hiei et al., 1997; Khanna and Raina 1999; Park et al., 1996). Additionally, the apical meristem resulted in fertile transgenic plants that had the original genotypic and phenotypic make-up intact. This was owing to the consistently dividing meristematic cells found in the shoot apex that contained transformation-competent cell type. Successful studies recorded similar results in rice transformation (Morel, 1972; Gould 1996; Park et al., 1996). The successes achieved by using the shoot apical meristem were compared to methods using other plant tissues.

Historically rice genetic transformation came as no surprise, since in 1986 Ou-Lee and his colleagues reported transient expression in the protoplasts of rice using the PEG-mediated gene transfer. In that same year (Uchimiya et al., 1986), made noted success when he recovered stable transformed rice callus from *japonica* rice using PEG fusion. In 1988 three groups reported the production of transgenic rice plants from the *japonica* sub species. Zhang and Wu (1988) were able to produce transgenic *japonica* rice through PEG-mediated gene transfer using protoplasts while Zhang et al 1988 and Toriyama et al 1988 produced transgenic rice plants through electroporation using rice protoplasts. These studies noted that the success of this technique came from the higher frequency at which transgenic plants were produced.

Furthermore, incorporating the use of super binary vector, such as LBA4404 (pTOK233), would eventually result in an increased efficiency rate of rice genetic transformation (Komari et al., 1996). Moreover, creating constructs of this nature is guaranteed to optimize shoot apex-mediated transformation.

Statement of Problem

Despite the success of the apex-mediated DNA transformation, improvements had to be made to optimize this type of rice transformation, since presently a repeatable methodology is not fully established. Protocols of the technique are readily available (Gould, 1996; Gould et al., 1991; Hiei et al., 1996, Smith et al., 1996; Park et al., 1996, Valdez et al., 1998, Khanna and Raina, 1999; Ratnayaka, 1999); however, since the technique is still in the initial stages changes to improve upon it are constantly being made. Also, a mutual agreement among the studies stated that there was a need to find ways to increase the yield of the transformed plants, in return increasing the efficiency of

the technique, thus establishing the use of this technique worldwide. This was the overall aim of this research.

The four objectives of this study were the following: (i) to develop an efficient and reliable method for extraction culture and regeneration of young vegetative shoot apices in rice (ii) to optimize the survival rate of shoot apices after co-cultivation and vacuum infiltration (iii) to establish a kill curve of the apices using hygromycin B as the selective agent and (iv) to develop engineered DNA vectors for apex-mediated DNA transformation. The first part of the study involved the evaluation and optimization of specific parameters that affected shoot apex transformation, while the second part concentrated on constructing DNA vectors with rice ubiquitin (RUBQ2) promoter and the hygromycin B selectable marker gene fused to the super-binary vector. The use of hygromycin B as a selection gene provided the means of establishing a permanent transgene expression.

The work reported in this thesis is presented in three chapters. Each chapter deals specifically with a phase of this study. The values and figures in this research should be used as a general guide in understanding the methods needed to optimize apex-mediated DNA transformation in rice.

CHAPTER III

***AGROBACTERIUM*-MEDIATED TRANSFORMATION OF RICE (*ORYZA SATIVA* L.) VIA THE SHOOT APEX**

Introduction

The use of *Agrobacterium tumefaciens* as a method for the transferring of genetic information in various plant species is widely becoming a standard laboratory technique (Liu et al., 1992, Janssen and Gardner, 1989), despite earlier beliefs that this method of choice was restricted solely to dicotyledonous plants. Since numerous efforts to improve upon this method, the range of infectious plant species have grown from the original dicotyledonous plants that shows or does not show gall formation to the non-cereal (Asparagus and Dioscorea) and cereal monocotyledonous (maize and rice) plants (Hernalsteen et al., 1984; Schafer et al., 1987; Gould, 1997).

The cereals are reported to be the last group to be transformed using *Agrobacterium* via methods of direct gene transfer. In 1990, Raineri and his colleagues were among the first to provide molecular evidence for the stable transformation of *japonica* rice by *Agrobacterium* into callus cells (Raineri et al., 1990). The strain used in this study was *Agrobacterium tumefaciens* A281 that contained the super virulent pTiB0542 vir plasmid. A study by Hood et al. (1986) was the first to show that fertile production of cereal plants was possible with *Agrobacterium* transformation and EHA101 (A281 pTiB05420). However, Gould et al., (1991a), was the first to report evidence of transgenic progeny demonstrating inheritance of *Agrobacterium* transferred genes made possible by a new method using shoot apical meristem inoculation and plant regeneration. Despite these numerous studies conducted to show *Agrobacterium*

transformation was indeed possible in monocotyledons, this method was still not taken seriously and efforts to develop the method into a routine laboratory technique as with the dicotyledonous plant species was at a standstill. This was due to the fact that the scientific community still considered cereals to be refractory to *Agrobacterium* (Vasil, 1994; Vijayachandra et al., 1995).

However, in 1994 Hiei and his colleagues convinced the scientific community that *Agrobacterium*-mediated gene transfer, including the genomic integration of transferred genes, actually occurred in cereals (Hiei et al., 1994). In this research Hiei was able to demonstrate transformation of rice scutellar tissue with the help of a specially developed super binary vector pTOK233 in the *A. tumefaciens* strain LBA4404. In addition he demonstrated the similar effectiveness of the newly constructed vector pTOK233 (LBA4404) to the previously used super virulent pTiB0542 (EHA101). Even though Hiei was not able to recover plant shoot apices inoculated with pTOK233 (LBA 4404), Park and his colleagues in 1996 recovered transgenic rice plants from shoot apices inoculated with EHA 101 (Hiei et al., 1994; Park et al., 1996). This significant breakthrough paved the way for the continuous development of *Agrobacterium* transfer of genes in cereals, and further studies by Gould et al., (1996) and Zhang et al., (1999) demonstrated that *Agrobacterium*-mediated gene transfer in the cereals via the shoot apical meristem was possible.

Using the apical meristem as a way to provide rapid regeneration in plants arose from the assumption that the clonal and genetic characteristics of the original plant are retained in the explant (Morel, 1972). Therefore, the concept that the cereals were indeed *Agrobacterium*-transformation competent was made credible because of actively dividing

meristematic cells found in the embryo and shoot apex, as shown in Figure 3.1. These were areas of importance since they contained cells that subsequently underwent differentiation to form mature tissues of the plant body, and because owing to their highly organized structure the shoot apex was able to provide genetically stable transformation (Kane, 1996). The apex's natural ability to provide these advantages over the previously used methods of direct gene transfer (microprojectile bombardment, electroporation and polyethylene glycol), made it worthwhile to improve and develop this new method into a more routinely used laboratory technique.

The objectives of this research were to optimize the apex-mediated DNA transformation in rice by: i) developing a reliable and efficient method of gene transfer in rice, by preparing an extraction culture and increase the survivability of young shoot apex during plant regeneration, ii) optimize various parameters of maximum gene transfer rates via co-cultivation of apices with *Agrobacterium*, which included size of the apex, concentration of the infecting bacteria, contents of the selective medium, use of acetosyringone and concentration of hygromycin B, and iii) to identify and compare the transient expression of the beta-glucuronidase (GUS) gene in the rice shoot apices inoculated with LBA4404 (pCL3) and LBA4404 (pCL4).

Materials and Methods

Plant Material

Cypress, a popular *javanica* type long grain rice (*Oryza sativa* L.) developed at LSU AgCenter Rice Research Station Crowley LA was used entirely for the duration of this study. Seeds used were selected from the seed lots of 1995 and 1996.

