

**Establishment of transformation systems using mature seeds of maize inbred lines**

by

**Juan Carlos Martínez Nicolás**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

**MASTER OF SCIENCE**

Major: Plant Breeding

Program of study committee:  
Kan Wang, Major Professor  
Michael Lee  
Philip Becraft

Iowa State University

Ames, Iowa

2008

Copyright © Juan Carlos Martínez Nicolás, 2008. All rights reserved

UMI Number: 1450155



---

UMI Microform 1450155

Copyright 2008 by ProQuest Information and Learning Company.  
All rights reserved. This microform edition is protected against  
unauthorized copying under Title 17, United States Code.

---

ProQuest Information and Learning Company  
300 North Zeeb Road  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

**DEDICATORY**

To life (or whoever responsible)... for letting me see the sunrise and the sunset every day...

To my family... because they are the engine of my existence

To my good friends... because friendship is the nourishment of my soul...

To all the people who have had a remarkable presence in my life...

## TABLE OF CONTENTS

LIST OF FIGURES .....	v
LIST OF TABLES .....	vi
ACKNOWLEDGMENTS .....	vii
ABSTRACT .....	viii
CHAPTER 1. INTRODUCTION .....	1
1.1 Importance of maize .....	1
1.2 Maize improvement using traditional plant breeding .....	2
1.3 Maize improvement using genetic engineering.....	4
1.3.1 Delivery methods .....	4
1.3.1.1 Agrobacterium tumefaciens.....	5
1.3.1.2 Biolistic. ....	5
1.3.2 Target tissues .....	6
1.4 Importance of inbred lines .....	7
1.5 Immature embryo vs mature seed.....	8
1.6 Current status in inbred transformation using mature seed as starting materials.....	9
1.6.1 Shoot meristems in maize transformation.....	10
1.6.2 Somatic embryogenesis in maize transformation.....	11
1.7 Research objectives and thesis organization .....	12
References.....	13
CHAPTER 2. OPTIMIZATION OF STERILIZATION TECHNIQUES FOR MAIZE MATURE SEED USED FOR IN VITRO CULTURE AND TRANSFORMATION .....	21
Abstract .....	21
2.1 Introduction .....	21
2.2 Materials and methods .....	22
2.2.1 Plant material .....	22
2.2.2 Seed sterilization methods .....	23
2.2.2.1 Plant-extracted essential oils sterilization method.....	23
2.2.2.2 Chlorine gas sterilization method .....	23
2.2.2.3 Liquid sterilization method.....	24
2.2.3 Plant tissue culture media.....	24
2.2.4 Mature embryo dissection .....	24
2.2.5 Seedling vigor test.....	25
2.2.6 In vitro callus induction.....	25
2.2.7 Statistic analysis.....	25
2.3 Results and Discussion .....	26
2.3.1 Evaluation of various seed sterilization methods .....	26
2.3.1.1 Plant extracted essential oils method .....	26
2.3.1.2 Chlorine gas method .....	26
2.3.1.3 Liquid sterilization method.....	27
2.3.2 Improvement of liquid surface sterilization method for maize seed.....	27
2.3.3 Further optimization of the liquid seed sterilization method.....	28

2.3.4	Effect of seed sterilization treatments on seedling vigor and in vitro callus induction. ....	29
2.3.4.1	Contamination rate assessment.....	29
2.3.4.2	Seedling vigor assessment.....	30
2.3.4.3	Callus induction assessment.....	30
2.4.	Conclusions.....	31
	Acknowledgements.....	31
	References.....	32
<b>CHAPTER 3. ESTABLISHMENT OF TISSUE CULTURE SYSTEMS AND GENETIC TRANSFORMATION FOR INBRED LINES USING MATURE SEEDS AS STARTING MATERIAL.....</b>		<b>42</b>
	Abstract.....	42
3.1	Introduction.....	42
3.2	Materials and Methods.....	43
3.2.1	Plant material.....	43
3.2.2	Seed sterilization.....	44
3.2.3	Shoot meristem culture (SMC) establishment and regeneration.....	44
3.2.4	Somatic embryogenic culture (SEC) establishment.....	45
3.2.5	Particle bombardment.....	45
3.2.5.1	DNA construct.....	45
3.2.5.2	Plant culture preparation.....	45
3.2.5.3	Particle bombardment conditions.....	46
3.2.6	Agrobacterium-mediated transformation.....	46
3.2.6.1	Agrobacterium culture preparation.....	46
3.2.6.2	Agrobacterium infection.....	47
3.2.7	Selection and regeneration for biolistic gun-mediated transformation on embryogenic callus.....	48
3.2.8	Histochemical GUS assay and herbicide resistance test.....	48
3.2.9	Progeny analysis.....	49
3.2.10	Statistical analysis.....	49
3.3	Results and Discussion.....	49
3.3.1	Shoot meristems culture (SMC) transformation.....	49
3.3.1.1	SMC establishment.....	50
3.3.1.2	Biolistic transformation.....	50
3.3.1.3	Agrobacterium-mediated transformation.....	51
3.3.2	Somatic embryogenic callus (SEC) transformation.....	52
3.3.2.1	Embryogenic callus initiation frequency.....	52
3.3.2.2	Genetic transformation.....	53
3.3.2.3	Transgenic plant analysis.....	54
3.4	Conclusions.....	55
	Acknowledgements.....	56
	References.....	56
<b>CHAPTER 4. GENERAL CONCLUSIONS.....</b>		<b>75</b>
<b>APPENDIX I.....</b>		<b>77</b>
<b>APPENDIX II.....</b>		<b>81</b>