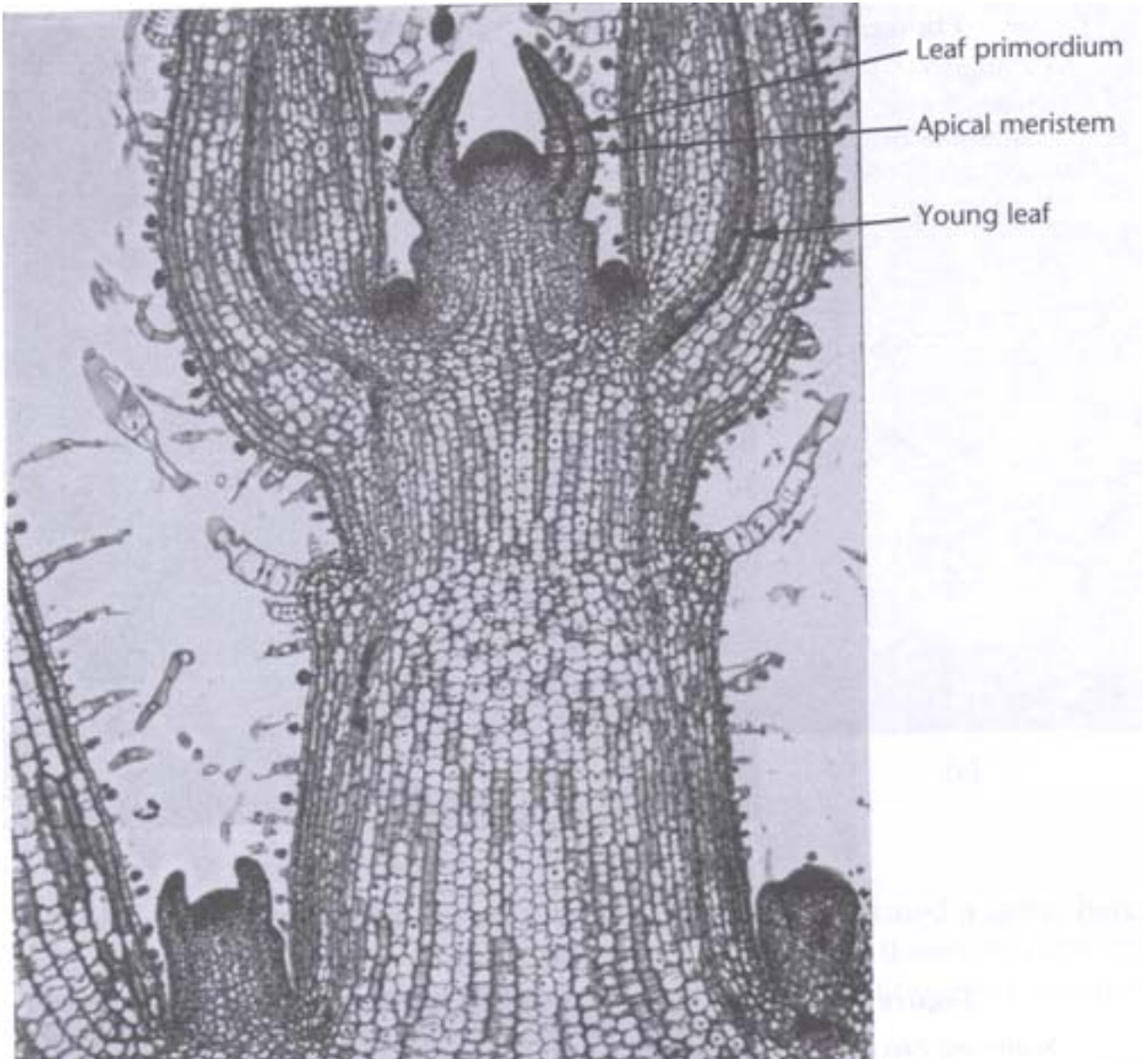


Figure 3.1. A microscopic view of the longitudinal section through the rice shoot apical meristem showing the location of the apex and the leaf primordia and the area containing the young leaves. Source: Plant Genetic Engineering: New Genes Old Crops Chrispeels and Sadava, p. 356, 1994.

Seed Surface Sterilization

Mature seeds were dehulled and sterilized in 20 mL of 0.1% Triton X-100 for 20 minutes and in 20 mL of 50% Clorox (v/v commercial bleach) with 50% 0.1 % Tween – 20 (surfactant) for 30 minutes with shaking. Seeds were washed three times in sterile deionized water and allowed to germinate in 16 mm petri dishes in the dark for 3 days and in the light for 4 days at 28° C. The petri dishes contained Rice Shoot Apex (RSA) medium which consisted of 4.3 g/L Murashige and Skoog (MS) salts (GIBCO BRL, Gaithersberburg, MD), 0.1 g/L myo-inositol, 0.002 g/L glycine, 30 g/L sucrose and 0.50 mg/L kinetin and 1.75 g/L phytigel.

Agrobacterium Strains and Culture

Agrobacterium strains LBA4404 (pCL3) and LBA4404 (pCL4) were grown in 50 $\mu\text{g mL}^{-1}$ kanamycin and 50 $\mu\text{g mL}^{-1}$ spectinomycin containing LB media (Difco Bacto Tryptone 10 g/L, Difco Bacto yeast extract 5 g/L and NaCl 10 g/L) solidified with 12 g of Bacto Agar at pH = 7 (GIBCO BRL, Gaithersberburg, MD) for 3 days at 28° C. Two days prior to inoculation, sterile wooden sticks were used to select single colonies of pCL3 and pCL4 and allowed to grow overnight in 50 ml of LB media with 50 $\mu\text{g mL}^{-1}$ kanamycin and 50 $\mu\text{g mL}^{-1}$ spectinomycin on a 200 rpm shaker at 28° C. After 24 hours, 0.5 ml of the overnight culture was re-suspended in 50 ml of LB without antibiotics and placed on a 200 rpm shaker at 28° C to be incubated overnight. The optical density (OD) of the bacterial solution was measured with a spectrophotometer at 600 nm. An OD value of 0.6-0.8 was taken as the optimal concentration. The restriction maps of the T-DNA region of pCL3 and pCL4 are given in Figures 3.2 and 3.3 respectively. LBA4404 (pCL3) and LBA 4404 (pCL4) were constructed and provided courtesy of Dr. Dawen

Liu, a visiting research scientist to Louisiana State University. *A. tumefaciens* LBA4404 has its T-DNA deleted but contains a functional *vir* region that is capable of transferring T-DNA into plant cells. The effect of the concentration of the *Agrobacterium* and the optimal OD reading were evaluated in one set of experiments.

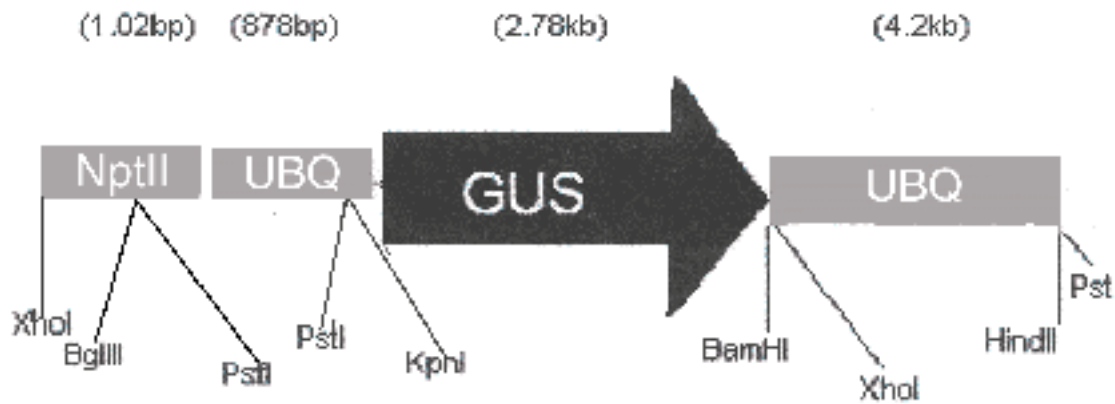


Figure 3.2. Restriction map of T-DNA region of the vector LBA4404 (pCL3). The arrow indicates the direction of transcription and sizes of individual fragments are placed in parentheses. NptII- neomycin phosphotransferase gene, UBQ - maize ubiquitin promoter, GUS- intron containing GUS gene and restriction enzymes – XhoI, BglIII, PstI, KpnI, BamHI, HindIII.

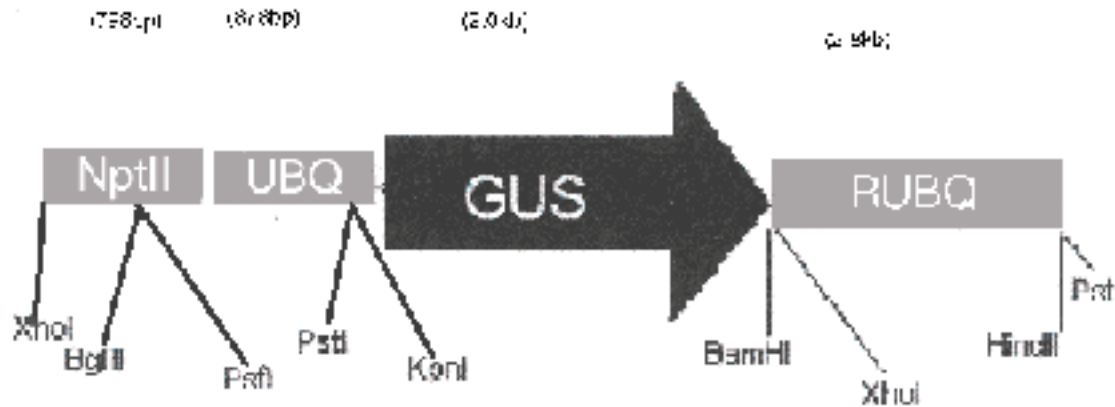


Figure 3.3. Restriction map of T-DNA region of the vector LBA4404 (pCL4). The arrow indicates the direction of transcription and sizes of individual fragments are placed in parentheses. NptII- neomycin phosphotransferase gene, UBQ- maize ubiquitin promoter, RUBQ- rice ubiquitin promoter, GUS- intron containing GUS gene and restriction enzymes – XhoI, BglIII, PstI, KpnI, BamHI, HindIII.

Shoot Apex Isolation

Shoots from 7 day old germinating seedlings grown on RSA medium were used for an apex extraction method modified from Gould et al., (1991a) (Figure 3.5a). The shoot apex is situated at the base of the seedling closest to the seed. A dissecting microscope at 15x magnification was used in conjunction with a beveled scalpel and forceps as shown in Figure 3.4, to isolate the apex. Working under the microscope, the endosperm is removed along with portions of the shoot, and all but a 1 cm basal portion of the stem referred to as the ‘log’ was left untouched (Figure 3.5b). The term ‘log’ is the word used to describe the area of the seedling containing the apex. The apex can be

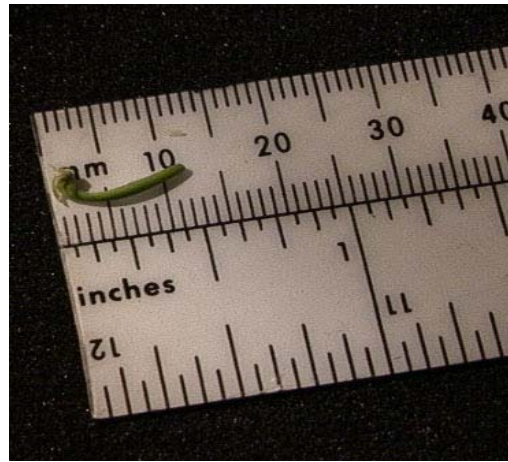
visually identified as a bulge within the log. It is then isolated from the ‘log’ by first making an asymmetrical cut along the entire length of the log with the green side up (log is green colored at the shoot end and white at the base). The apex was opened and exposed by cutting through the apical meristem longitudinally (Gould, 1997). The leaf primordia and the innermost elongating leaves were not removed since they aid the apex to grow and survive on the RSA medium. The apex was trimmed to a length of 1 mm with the base intact (Figure 3.5c). Apices were always plated in the upright position. The importance of size to the survival rate of the apices was evaluated in a two sets of experiments and the results shown in Figure 3.10. The growth stages of the shoot apical meristem are presented in Figure 3.6.



Figure 3.4. The apparatus needed for shoot apex isolation procedures performed entirely under a laminar hood.



A



B



C

Figure 3.5 (A-C). Pictorial presentation of the phases of apex isolation procedures showing a) 7-day old rice seedling, b) the 'log' containing the apex and c) the isolated apex with the leaf primordia intact to aid in survival.

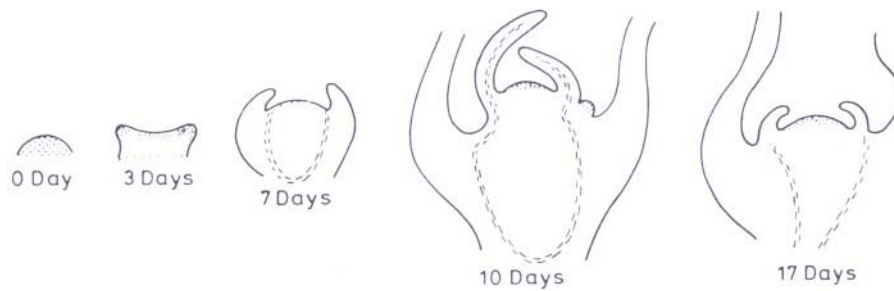


Figure 3.6. An illustration of the gradual development of the shoot apical meristem before and after shoot apex isolation. At 0-3 days apex is < 1.0 mm, 7 days 1.0-1.2 mm and the root and shoot axis are well established, between 10-17 days the root and shoot develops and averages > 4 cm. Source: Plant Tissue Culture: Applications and Limitations, p.171, 1990.

Inoculation and Co-cultivation

Immediately after the apices were isolated, they were inoculated by placing them in 5ml of the *Agrobacterium* suspensions of pCL3 or pCL4 in LB medium. The *Agrobacterium* suspensions were rocked for 5 minutes at 5 rpm at room temperature with a desktop rocker. The inoculated apices were vacuum infiltrated for 5 minutes at 20 Hg. After co-cultivation in these suspensions, the apices were blotted dry on sterile filter paper, to remove excess moisture and then plated on culture plates containing 4.3 g/L Murashige and Skoog (MS) salts, 0.1 g/L myo-inositol, 0.002 g/L glycine, 25g/L sucrose, 0.50mg/L kinetin, 0.050 g/L arginine, 1 mL B5 vitamins, 100 ppm acetosyringone and 1.75 g/L phtyagel. The above chemicals were purchased from Sigma or Sigma Aldrich, St. Louis, MO and Milwaukee, WI., respectively. Plated apices were then co-cultivated for 2-3 days in the dark at 25° C. After 3 days the explants were removed from the co-cultivation media, and rinsed 2-3 times sterile water containing 150mg/L timetin, before being assayed for GUS activity. The following parameters were evaluated in this research, which were reported in previous studies to have an effect on apex-mediated

DNA transformation. They are (i) the effect of acetosyringone to the co-cultivation media, (ii) the effectiveness of vacuum infiltration and the level of mercury (Hg) to use, and (iii) the minimum lethal concentration for hygromycin B (selective agent).

Assay of GUS Activity

Histochemical assays were based on the method described in Jefferson (1987), with the following modifications. The explants were cut into apices, stem, leaves and roots and incubated at 37 °C, in 1 ml of GUS substrate buffer (5 mM potassium ferrocyanide, 0.1M sodium phosphate buffer pH 7.0, 20 µl of 0.3 % v/v Triton X-100) for 25 minutes and then vacuum infiltrated for 5 minutes at 20 Hg. The chemical 5-bromo-4-chloro-indolylgluc-uronide (X-gluc) was added to the substrate buffer and explants were vacuum infiltrated for an additional 7 minutes at 20 Hg. The explants were removed from the vacuum and placed in 37° C for 24-38 hours. Within 24 hours explants were examined under the microscope for (GUS) gene expression, observed as blue spots.

Experimental Design

All experiments were conducted using five treatments and one control per set of treatment. Each experiment was replicated three times. For each sterilization procedure, 80 seeds were sterilized and placed 15-20 seeds per 16 mm Petri dish. Thirty-five apices were isolated per set with 5 apices per 16 mm Petri dish. For GUS histochemical assays, control and treatments were divided into roots, leaves, apices and stem and tested separately for the presence of blue coloration.

The treatment parameters were based on the following factors: (i) size of the apex, (ii) concentration of *Agrobacterium*, (iii) use and proper concentration of acetosyringone, (iv) the most suitable vacuum infiltration level, (v) minimum lethal

concentration (kill curve) of selective agent hygromycin B, (vi) optimal OD for *Agrobacterium*, and (vii) the most suitable *Agrobacterium* strain.

Data Analysis

Mean and standard error calculations, in addition to ANOVA and Duncan's Multiple Range test (at 5% level) comparisons were performed using the computer based statistical program SAS (SAS Institute Inc. version 8, 1999). Significance values were tested at $LSD_{0.05}$.

Results and Discussion

Sterilization of the Plant Material

In this study, Cypress performed better over Bengal, because it was proven in this study to have a higher germination rate in a 7 day period. Bengal's slow germination rate was attributed to the surface sterilization process, which contained a mixture of commercial bleach that decreased the germination rate. On the other hand, the sterilization process did not affect Cypress and its germination rate was basically similar before and after sterilization. The contrast between both varieties showed a general tendency for reduction, but lacked statistical difference. By comparing the effect of sterilization on the seeds of both Cypress and Bengal rice, it was clearly understood that timing was very important in this process because the germination rates of both seed types were significantly decreased when seeds were left in the sterilization solution (20 ml of 50% Clorox bleach and 50% 0.1 % Tween 20) longer than 30 minutes. In Figure 3.7 it is shown that as time increased over a period of 25 minutes, the percentage germination rate of the seeds decreased. Therefore, if the seeds remained in the

sterilization solution longer than 25 minutes the germination rate of Cypress was lowered to ~ 60% while Bengal's was lowered to ~35%.

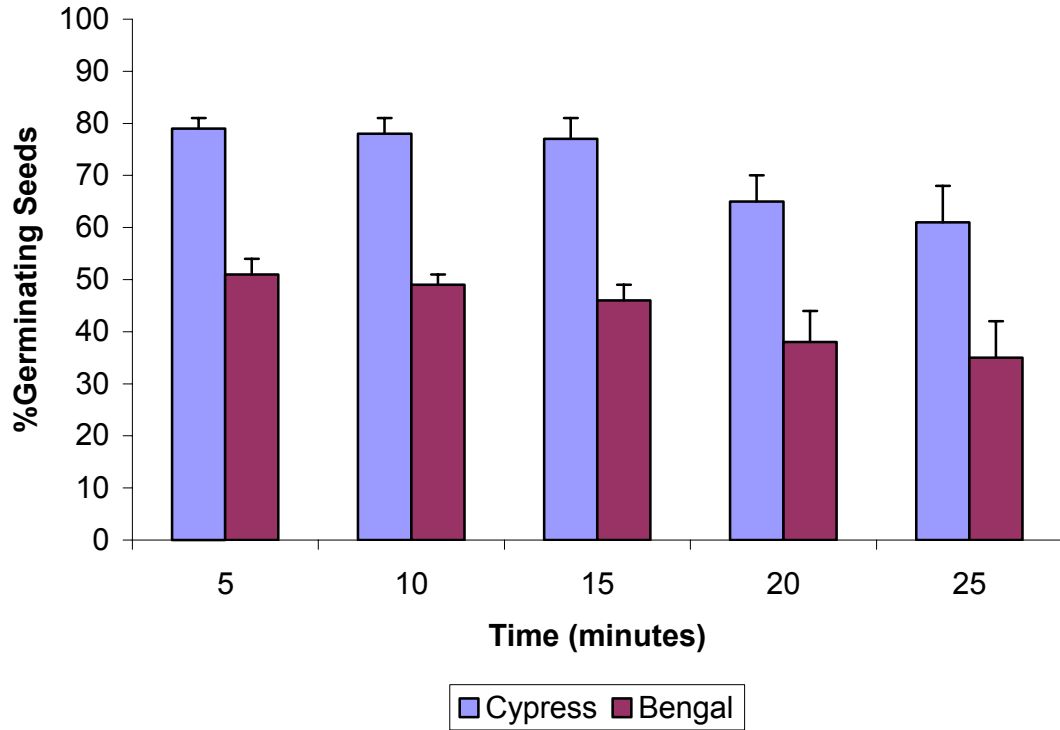


Figure 3.7. The percentage of germinating seeds versus the surface sterilization time of Cypress and Bengal seeds. Vertical bars represent standard error of the means.