## LIST OF FIGURES

### CHAPTER 2

Figure 1. Hand-made strainers used for mature embryo sterilization.....	34
Figure 2. Contamination ratios of different modifications for liquid seed sterilization method.....	35
Figure 3. Best B73 plantlets for each treatment of seed sterilization optimization experiment .....	36
Figure 4. Contamination and abnormal plantlet rates for seed sterilization optimization experiment on B73 .....	37
Figure 5. Contamination and abnormal seedling rate in seedling vigor experiment .....	38
Figure 6. Correlation between SGR and callus induction .....	39

### CHAPTER 3

Figure 1. Plasmid pBU-B35S.IG used for genetic transformation .....	60
Figure 2. Tissue culture system for SMCs .....	61
Figure 3. Somaclonal variation in B73 inbred line after regeneration from SMC .....	62
Figure 4. Response to SMC induction on different inbred lines .....	63
Figure 5. GUS gene expression for bombardment experiments .....	64
Figure 6. Transient GUS expression in SMC's after 3 days infection with <i>Agrobacterium</i> .....	65
Figure 7. Morphology of embryogenic callus initiated from mature seeds of different inbred lines and hybrid Hi II .....	66
Figure 8. Transient GUS expression of embryogenic bombarded callus of different inbred lines .....	67
Figure 9. Stable <i>gus</i> gene expression on embryogenic callus of H99 inbred line after 8 weeks selection for biolistic experiments .....	68
Figure 10. Phenotypic expression of the <i>bar</i> gene after herbicide spraying .....	69
Figure 11. Stable <i>gus</i> gene expression for T0 plants of inbred line H99 .....	70
Figure 12. Levels of somaclonal variation in tassels of T0 plants for inbred line H99 .....	71

**LIST OF TABLES****CHAPTER 2**

Table 1. Treatment description for seed sterilization using chlorine gas .....	40
Table 2. Treatments used for optimization seed sterilization method .....	41
Table 3. Effects of two different seed liquid sterilization techniques and no sterilization (control), on seedling vigor (SGR) .....	42

**CHAPTER 3**

Table 1. Transient expression after transformation of different inbred maize genotypes using <i>Agrobacterium</i> -mediated and SMC's .....	72
Table 2. Callus initiation frequency for different inbred lines .....	73
Table 3. Bialaphos resistance callus frequency (BRCF) of bombarded embryogenic callus of different inbred lines .....	74
Table 4. Segregation analysis for bar and <i>gus</i> gene expression on T1 progeny plants .....	75

## ACKNOWLEDGMENTS

With all my appreciation and gratitude:

I'm extremely thankful to Dr. Kan Wang for the opportunity to do my studies at the Plant Transformation Facility and for all the scientific and personal guidance she provided to me along these years. Thank you very much Dr. Wang. To Bronwyn Frame for all the scientific assistance and great ideas she shared with me. To Margie Paz for all the knowledge she transmitted to me while I was working under her supervision in the Soybean Transformation Team. To my scientific committee: Dr. Michael Lee and Dr. Becraft for their support. To all past and present personnel in the PTF lab: Patricia Bordallo, Helene Eckert, Javier Garcia, Sule Karaman, Jennie Lund, Diane Luth, Marcy Main, Lise Marcell, Ksenija Markovic, Jennifer McMurray, Lorena Moeller, Tina Paque, Andrea Scarpa, Raye Taylor, Francois Torney, Xing Xu, and Jessica Zimmer who directly or indirectly contributed to this work.