Optimizing the Survival Rate of the Apices

Improving on the survival rate of the apices under standard conditions was very important, since the reported rate of survival was 40% by Gould, (1997). In the Fall of 1998, the first experiments conducted for this reason, 20 sets of apices being isolated, which had 4 treatments and 1 control per set. Apices were allowed to grow on Rice Shoot Apex (RSA) medium consisting of 4.3 g/L Murashige and Skoog (MS) salts (GIBCO BRL), 0.1 g/L myo-inositol, 0.002 g/L glycine, 30 g/L sucrose and 0.50mg/L kinetin. On day 7, growing apices were transferred to RSA medium without hormones. Explants were observed over a 12-day period and size and color of the apex, and the presence of leaf and /or roots were recorded. The first two sets were discarded because of the apices' lack of ability to grow and survive on the medium. This decline in survivability was soon attributed to the inconsistencies with the starting size of the apex. It was observed that if the starting size of the apex was much greater than 1.5 mm, it meant that not enough leaf tissue was removed and the explant gave rise to multiple shoots, which was not a desirable characteristic for this study. An apex smaller than 1 mm, had too much of its leaf tissue removed and/ or the apex itself was be damaged. Inevitability within hours of isolation, this apex would die, as the factors to aid in growth and survival had been removed. Apices that were between 1.0mm - 1.2 mm survived better under laboratory experimental conditions as shown in Figure 3.10, where the results of Fall 1998 were compared to a similar experiment conducted in Summer 2001. Another problem was soon observed, as early transfer of the apices from RSA with hormones to RSA without hormones would prove to be fatal. Cytokinin (kinetin-growth hormone) was used to promote cell division and aid in the regeneration of the apical meristem. Once shoot

growth was not sufficient, the apices ability to survive on medium without a growth hormone was zero. These were the only two problems that affected this experiment as steps to prevent bacterial and fungal contamination were strictly adhered to. However, by constantly correcting these problems, 18 sets of experiments showed gradual increases in the survival rate for the duration of the experiment as shown in the Figure 3.10. After the 16th set, the number of surviving apices was equal to the number of apices isolated. After 500 apices, 447 survived giving a cultivation rate of 90.1%. These results showed a 40% increase over similar results reported by Gould et al., (1997).

Culturing of the *Agrobacterium* Strains

Timing and temperature greatly affected virulence of the strains LBA4404 (pCL3) and LBA4404 (pCL4). These strains had to be promptly removed from culture within 48 hours. When these strains were left for a more extended period of time, the ability of the bacteria to cause transformation within the shoot apex decreased. Temperatures higher than 28° C also proved to be detrimental. Making fresh *Agrobacterium* suspensions for each experiment was routinely done since it aided in maintaining competency of the *Agrobacterium* suspension and increased chances of successful transformation.

Optimal Optical Density (OD)

In finding the most suitable level of OD for the *Agrobacterium* used in this experiment, seven different levels were tested; 2.0, 1.8, 1.5, 1.2, 1.0, 0.8, 0.6, and 0 (control). The optimal OD was determined by using histochemical analysis. This was done by observing the number of blue spots in the apex transformed by the *Agrobacterium* of choice as shown in Figure 3.8, and also by the number of apices that exhibited this blue color for that particular OD measurement. The OD was not the same

for the *Agrobacterium* strains used: this is also given Figure 3.8. For example, when using the *Agrobacterium* LBA4404 (pTOK233), the highest number of GUS expressing apices were observed between OD readings of 1.5-2.0, therefore this OD was used for this strain. However, a difference was seen with LBA4404 (pCL3) and LBA4404 (pCL4), as the highest number of GUS expressing apices were observed at OD 0.6-0.8. This difference was attributed to the nature of *Agrobacteria* strains. During the study it was observed that pTOK233 had a tendency to have relatively few colonies after 72 hours of culturing, while pCL3 and pCL4 already had a bacterial lawn (large amounts of tiny colonies) within 36 hours. Using *Agrobacterium* at OD of 0.6-0.8 was coincidental with the fact that most *Agrobacterium* strains would be experiencing the early log (exponential) phase during which active cell division occurs at 0.4-0.8 OD (Janssen and Gardner, 1989). These results were further evaluated to find the most suitable *Agrobacterium* strain for apex-mediated transformation. It was decided to use pCL3 and pCL4 because they produced colonies in as little as 36 hours. This decision was reinforced by Dawen Liu's unpublished data stating the effectiveness of the strains in the transformation of sugar cane. All OD readings were measured at an absorbance of 600 nm.

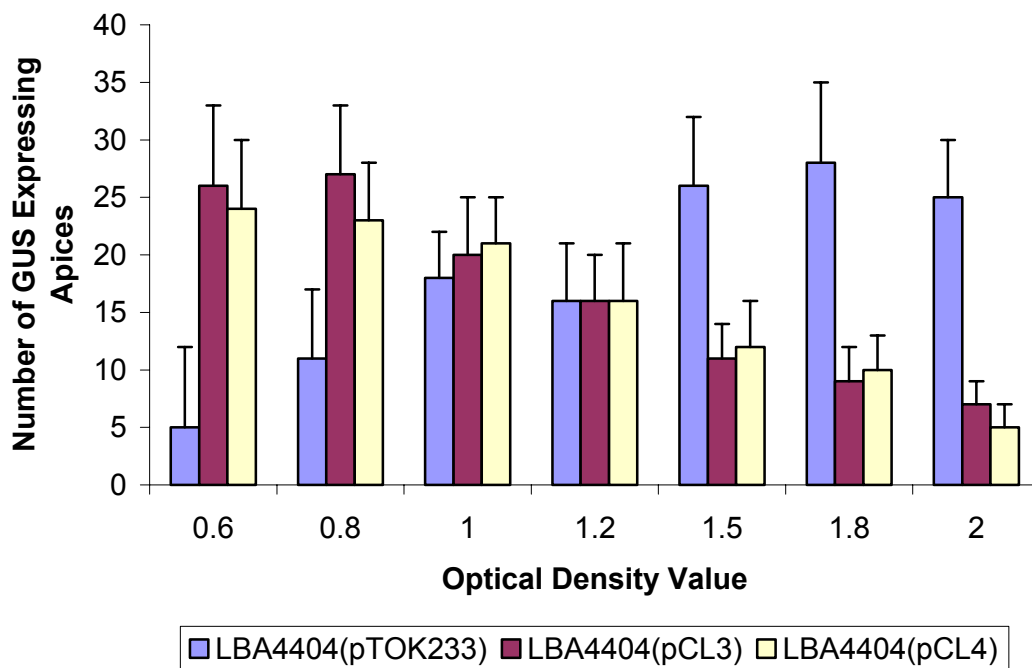


Figure 3.8. Optimal optical density of pTOK233, pCL3 and pCL4 *Agrobacterium* engineered strains as measured by the number of GUS expressing apices. Vertical bars represent standard error of the means. Optical density (OD) was measured at 600nm.

Effect of Acetosyringone During Co-cultivation

Various studies (Khanna and Raina, 1999; Gould, 1997), examined the importance of acetosyringone to *Agrobacterium* co-cultivation procedures. These studies stated that without it, there would be a complete absence of GUS expression, the quickest and most visual proof of transformation. However, the problem was with the level of acetosyringone to use since concentrations to use ranged from 30 ppm – 1000 ppm from different studies. Therefore, five different concentrations were tested; 50 ppm, 100 ppm, 400 ppm, 600 ppm and 1000 ppm using 30 apices per concentration. The effectiveness of the concentration of acetosyringone was observed by the presence of blue color from GUS assays performed on the apex. The results are presented in Figure 3.9. The results show that as the concentration of acetosyringone increased, the number of blue GUS spots/ apices also increased. However, between 400 ppm and 1000 ppm the results (i.e. the number of blue spots) were not significantly different. Therefore, using acetosyringone at 1000 ppm was decided upon for the co-cultivation medium. For this study a stock solution for acetosyringone was prepared using the following method: -

1ml of 95% ethanol with 20mg/ml of acetosyringone.

Kill Curve for Hygromycin B

Hygromycin B (Hph) as a selective agent comes from the fact that it is an aminocyclitol antibiotic that inhibits protein synthesis in both prokaryotes and eukaryotes. This ability is useful in enhancing plant transformation because the hygromycin (*hph*) gene is particularly effective in stringent selection of transformed rice cells (Zheng et al., 1991). Six concentrations were tested; 25 mg/L, 12.5 mg/L, 7.5 mg/L, 5.0 mg/L, 2.5 mg/L and 0 mg/L (control). During the course of the experiment, it became

quite obvious that the apices did not have the ability to survive on Hph 25 mg/L. After a month of careful observation the use of Hph at 25 mg/L was discontinued.

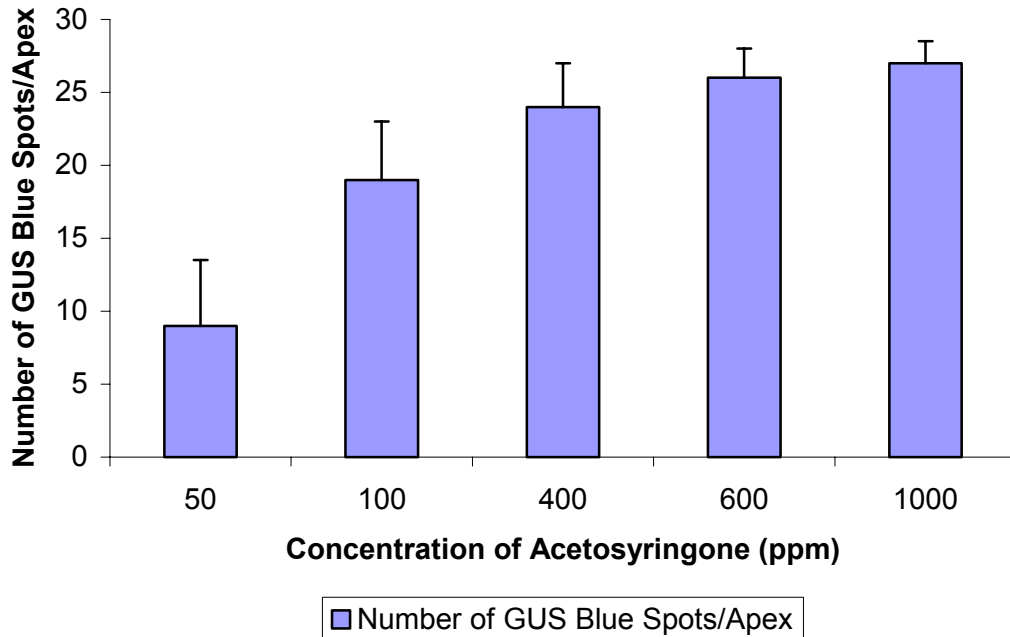


Figure 3.9. Effect of acetosyringone on GUS activity as measured by the number of blue spots in the apex. Vertical bars represent the standard error of the means.

Hph 12.5 mg/L and 7.5 mg/L were soon discontinued for the same reason, since after the fourth set they were no surviving apices. Hph 5.0 mg/L was determined to be the minimum lethal concentration with the ability to kill the apices for this study, since all apices started to brown after 3 days and were all dead within 2 weeks. The results of this experiment are shown in Table 3.1, with the number of survivors recorded at 7 and 14 days.

Table 3.1. The number of surviving apices grown in the presence of 0-25 mg/L Hygromycin B for 7-14 days. Hph- hygromycin. Each experiment was replicated at least three times.

Concentration of Hygromycin	No. of Apices Plated	No. of Survivors at 7d	No. of Survivors at 14d
Hph 0 (control)	100	70 ^a	65 ^a
Hph 2.5 mg/L	100	40 ^b	15 ^b
Hph 5.0 mg/L	100	40 ^b	0 ^c
Hph 7.5 mg/L	100	30 ^c	0 ^c
Hph 12.5 mg/L	100	1 ^d	0 ^c
Hph 25.0 mg/L	100	1 ^d	0 ^c
TOTAL	600	182	80

^{a-d} Data with the same superscript letters are not significantly different from each other at the 5% level based on Duncan's Multiple Range test.

Effectiveness of Vacuum Infiltration

Vacuum infiltration is very important to transformation, however for this study the optimal level at which to vacuum infiltrate had to be evaluated. Four levels of vacuum were tested at 5 Hg, 10 Hg, 15 Hg, and 21 Hg. The effectiveness of vacuum infiltration at the different levels Hg was reflected by the presence of blue spots in the apices after GUS assays. The number of apices with the same intensity of blue spots was recorded as shown in Table 3.2. The ratio of transformation was highest in Treatment 5, which showed 91 % of GUS activity in the apices when AS at 1000 ppm was used. Treatment 5 led us to believe that 21 Hg was the optimal level at which transformation would be greater than 90%. Co-cultivation was for 3 days and selection began after 3 days.

The Efficiency of Transformation

The results of all the factors tested (acetosyringone, vacuum infiltration, optical density (OD), concentration of hygromycin B) were all combined in one final experiment to evaluate the effectiveness of these factors on enhanced transformation and growth rates of treated apices. The results are given in Table 3.3, were the total number of apices used

was 80 for each treatment. These results reinforced that vacuum infiltration at 21 Hg and the use of acetosyringone at higher levels aided in increasing the number of transformed apices to be transferred to the selection medium. Moreover a survival rate of 90% and greater was also maintained from co-cultivation to selection at a 7 day interval and a survival rate of ~85% was achieved at 14 day intervals.

Table 3.2. Gus expression in shoot apex and roots under different levels of vacuum, co-cultivation, and acetosyringone.

Treatments	No. of apices with Blue spots	No. of roots with Blue Spots	No. roots/apices Transformed
Treatment 1	0 ^a	0 ^a	0/0=0
Treatment 2	4 ^b	2 ^b	2/4=0.5
Treatment 3	9 ^c	7 ^c	7/9=0.78
Treatment 4	17 ^d	12 ^d	12/17=0.71
Treatment 5	21 ^e	19 ^e	19/21=0.91
TOTAL	51	40	40/51=0.78

Treatment 1- no Agro; no VI; Co on RSA (I/K/AS 100ppm); Sel. (RSA)

Treatment 2 – pCL3; VI 5 Hg; Co on RSA (I/K/AS 400 ppm); Sel. (RSA)

Treatment 3- pCL3; VI 10 HG; Co. RSA (I/K/AS 400ppm); Sel. (RSA/Hph)

Treatment 4- pCL3; VI 15 Hg; Co. RSA (I/K/AS 600ppm); Sel (RSA/Hph)

Treatment 5 –pCL3; VI 21 Hg; Co RSA (I/K/AS 1000ppm); Sel (RSA/Hph)

Agro-Agrobacterium;VI-vacuum infiltration;Co-cocultivation; RSA- Rice Shoot Apex medium; I-Indoleacetic acid IAA; K-Kinetin; AS-acetosyringone; Sel-Selection;Hph-hygromycin B

^{a-e} Data with the same superscript letters are not significantly different from each other at the 5% level based on Duncan's Multiple Range test.

Table 3.3. Transformation efficiency of shoot apex under optimized vacuum infiltration and co-cultivation conditions. Each treatment was replicated at least three times.

No. of Treatments	No.of transformed Apices after 7d	No.of transformed Apices after14 d	Transformation efficiency of apex at 7d/80	Transformation efficiency of apex at 14d/80
Trt 1	0 ^a	0 ^a	0/80=0	0/80=0
Trt 2	75 ^b	71 ^b	75/80=0.94	71/80=0.89
Trt 3	73 ^c	66 ^c	73/80=0.91	66/80=0.83
TOTAL	148	137	148/160=0.93	137/160=0.85

Treatment 1: no VI, no Agro (control), treatment 2 VI at 21 Hg; pCL3 and pCL4; Treatment 3 VI at 21 Hg; pTOK233. Total number of seeds per treatment was 80.

VI-vacuum infiltration, *Agro-Agrobacterium*.

^{a-c} Data with same superscript letters are not significantly different from each other at the 5% level based on Duncan's Multiple Range test.

Inoculation of Apices with LBA4404 (pCL3) and LBA4404 (pCL4)

Seven sets of inoculation experiments were conducted on apices with constructs pCL3 and pCL4, respectively. Apices began to brown 3-4 days after inoculation and the number of survivors was recorded 7 days after inoculation. A total of 270 explants were recovered from the apices and the survival rate is shown in Figure 3.10. The comparison between the inoculation of pCL3 and pCL4 were also analyzed and recorded in Table 3.4. Leaf, root and apex samples from all the explants were tested for GUS activity (Jefferson et al 1987). Photographic representations of the results are shown in Figures 3.11-3.14. This method was used to detect possible transformed plants. This method was repeated thrice for each sample to obtain repeatable results. The overall condition of the explants was recorded and presented in Tables 3.5.

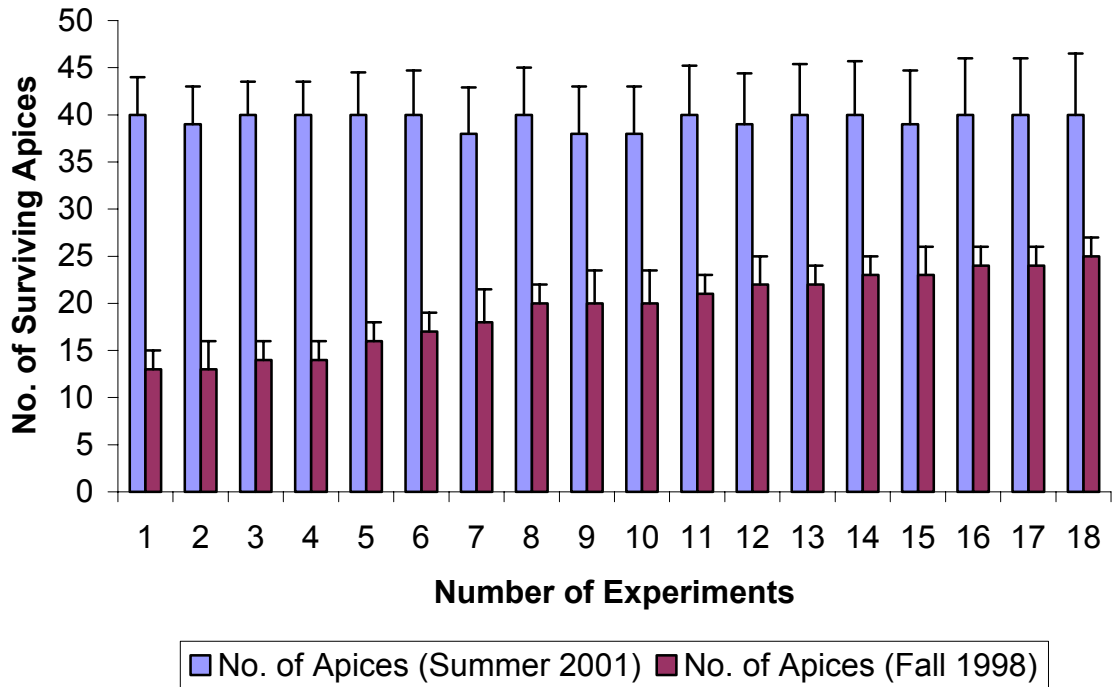


Figure 3.10. Comparison of the survival rate of the extracted apices in Summer 2001 between the survival rates achieved in Fall 1998 under similar experimental conditions. Total number of apices isolated for Fall '98 was 25, while the total number of apices isolated for Summer '01 was 40. Vertical bars represent the standard error of the means.

Table 3.4 Inoculation of apices and plant regeneration efficiency using LBA4404/pCL3 and LBA4404/pCL4. Each experiment was replicated three times.