I also want to thank the Mexican Government for all the financial support as well as the National Science Foundation. Plant Genome Program (DBI 0110023).

## ABSTRACT

Maize is one of the most important crops for plant biologists not only for its tremendous values for agriculture but also for its wealthy genetic information for biology. Recent years, the advent of biotechnology has further expanded the possibility of this crop. One of the most important tools for crop improvement and basic biological studies is genetic transformation. While plant genetic transformation using either *Agrobacterium tumefaciens* or biolistic gun as delivery systems has been available for more than 20 years, genetic transformation of many plant species, especially cereal crops, is still a challenging task for most laboratories.

The efficiency in genetic transformation depends upon the establishment of a robust and reproducible plant tissue culture system as well as an efficient delivery system for transformation. For current maize transformation system, immature embryos are routinely used as starting material for *Agrobacterium* infection or particle bombardment. The major problem for using the immature embryos is that it requires maize plants to be grown in the greenhouse year-round to meet the research demands. This practice requires large greenhouse spaces, quality growth conditions and experienced supporting staff.

One other problem with the current maize transformation system is that only Hi II, a hybrid germplasm bred specifically for tissue culture purposes, is amendable for transformation. For the maize community, inbred lines are the most desirable target for transformation due to their genetic background and agronomic importance in crop improvement.

This research program aimed at developing a transformation system using maize mature seeds as starting material for inbred lines B73, B104, H99, Mo17 and W22.

The first challenge in this study was to obtain sterilized mature embryo material for callus initiation. We have established an efficient and optimized seed sterilization protocol for maize seeds harvested from field-grown plants which have an increased amount of seed-borne pathogens. Our protocol ensures 98-100% sterility of plant material without noticeable compromise of the vigor and callus initiation.

Two different approaches: shoot meristematic cultures (SMC) and somatic embryogenic cultures (SEC) have been evaluated to obtain transformation-target tissue. Although SMC of different inbred lines could be obtained in a non-genotype dependent fashion, and GUS expression was detected when performing genetic transformation using *Agrobacterium*-mediated and the biolistic gun, no plants were recovered using this tissue culture system. On the other hand SEC was observed in all inbred lines at different levels, however just H99 responded with high frequency on callus induction medium (71 %). Bombardment of SEC of inbred line H99 resulted in fertile transgenic plants. Analysis of progeny indicated that both *gus* and *bar* transgenes have been transmitted to the next generation. Bialaphos resistant callus frequency (BRCF) ranged from 24.1 % to 74 % with this system.

## CHAPTER 1. INTRODUCTION

### 1.1 Importance of maize

Maize or corn (*Zea mays* L. ssp. *Mays*) is the third most cultivated crop in the world (after wheat and rice) (Torney et al, 2007). It is an important crop for alimentary, cultural, economic, and scientific reasons. From ancient to modern cultures, maize has been a staple food in several regions in America and Africa (Barreiro, 1989) and has thousands of uses. It is the most important crop used for food and feed around the world (Torney et al, 2007). Maize is responsible for 15-20% of the total daily calories in the diets of more than 20 developing countries in Latin America and Africa (Downswell, et al, 1996). It was considered as a sacred plant in ancient mythology of the Pre-Columbian Americas especially about its origin (Fusell et al, 1992). Modern maize and its ancestor teosinte have drastically different morphology in many aspects, making this plant culturally and scientifically intriguing.

In addition to being major feed and food sources, maize is used for many other industrial purposes, including starch plastic, sweeteners, paper, adhesives, additives, etc (Johnson, 2000). Lately, probably the area that draws most attention is the production of ethanol from maize to be used as a biofuel (Torney et al, 2007). Today, the United States, China, the European Union, Brazil and Mexico are the world's largest producers of maize (Meng et al, 2000). The US and China are the producers of nearly 60% of the total production of maize (Smith et al, 2004). Around 68% of the land used to produce maize is located in developing countries, however only 46% of maize production occurs there, indicating that there is a need for improvement of yields in those countries where it is a major source of direct human consumption (Pinglai, 2000). The United States is the country that produces and consumes more corn than any other. The annual production is around  $257 \times 10^6$  tons and the consumption is approximately  $210 \times 10^6$  tons (<http://www.ers.usda.gov/Briefing/Corn/>).

In the scientific field, maize has been one of the most explored crops over the last 70 years. It has some particular features that cannot be found in any other cereals. It is a C4 plant with higher yield when compared to other crops, it has a wide adaptation and natural

genetic variability and it is considered as a unique plant for cytogenetics research (Vasal et al, 2006). The development of sophisticated tools in molecular biology has accelerated the improvement of this crop. Tools such as molecular markers have been remarkable in fingerprinting maize genotypes (Smith et al, 1997; Senior et al, 1998; Warburton, 2001) for maize breeding programs and for other areas such as improvement of maize tissue culture response (Armstrong et al, 1992), gene mapping (Burr et al, 1988; Austin et al, 2000) study of transposable elements (Casa et al, 2000), stress (Quarrie et al, 1995), and lately, genetic engineering which has made possible the development of enhanced genotypes of maize with resistance to biotic stress (fungi, bacterial, virus, insects) (Castle et al, 2006) and abiotic stress (cold, drought, salinity) (Vinocur and Altman, 2005).

These tools have made more efficient the study and improvement of maize and is leading the evolution of traditional plant breeding.

## **1.2 Maize improvement using traditional plant breeding**

Plant breeding has been part of the human life for centuries and it has evolved together with mankind. The ultimate goal of plant breeding is to cultivate crops suitable for human's usage in the fashion of improving his life. Maize was probably one of the first plants which underwent breeding and actually it is also the biggest example of the potential of this field since it has evolved in such a tremendous manner that not even the suggested ancestor (teosinte) is similar to the modern maize plant. There is an enormous amount of information generated through the path of maize breeding along the years (Galinat, 1978).

Maize is a cross-pollinated crop and naturally shows higher heterozygosity and genetic variability (Phoelman and Sleper, 2003). Many breeding methods have been applied to enhance maize genotypes. Specific methods such as recurrent selection and its variants have been probably the most widely used in corn. It consists in a systematic selection of superior individuals from a population followed by their recombination to produce a new different population; the process is repeated in a recurrent manner (Fehr, 1987). Another strategy used for maize breeding has been the development of synthetic cultivars, which are created by intercrossing selected inbred lines. While synthetic cultivars are used in some countries, they are not used in the United States where hybrid cultivars are preferred (Fehr, 1987).

Among the breeding methods used to improve maize, the development of hybrids has been probably the highest achievement in maize breeding (Shull, 1909; Crow, 2001). Hybrids are developed by the crossing of two or more inbred lines. Hybrid maize produce higher yields, they are more vigorous due to their ability to generate heterosis or hybrid vigor (Duvick, 2001). Presently, hybrid maize is predominantly used in the United States for commercial purposes.

The primary goal of plant breeding science is and has been to increase crop yield through improving disease and pest resistance, abiotic stress (salinity, draught, high and low temperatures), protein content, starch composition etc. Selection and improvement against maize diseases have been practiced and evolved through the years (Jeffers et al, 2000). Efforts in pest resistance have also been conducted mainly for worms affecting different parts of the plant (Mihm et al, 1997, Bergvinson, 2002, Martin et al, 2004, Jauhar, 2006). Development of germplasm to resistant to abiotic stress such as drought (Rosielle and Hamblin, 1981; Edmeades et al, 2000; Bruce et al., 2002.), heat, extreme temperatures, high-velocity winds, frost, salinity, etc (Jauhar, 2006) are also major part of maize breeding programs.

Plant breeding methods have been powerful in generating superior maize cultivars. However there are some limitations. First of all, it is a long process to acquire the desirable genotype and to express the desirable traits. For example in pest control, the incorporation of resistance to European corn borer (ECB) was achieved after 12 years of conventional breeding (Vasal et al, 2006). Some traits can be difficult to establish to satisfy the field performance. Corn rootworm (CRW) is one of the most severe pests in the US Corn Belt (Lamkey and Lee, 2006). Development of resistance to CRW was approached through conventional breeding. However after decades of breeding (Rogers et al, 1975) the tolerance achieved was insufficient to combat the insect in the field (Vasal et al, 2006). The second limitation of the conventional breeding is the incorporation of undesirable traits along with the targeted trait during the breeding process. To fix a desired trait and remove the undesired trait, it takes many generations of backcrosses that can be time and resource consuming. A third disadvantage is that the conventional plant breeding does not allow to introduce useful

traits from unrelated species (Jauhar, 2006). The development of modern technologies such as genetic engineering has made possible the overcoming of these difficulties.

### **1.3 Maize improvement using genetic engineering**

Genetic engineering is defined as the modification of the genetic structure of an organism, involving isolation, manipulation and reintroduction of DNA into cells of an organism usually with the purpose to express a protein (Schouten et al, 2006). In plants, genetic transformation is a critical technology to study the functions of genes (i.e. complementation, overexpression, gene silencing) and also an important tool in crop improvement.