Agro strain	No. Apices Inoculated	No. of Plants Regenerated	No. of Plants/Apices Inoculated
LBA4404/pCL3	40	39 ^b	39/40=97.5
	40	38 ^c	38/40=95.0
	40	39 ^b	39/40=97.5
	40	37 ^d	37/40=92.5
	40	39 ^b	39/40=97.5
	40	38 ^c	38/40=95.0
	40	37 ^d	37/40=92.5
LBA4404/pCL4	40	40 ^a	40/40=100.0
	40	38 ^c	38/40=95.0
	40	37 ^d	37/40=92.5
	40	39 ^b	39/40=97.5
	40	39 ^b	39/40=97.5
	40	38 ^c	38/40=95.0
	40	39 ^b	39/40=97.5

^{a-d} Data with the same superscript letters are not significantly different from each other at the 5% level based on Duncan's Multiple Range test.

Table 3.5. Analysis of apices after co-cultivation with (pCL3) or (pCL4) at 3, 7 and 14 day intervals. A total of 280 apices were evaluated for pCL3 and pCL4.

Agro Strain used	Parameters Evaluated	Days After Treatment		
		3	7	14
LBA4404/pCL3	Length	0.5 ^a cm	1.5 ^b cm	3.5 ^c cm
	Color	Green	Green	Green
	Roots	-	+	+
	Leaves	-	+	+
LBA4404/pCL4	Length	0.4 ^a cm	1.3 ^b cm	3.1 ^c cm
	Color	Green	Green	Green
	Roots	-	+	+
	Leaves	-	+	+

- = explants without leaves or roots

+ = explants with leaves or roots

^{a-c} Data with the same superscript letters are not significantly different from each other at the 5% level based on Duncan's Multiple Range test.

Figure 3.11. Comparison of control (left) and GUS treated roots (right), showing no difference in GUS activity. Results show that LBA4404 (pCL3) and (pCL4) are shoot apex specific.

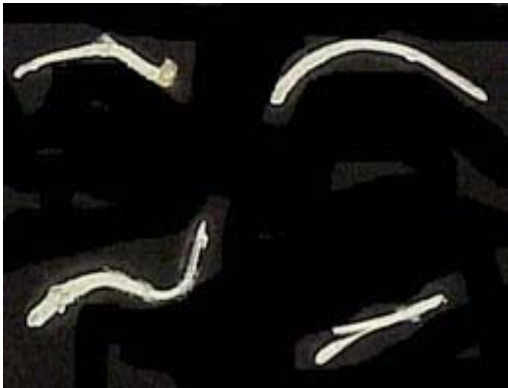
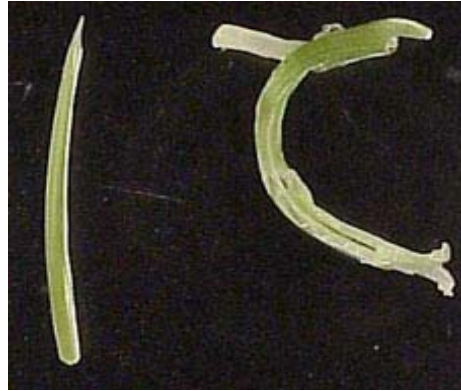


Figure 3.12. Comparison of control (left) and GUS treated roots (right), showing no difference in GUS activity. Results proving that LBA4404 (pCL3) and (pCL4) are shoot apex specific.

Figure 3.13. GUS staining of shoot apex transformed with LBA4404 (pCL3) after 4d of co-cultivation.



Figure 3.14. GUS Staining of shoot apex transformed with LBA4404 (pCL4) after 4 d of co-cultivation.

Conclusion

This study demonstrated the transfer of foreign genes to rice via *Agrobacterium tumefaciens* LBA4404 (pCL3) and LBA (pCL4) by the shoot apex are indeed credible. The interest in developing this method came from the first report of *Agrobacterium*-mediated cereal transformation in maize by Gould et al., (1991a), which caused the issuance of an US patent to the Texas A&M University System for this invention (Smith et al., 1992). The method has proven to be valuable because it is genotype-independent, plant regeneration is direct and rapid on a simple medium, and also the expected culture induced mutation is low (Ratnayaka, 1999).

Various studies by Gould et al., (1991) and Park et al., (1996) demonstrated that shoot apex-mediated DNA transformation using EHA 101 occurred in rice. However, the incidence of transformation was extremely low, approximately 5 plants per 721 inoculated shoots or simply stated a 0.7% survival rate, and Gould et al., (1991) had difficulties fully establishing the transformation of maize by shoot apices. Therefore, the attempt of our research was to once more reinforce that (a) apex-mediated DNA transformation does occur in rice and (b) to provide ways in which to improve the frequency of transformation. Our research was then geared towards improving upon this transformation rate by engineering vectors that would greatly enhance transformation owing to their construction. In addition, increasing the survival rate of the apex by improving the factors that directly affected the apex (i) size of the apex, (ii) level of vacuum infiltration, (iii) concentration of acetosyringone for co-cultivation, (iv) optimal OD of *Agrobacteria*, (v) concentration of selection agent hygromycin B and (vi) suitable vectors via construction (pCL3 and pCL4).

Our study determined that the paying attention to the size of the apex was very important to the cultivation rate of the apex. An apex ranging between 1.0-1.2 mm would be able to survive transformation, giving rise to true transgenic plants free of multiple shoots. Using acetosyringone would greatly enhance the transformation rate and vacuum infiltrating at 20-21 Hg is very significant in transformation as it assisted in increasing the transformation rate of the apex. Bacterial virulence was of utmost importance and it was found that using the most suitable OD for a specific strain of *Agrobacterium* would determine the success or failure of the genes to be transferred to the apex.

By consistently testing to achieve reliable and repeatable results, our research produced the first example of rice shoot apex transformed by pCL3 and pCL4. We were able to increase the survival rate to over 90%. The transformation rate was subsequently increased, since transformation of the apex is directly dependent on the ability of the apex to survive culturing. In comparison to EHA 101 used in previous studies, pCL3 and pCL4 indicated that with further research would bring about fertile transgenic plant from the shoot apex. The results indicated that the gene transferred would be more active in the apex and would show least activity in the roots, an advantage over other previously used constructs. The level of GUS activity showed the superiority of the rice ubiquitin promoter (RUBQ2) in the shoot apex via pCL3 and pCL4. These results reinforced the inefficiencies now surrounding the popular and widely used 35S or cauliflower mosaic virus (CaMV) promoters. Overall, timing and temperature were extremely important, since failure to comply with appropriate and suggested time and temperature of culturing bacteria, inoculating apices would prove to be fatal to the success of any research.

CHAPTER IV

CONSTRUCTION OF ENGINEERED DNA VECTORS

Introduction

Genetic engineering involves the transfer of genes between organisms. Historically gene transfers are of a result of sexual crosses that took place during plant evolution (Chrispeel and Sadava, 1994), and were often times restricted to organisms of the same species. This natural occurrence led scientists to manipulate transfer between *Agrobacterium tumefaciens* and certain plants to develop techniques to efficiently and permanently transform plants. The use of *Agrobacterium*-mediated transformation is most convenient as it allows scientists to construct DNA vectors of interest and use the naturally occurring gene transfer system in plants to produce transgenic plants (Hooykaas, 1989; Chrispeel and Sadava, 1994).

Prior to the discovery of plant transformation, natural hybridization or heroic efforts of plant breeders would be the only way possible for crop plants to acquire large number of economically important genes from wild species. The advent of plant transformation has allowed the break down of sexual barriers, so that any gene natural or artificial from any organism can be introduced into the plant of interest. However, for the gene to be useful it has to be expressed correctly at the right time, in the right organ, tissue or cell, and also the protein that the gene encodes must have the correct function. Therefore with this in mind, the ability to identify useful genes and to introduce difficult genes into crops of interest is always a factor of utmost importance.

Presently, *Agrobacterium* transformation lessens the challenges of such transfers as it guarantees that the gene to be transferred will be stably integrated into the

chromosome of a single plant cell and will eventually be inherited by all the progeny of this cell. Basically the transfer of genes via *Agrobacterium tumefaciens* transforms the plant. This occurs because a bacterial plasmid, which is a circular DNA molecule, carries the genes transferred from the bacterium to the plant cell. Transformation of plants is made possible by binary vectors that consist of a disarmed Ti plasmid (tumor inducing helper plasmid) and a smaller plasmid that carries the T-DNA (transfer DNA) (Hiei et al., 1994; Komari et al., 1996).

The development of super binary vectors such as LBA4404 (pTOK233) and LBA4404 (pSB324) have led to much improved transformation technologies for monocotyledons (Komari et al., 1996). These super binary vectors are different from normal vectors since they contain virulence (*vir*) genes that are responsible for the transfer of the T-strand into the plant cell, mobilization of transferred DNA into the nucleus and subsequent stable integration into the host genome (Janssen and Gardner, 1988).

However, even with the use of a super binary vector in vector constructs, the most common hindrance to the success of the transformation was the ability to properly detect actual transformation in the plant tissues and not in the bacterial cells, since most indicator genes would already be active in the *Agrobacterium* (Vancanneyt et al 1990; Janssen and Gardner, 1989). Deliverance from this problem came from using the chimaeric gene construct that expressed B-glucuronidase (GUS) in transformed plant tissue and not in bacterial cells (Vancanneyt et al., 1990; Janssen and Gardner, 1989).

This GUS gene is a reporter gene containing a plant intron that has the natural ability to splice very efficiently, is portable (has the ability to be easily inserted into

different sites of any gene of interest) and contains stop codons in three possible reading frames that is excluded in agrobacteria. The overall benefit of using this reporter gene is that it causes GUS enzymatic activity through a splicing apparatus not present in prokaryote. This activity is evident from the blue color observed when histochemical assays are conducted on transformed plant tissue. Basically this blue expression can be observed as early as 18-24 hours after *Agrobacterium* infection in the transformed plant tissue. (Vancanneyt et al., 1990; Janssen and Gardner, 1989).

The objective of this research was to construct a series of Ti-plasmids containing the GUS gene (2080 bp) with intron and the hygromycin gene (1100 bp) and driven by the rice ubiquitin promoter (RUBQ2) (2750 bp).

Materials and Methods

Minipreparation of DNA and DNA Screening

An alkaline protocol provided by Dr. Svetlana Oard was followed completely along with a Qiagen Purification kit and a Zymo Gel DNA Recovery kit purchased from Qiagen Inc. Valencia, CA., and Orange, CA., respectively. All DNA screening was done using gel electrophoresis and Lambda (λ)/HindIII as a marker and low mass DNA marker.

Cloning Procedures

This was conducted from protocols developed by Dr. Svetlana Oard (Louisiana State University). It consisted of digestive reactions that followed this general example: (DNA 5 μ l, RNase 0.2 μ l, Buffer - React 1/2/3 2.0 μ l, water 10-12 μ l and specific digestive enzymes 0.1-0.5 μ l.), ethanol precipitations and blunt end overhangs using DNA

20 µl, 10 µl, 5 x T4 Polymerase, 1 µl 10mM dNTP's, 50µl sterile water and 5µl T4 DNA Polymerase. All ingredients used were purchased from GIBCOBRL Life Technologies.

Histochemical (GUS Assays)

To detect the activity of the plasmid/ vector constructed in the shoot apex, GUS assays performed as described previously in Chapter III – Materials and Methods. A schematic illustration of the general steps involved in the construction of Ti plasmids to be reintroduced into *Agrobacterium tumefaciens* can be seen in Figure 4.1.

Results and Discussion

Initially the basic plasmid pLIT-G1 (4850 bp) containing the GUS intron was first constructed from the binary vector pCNL65 cloned into LITMUS 28 at the restriction sites NcoI and EcoRI. Gel electrophoresis confirmed pLIT-G1 as shown in Figure 4.2. pLIT-G1 was then cloned into pRGL210 resulting in pRQGus 7780 bp fragment consisting of 2750 bp RUBQ2 promoter, 1100bp GUS intron and 300 bp Nos 3' termination end (Figure 4.3). pRQHg a 6800 bp fragment was also constructed. It was similar to pRQGus instead it had a 1100 bp Hph region attached to the 300bp Nos 3' termination end (Figure 4.4).

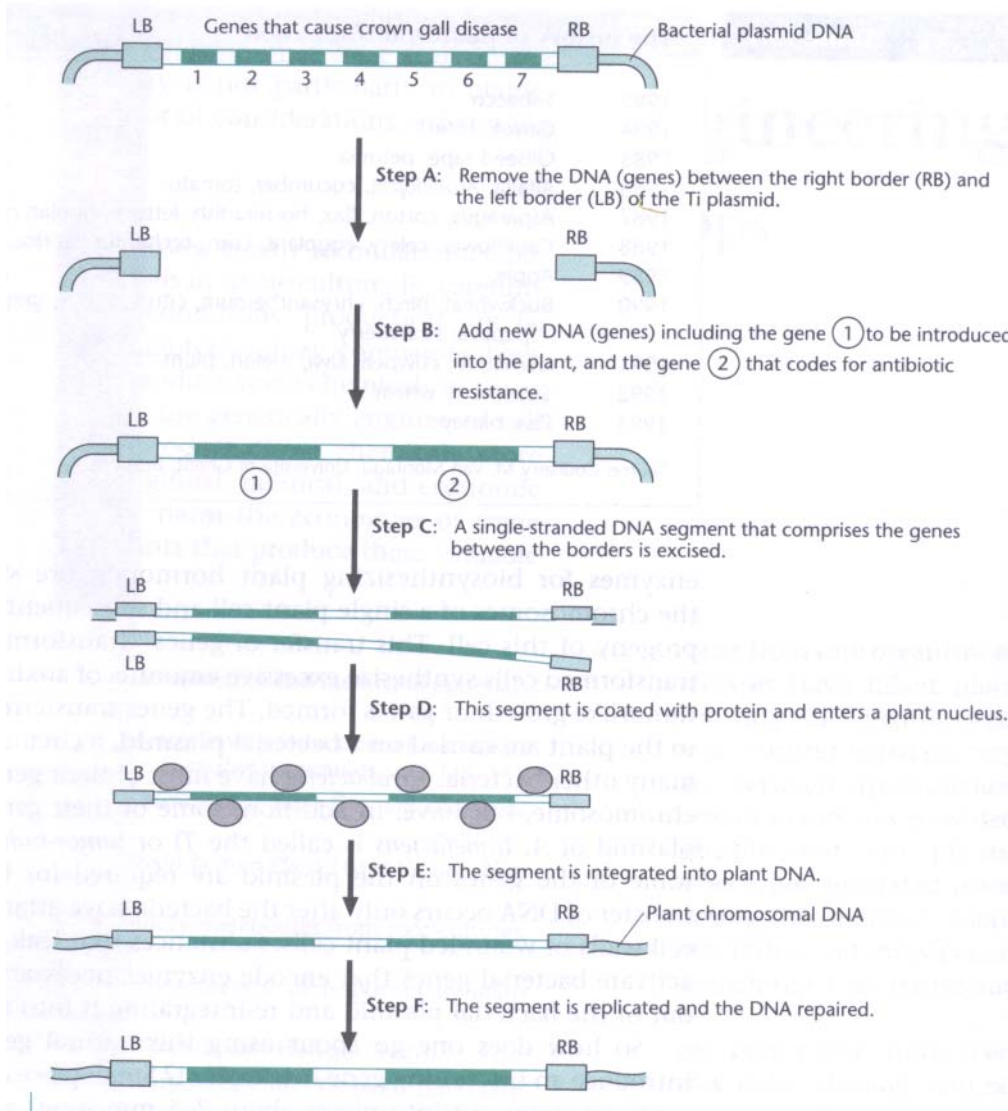


Figure 4.1. The general steps involved in the manipulation of Ti plasmid DNA to be introduced into new genes (steps a and b) and the transfer of the DNA into the plant DNA (steps c-f). Source: Plant Genetic Engineering, Chrispeel and Sadava, p. 456, 1994.

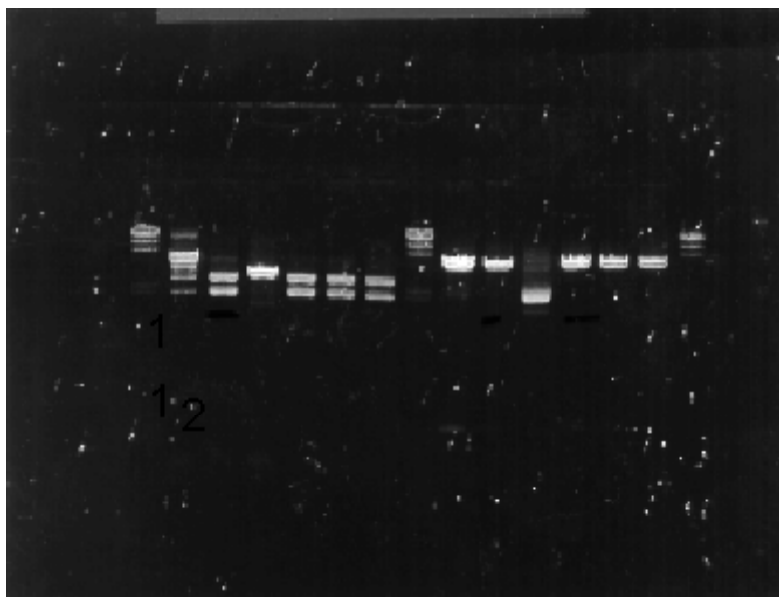


Figure 4.2. Gel image confirmation of the preliminary plasmid pLIT-G1, using 1 kb ladder as a marker and EcoRI and NcoI as the digestive enzymes. Lanes (l-r) 1= λ /HindIII, 2= pLIT-G1, 3-7= pLIT-G1/EcoRI, 8 & 16= λ /HindIII, 9=pLIT-G1, 10-15 pLIT-G1/NcoI. Giving an expected fragment size of 4480bp.

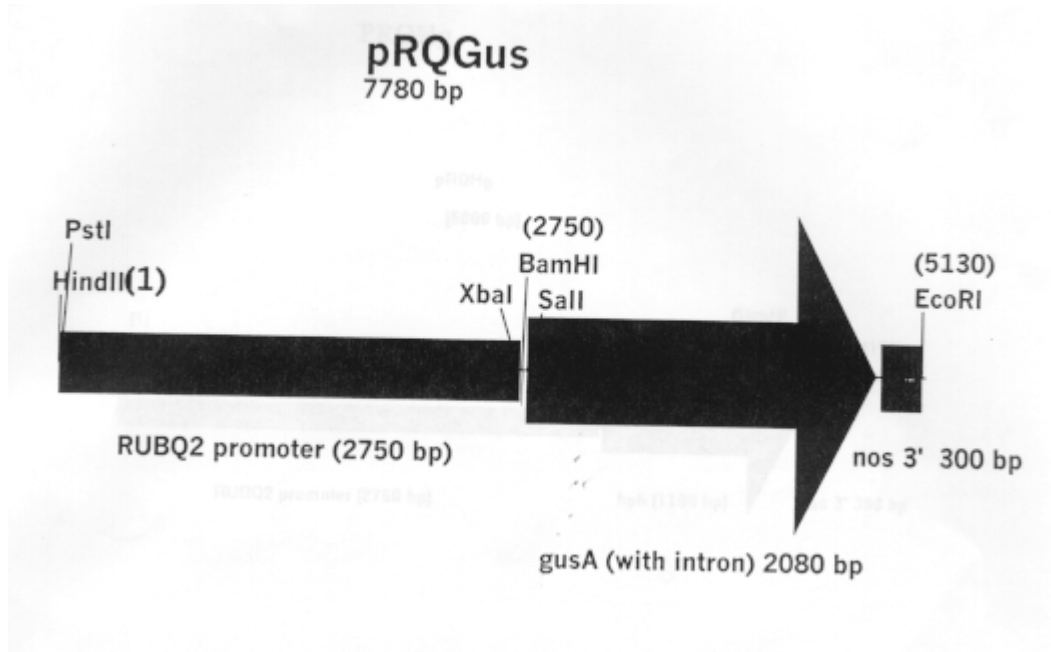


Figure 4.3. The T-DNA region of the construct pRQGus(7780bp). Drawing courtesy of Dr. Svetlana Oard.