In maize, the first commercialized product carrying a desirable trait incorporated through genetic engineering, the Bt corn (European Corn Bore resistant), was released in 1996 and by 2005, 35% of the total US crop planted was Bt corn (Brookes and Barfoot, 2006). Another example of successful and applicable maize transformation is the development of herbicide tolerant maize which has been commercially used since 1997 and in 2005, half of the total US maize crop was planted with this enhanced corn (Brookes and Barfoot, 2006). A third example is the development of corn rootworm resistance which was planted in 5 % of the total maize planting (Brookes and Barfoot, 2006).

While genetic transformation technology has been used widely in basic and applied plant researches, the successful frequencies for different plant species or even different genotypes within a species can be very different. There are three main factors that have to be considered to perform efficient transformation: delivery method, target tissue and selection system. In this chapter delivery methods and target tissues will be emphasized.

#### **1.3.1 Delivery methods**

One of most important issues in delivering DNA into plant cells is to overcome the barrier of plant cell walls. Numerous techniques have been examined during the last 20 years. Basically, two types of delivery system have been used. One system is the natural delivery system, that is, the use of a soil bacterium *Agrobacterium tumefaciens* to deliver DNA into plant cells. The other system is the physical delivery system, that is, the use of various physical or chemical means to introduce DNA into plant cells. These methods

include: biolistic gun, polyethylene glycol solution (Armstrong et al, 1990; Morocz et al, 1990; Golovkin et al, 1993; Omirulleh et al, 1993), electroporation (Fromm et al, 1986; Rhodes et al, 1988), silicon carbide whiskers (Kaepler et al, 1992; Frame et al, 1994), etc. To date, the most successful and popular methods for maize transformation is the biolistic gun and *Agrobacterium*-mediated methods.

#### 1.3.1.1 *Agrobacterium tumefaciens*.

*Agrobacterium tumefaciens* is a tumor-inducer bacterium in plants (Smith and Townsend, 1907). Its oncogenic capacity (ability to produce tumors) is ascribed to the ability of transferring a portion of DNA into the host genome. The first evidence that a disarmed strain of this bacterium was able to transfer the T-DNA with a modified portion of DNA was described by Bevan et al, 1983, Fraley et al, 1983, Herrera-Estrella et al, 1983.

The T-DNA penetration from the bacteria to the plant is efficient to produce transgenic plants; however the mechanism of this delivery method is still unclear. The T-DNA region, which is the part of *Agrobacterium* Ti (tumor-inducing) plasmid DNA transferred to the plant cell, is bracketed by two 25-bp border sequences. Virulence (*vir*) is another critical factor on the Ti plasmid; this region is located in the outer part of the DNA. The *vir* genes produce enzymes and proteins to facilitate the T-DNA transfer. It is believed that the T-DNA is cleaved from the Ti plasmid a single stranded molecule (T-strand) is coated by virE1 and E2 proteins and capped by virD2 protein in the bacteria. The process of transferring the nucleoprotein complex into the plant cell is similar to conjugation. *A. tumefaciens* uses a type IV secretion system to transfer the nucleoprotein complex into the plant cell (Gelvin, 2000). Recently, efforts to improve genetic transformation are based on modifying bacterial components. Transformation of maize was reported by several groups (Schlappi and Hohn, 1992; Ritchie et al, 1993; Shen et al, 1993). Transgenic plants carrying the *gus* and *nptII* genes were obtained using this technology by the infection of shoot apices (Gould et al, 1991) and the first robust method for maize *Agrobacterium*-mediated transformation was reported in 1996 (Ishida et al, 1996).

#### 1.3.1.2 Biolistic.

The biolistic gun system is an apparatus capable to deliver high-velocity micro-particles. This system relies in the acceleration of microparticles coated with DNA, which are

forced to penetrate into the target tissue (Torney et al, 2007). The gun used for biolistic was reported for the first time by Klein et al (1987) and later fertile plants were recovered using this system (Gordon-Kamm et al, 1990). This system has shown to be reproducible and robust to transform different types of explants of maize. The use of this technology was rapidly adopted and led to the creation of the Bt corn, the first commercial transgenic product released after six years from the first work reporting the attainment of fertile maize plants (Fromm et al, 1990; Gordon-Kamm et al, 1990). In 1989, Klein obtained stable transformants using BMS cell suspension. Stable transgenic plants were also achieved using embryogenic maize suspension cultures (Fromm et al, 1990; Gordon-Kamm et al, 1990; Register et al, 1994), from type I callus (Wan et al, 1995), type II callus (Fromm et al, 1990; Walters et al, 1992; Armstrong et al, 1995; Pareddy and Petolino, 1997; Frame et al, 2000) from immature zygotic embryos (Koziel et al, 1993; Songstad et al, 1996; Brettschneider et al, 1997; Frame et al, 2000). Some attempts have been made to use this technology with shoot apical meristem-derived cultures (Lowe et al, 1995; Zhong et al, 1996; O'Connor-Sanchez et al, 2002; Zhang et al, 2002) but they have been inefficient.