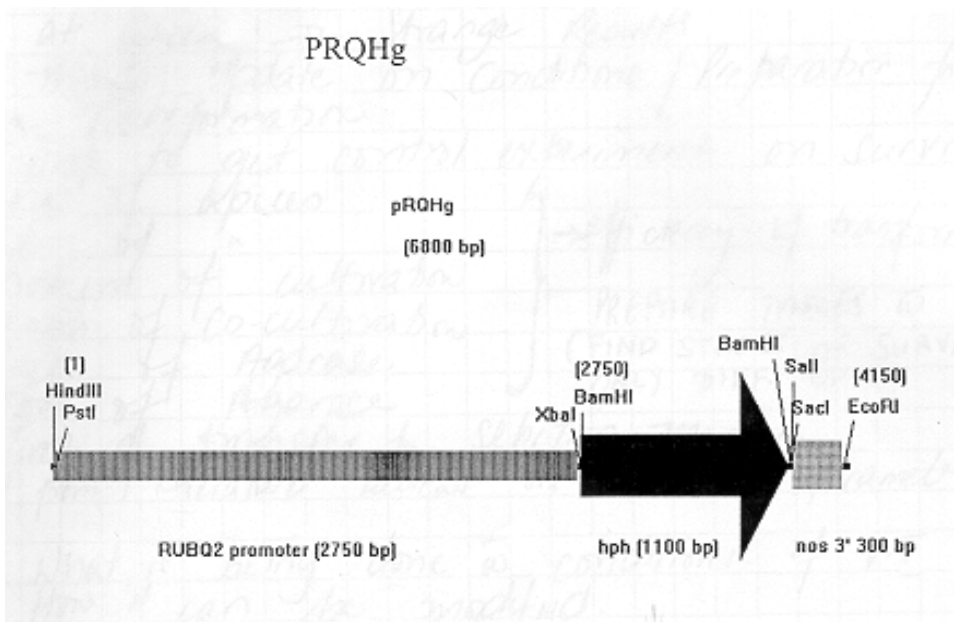


Figure 4.4. The T-DNA region of the construct pRQHg (6800bp). Drawing courtesy of Dr. Svetlana Oard.

The insertion of pRQGus into pRQHg resulted in the pSB41; confirmation of this plasmid can be viewed in Figure 4.5

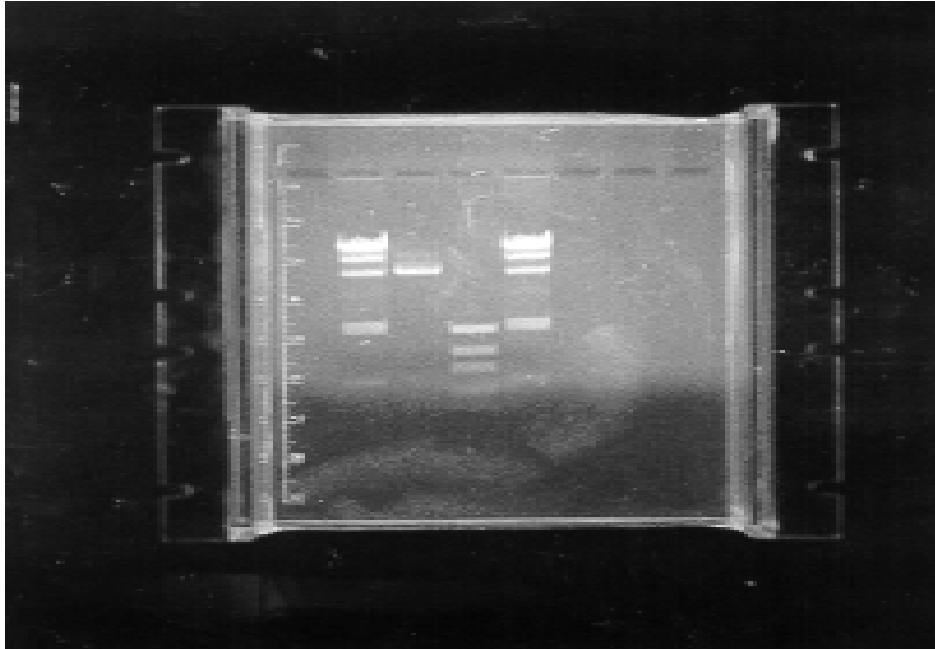


Figure 4.5. Gel image confirmation of pSB41, with 1kb ladder as a marker. Lanes (l-r) 1&4= λ HindIII, 2= pSB41, 3=pSB41/pST1

Gus Assays

The entire construct showed GUS activity in the shoot apical meristem after histochemical treatments. This was observed by a blue coloration as described by Jefferson, 1987.

Conclusion

This study was able to develop constructs that resulted in major improvements to the efficiency rate of *Agrobacterium* transformation, proving and reinforcing that apex-mediated DNA transformation is indeed a possibility. The rationale in constructing these plasmids of interest had to do with the understanding of how to construct binary vectors stated in various studies (Hiei et al., 1996, Komari et al., 1996; Vancanneyt et al., 1990). It was also observed that apices treated with pCL3 developed faster than apices inoculated with pCL4. Apices treated with pCL4 expressed GUS activity faster than apices treated with pCL3; this was due to the rice ubiquitin promoter in pCL4, which was not present in pCL3.

Theoretically constructing plasmids wherein all the constituents had been proven to increase the efficiency rate of *Agrobacterium* transformation either partially or wholly, should increase the overall transformation efficiency. Therefore, combining the rice ubiquitin promoter (RUBQ2), to drive the GUS and the Hph gene in a construct would demonstrate this theory practically. The use of gel electrophoresis aided in confirming the expected results of the constructs. Using the Hph gene resulted in the permanent genetic expression over the temporary genetic expression from the GUS gene.

CHAPTER V

SUMMARY AND FINAL REMARKS

The use of rice shoot apical meristems for *Agrobacterium* transformation is not yet a widely used technique. Except for one report by Park et al., (1996), all genetic transformations have been carried out using callus-based protocols. From our research it appears that failure to exploit the shoot apical meristem came from the fact that few researchers attempted to use this technique. This research was the first to combine all the parameters affecting the efficiency rate of *Agrobacterium* transformation in one complete study.

Through our study we have demonstrated that the size of the apex plays an important role in the success of apex-mediated transformation, we were able to standardize the size of the apex at 1.0-1.2 mm. Premature transfer of the apices to media free of hormone must be avoided so as to increase its chances of survival. Bacterial virulence was optimized when pCL3 and pCL4 were read at an OD of 0.6-0.8, and also when cultures were freshly prepared. Optical density also varied between different *Agrobacteria* strains. Using high levels of acetosyringone helped in the better expression of GUS activity in transformed cells in the apex. However, using concentrations between 400-1000 ppm had no significant differences on the intensity of GUS expression. It was also observed that there was positive growth in apices treated with acetosyringone prior to co-cultivation, thus confirming similar results achieved Khanna and Raina, (1999) by use of 'logs'.

For this experiment the minimum lethal concentration was 5.0 mg/L, this low reading could be attributed to the small sample size of the treatments used and the various conditions at which the apex was being exposed simultaneously. Vacuum infiltration had some effect on the transformation rate of the apices as higher survival rates were noticed when vacuum levels of 20 or 21 Hg were used. Additionally, the survival rate of the apices did not decline significantly during the co-cultivation period. Overall the survival rate of the apices was increased when all the parameters were standardized and combined. This 90% survival rate of the apices was indicative of the higher transformation rates that would be achieved once these apices are inoculated.

This study was the first to attempt constructing plasmids using the RUBQ2 promoter. Employing the use of the RUBQ2 promoter eventually would lead to an increased transformation efficiency since Wang et al., 1999, was able to show its superiority over 35S CaMV promoter in rice. By using the new constructs LBA4404 (pCL3) and LBA4404 (pCL4), we were able to detect GUS activity in the apex of the transformed plants. This was an extra advantage since these constructs specifically target the shoot apex, a characteristic not seen when using the super binary vector LBA4404 (pTOK233).

Further research on using the pSB41 construct for apex-mediated transformation would be welcomed since this study had difficulties in doing so. In addition actually producing transgenic plants from this study would be another area of interest. Since this study was only able to prove optimize apex-mediated DNA transformation in the apex itself and not a transgenic plant. In addition getting a better understanding in the

differences in results with apices inoculated with pCL3 and pCL4 would be a fact worthwhile knowing.

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VITA

Marsha Natalie Samuels was born in May Pen, Clarendon, Jamaica, on January 8th 1976. She graduated from Clarendon College, in Chapelton, Jamaica, with a high school diploma in July, 1992. At the age of 16, she entered College of Agriculture, Jamaica and graduated May 1995 with an Associate of Science degree in Agriculture. In May 1998, she received her Bachelor of Science degree in plant and soil system from Louisiana State University, Louisiana. In the Fall of 1998, she entered the Department of Agronomy at Louisiana State University to read for her Master of Science degree in agronomy. After enduring all of the obstacles successes and failures, she is still alive and kicking!