### **1.3.2 Target tissues**

There are two important aspects to be considered when selecting a tissue to be suitable for genetic transformation: competence and regenerability. Competence reflects the ability of a cell/tissue to be transformed by receiving DNA and then regenerate in a full fertile plant suitable to inherit the trait for which it was transformed to the descendants (Torney et al, 2007). In general, the desirable target tissue would be the one from which genetic transformation can be achieved on the germ-line cells and also that it can be maintained for a shorter period on tissue culture conditions since it has been observed that an extended period is associated with an increase of somaclonal variation (Lee and Phillips, 1987).

Unlike plant regeneration in tobacco, it is difficult to regenerate plants in maize from any mature tissues such as leaf or roots. In maize the immature zygotic embryo has been the preferred starting material and it has been also used to develop other target tissues as embryo-derived scutellar callus and callus derived liquid suspension. It has been used directly as a target for transformation as well, using either particle bombardment (Koziel et

al, 1993; Songstad et al, 1996; Frame et al, 2000) or *Agrobacterium*-mediated transformation (Ishida et al, 1996, 2003; Zhao et al, 1998, 2001; Negroto et al, 2000; Frame et al, 2002).

#### **1.4 Importance of inbred lines**

Inbred lines are defined as individuals produced by mating, that are closely related. The extreme form of inbreeding occurs when one individual is hybridized to itself. When mating related individuals, the homozygosity is increased by bringing together identical alleles at a locus; this allows the expression of recessive alleles that may be hidden by a dominant allele in the parents. If the recessive alleles are less favorable than the dominants, the performance of the individuals decrease causing inbreeding depression, this phenomenon is more visible in cross-pollinated species such as maize in which inbred lines have lower performance, and because of that they are not used for commercial production other than just for inbred seed production (Fehr, 1989). On the contrary hybrids (which are commonly the first generation of the crossing of two or more inbred lines) show hybrid vigor (heterosis) which is reflected in the enhancement of desirable traits in a homogenous manner.

While inbred lines are not suitable for field production, it is an essential resource for plant breeding and other studies in genetics (Liu et al, 2003). They are mostly used for hybrid corn development and production (Anderson and Brown, 1952; Troyer, 2001) and also extremely important for genetic studies such as development of linkage gene maps (Burr et al, 1988, Patterson et al, 1995; Yu and Buckler, 2006), quantitative trait locus mapping (Edwards et al, 1987, Kaepler et al, 2000, Austin et al, 2001; Bruce et al, 2001; Ming-Zhang et al, 2005), molecular evolution (Henry and Damerval, 1997; White and Doebley, 1999; Ching et al, 2002), developmental genetics (Poethig, 1988; Fowler and Freeling, 1996, Che et al, 2006; Ma et al, 2007) as well as in physiological genetics (Crosbie et al, 1978) and to estimate linkage disequilibrium in maize (Remington et al, 2001; Tenailon et al, 2001, Ching, 2002) and lately the genetic diversity and structure among some inbred lines were reported (Benchimol et al, 2000; Liu et al, 2003, Xia et al, 2004).

Due to the importance of inbred lines in crop improvement and genetic studies, it will be desirable to develop a robust system for maize transformation using this type of material. However, most inbred lines, with the exception of some inbred lines such as A188 (Armstrong and Green, 1985), are poor in tissue culture response and recalcitrant in genetic

transformation (Carvalho et al, 1997). There are some reports of genetic transformation of inbred immature embryos using the biolistic (Koziel et al, 1993; Brettschneider et al, 1997; Wang et al, 2003) and *Agrobacterium*-mediated transformation (Ishida et al, 2003; Frame et al, 2006; Wang and Wei, 2005). Lately it has been reported successful transformation through seedling-derived maize callus and mature seeds as starting material using *Agrobacterium*-mediated transformation as a delivery system (Huang and Wei, 2005; Sidorov et al, 2006) and particle bombardment (O'Connor-Sanchez et al, 2002).

### **1.5 Immature embryo vs mature seed**

To date, the most widely used target tissue for maize transformation is the immature zygotic embryo. Most of maize transformation protocols (using either the biolistic gun or *Agrobacterium*-mediate method) use immature embryos 10-14 days after pollination. They are placed into a media containing auxins to induce callus formation from the scutellum cells. After 2-3 days, embryogenic callus can be observed in the abaxial, basal area of the scutellum (Franz and Schel, 1990). Somatic embryogenic callus is categorized as type I and type II, these types of callus were first observed in inbred line A188. Type I callus is compact, strengthen, with higher degree of association between lobes, type II is a friable callus with independent and defined embryogenic structures (Armstrong and Green, 1985). The first transgenic maize plants obtained using biolistic and immature zygotic embryos were derived from suspension cultures acquired from highly embryogenic type II callus (derived from the cross of A188 x B73) (Gordon-Kamm et al, 1990). Subsequently transformation of type II callus derived from immature embryos using biolistic method was reported (Fromm et al, 1990, Walters et al, 1992; Pareddy and Petolino, 1997; Frame et al, 2000). In our lab (Plant transformation facility) the transformation efficiencies using *Agrobacterium*-mediated and particle bombardment with immature zygotic embryos are 1-21% and 8-41% respectively, using the standard binary vector (Frame et al, 2000).

The major problem for using the immature embryos is that it requires maize plants to be grown in the greenhouse year-round to meet the transformation demands. This practice requires large greenhouse spaces, quality growth conditions and experienced support staff. When multiple inbred lines are grown at same time in one greenhouse environment, it can be challenging to prevent pollen cross contamination among inbred lines. Because these logistic

issues, it is desirable to produce transgenic plants using the ready to use mature seed as starting materials.

The use of mature seeds as starting material for transformation either with biolistic or *Agrobacterium*-mediated methods has been popular in a number of small grain cereals such as rice (Chen et al, 1998; Dai et al, 2001), triticale (Rashid, 2001), wheat (Kim et al, 1999), barley (Cho et al, 1998), oat (Cho et al, 1999), millet (Rashid, 2002), and some grasses (Ke and Lee, 1996; Ha et al, 2001). Typically, two types of tissue culture can be induced from mature seeds, embryogenic callus from mature embryos or scutellum, and meristematic cultures from shoot apices of germinating seedling.

Both immature embryos and mature seeds have been used for maize inbred line transformation. Transformation success has been reported in inbred lines using immature embryos and *Agrobacterium*-mediated transformation system: Huang and Wei (2005) obtained stable transformation in four inbred lines, including Mo17 with a transformation frequency between 2.35 to 5.2. Frame et al (2006) reported a transformation frequency ranging from 2.8 % to 8 % in four inbred lines including B104.

Stable transformation using organogenic calli from mature seeds and particle bombardment on tropical and subtropical inbred lines has also been reported (O'Connor-Sanchez et al, 2002). Zhang et al (2002) used two methods for inbred line transformation: sector proliferation method (involving in vitro induction and proliferation of SMCs from transformed sectors derived from the bombardment of SAM) and direct bombardment of SMCs, they included the elite inbred line B73.

Lately, successful *Agrobacterium*-mediated transformation using somatic embryogenic callus developed from different inbred lines and mature seeds (Sidorov et al, 2006) was reported.

## **1.6 Current status in inbred transformation using mature seed as starting materials**

To date maize inbred line transformation using mature seeds has been achieved by using either shoot meristem cultures or embryogenic cultures (Torney et al, 2007). In most cases, the biolistic gun was used as the delivery system (Wang et al, 2003). However, most recently *Agrobacterium*-mediated mature seed transformation has also been reported (O'Connor-Sanchez et al, 2002; Sidorov et al, 2006).

### **1.6.1 Shoot meristems in maize transformation.**

The shoot apical meristem (SAM) is the most distant structure involved in cell division and differentiation and it contains the germline cells for tissue and organ formation (Medford, 1992). It is a complex region composed by three cell layers (L1, L2 and L3). Cereal crops only have L1 and L2 layers. L2 layer will develop the reproductive structures that lead to the formation of gametes, thus it is the especially important in achieving stable genetic transformation since it will pass the genetic information to the offspring (Zhong et al., 1996a; Simmonds, 1997; Maqbool et al., 2002; Chandra and Pental, 2003).

The first attempt to perform genetic transformation in history was using shoot apical meristems of maize seedlings (Coe and Sarkar, 1966), in this experiment, the total DNA from purple red-anthered maize plants was extracted and injected directly into apical meristems of maize seedlings.

Success in using this tissue as starting material for maize transformation was not reported until 1996 when Zhong et al (1996) produced transgenic maize from a number of inbred lines.

The difficulty of using this type of explant for transformation is that the DNA has to be delivered into the germ-line cells or the media has to be manipulated pre and post transformation to reprogram the cell to assure stable transformation (maintenance and multiplication of the transformed SMC's for 2-3 months) (Sticklen and Oraby, 2005). The failure in doing that could lead to the generation of chimeric plants that may not have the transgene integrated in the germ-line cells to be transmitted to the offspring. It is believed that this type of cultures could be excellent target for transformation since they have continuous growth of the shoot and reproductive organs (Sticklen and Oraby, 2005). The generation of such culture seemed to be genotype-independent to certain extent. Li et al (2002) reported that SAM culture could be produced in 70 % of 45 temperate-zone inbred lines and hybrids.

Zhong et al (1996) demonstrated that the SMC could be used for achieving transgenic plants in 16 inbred lines using the biolistic gun and bar gene as selectable marker. Their frequencies ranged between 1-7%. It has also been reported that B73, a well known

recalcitrant inbred line, has been transformed using SMC's and biolistic (Zhang et al, 2002) but with low frequency 1.3 %).

SMC's are also considered as elastic explants since it was observed that by altering the concentration of cytokinins and auxins, somatic embryogenesis can be induced and inter-conversion embryogenic-organogenic callus of tropical and subtropical maize achieved (O'Connor-Sanchez et al, 2002).

### **1.6.2 Somatic embryogenesis in maize transformation.**

Somatic embryogenesis through callus initiation involves the formation of asexual embryos from a single cell or group of cells (Tisserat, 1985). Somatic embryogenesis consist on establishing an explant in tissue culture conditions, subsequent proliferation of callus and initiation of pro-embryos (normally on tissue culture media containing a high concentration of auxins). Those pro-embryos can be induced to independent embryos by cultivating them in media without hormones. The somatic embryo is an independent bipolar structure; those embryos can further develop and germinate to generate a plantlet. Embryogenic callus is heterogeneous and may be composed by different types of cells. When callus is transferred to a low auxin levels further embryogenesis can occurs to give rise more pro-embryos and pre-formed pro-embryo initials to develop into bipolar embryos in a non-synchronized fashion (Tisserat, 1985).

Transformation systems using somatic embryogenic callus originated from mature seeds have been reported in some cereals such as rice and triticale using biolistic and *Agrobacterium*-mediated (Chen et al, 1998; Dai et al, 2001; Rashid 2001). In maize, embryogenic tissue was obtained deriving from mature seeds of seven inbred lines and immature zygotic embryos (Huang and Wei, 2005) and genetic transformation was reported in four Mexican subtropical inbred lines using particle bombardment and organogenic/embryogenic callus, the selectable marker used was the *bar* gene but the transformation frequency was low (O'Connor-Sanchez et al, 2002). Another work reported stable transformation in nine inbred lines using *Agrobacterium*-mediated transformation in seedling-derived maize embryogenic type I callus and the *CP4* gene as a selectable marker which confers resistance to glyphosate. The overall transformation efficiency was 5.2 % (Sidorov et al, 2006).

## **1.7 Research objectives and thesis organization**

The goals of this research project are: 1) to explore genetic transformation methods for maize inbred genotypes and 2) establish transformation protocols using mature seeds as starting materials.

This thesis begins with a review of the current literature of maize transformation which is addressed in chapter I. In this chapter, the importance of maize is the first point described as well as the advances in maize improvement using traditional plant breeding followed by the improvement using genetic engineering and tissue culture systems. Chapter 2 describes the establishment of an improved seed sterilization method for mature seeds with high seed-borne pathogen for in vitro culture purpose. Chapter 3 presents the two different approaches used for maize tissue culture as well as describes the procedures for genetic transformation using different inbred lines and mature seeds. Chapter 4 presents the general conclusions of this work. Finally, appendix I is the optimized system developed to sterilize maize mature seeds from field-grown harvested seeds and appendix II is a manuscript that we published in regards of an improved method of soybean transformation where I'm the second author.

Dr. Kan Wang is my major professor, and together with Bronwyn Frame provided me the instruction and direction for the entire project. Bronwyn Frame also provided me with the initial SMCs described in chapter 3. Dr. Jose Luis Cabrera from the Centro Internacional de Investigaciones y Estudios Avanzados (CINVESTAV) Mexico, provided input and ideas for this project. Javier Garcia was an undergraduate student from Mexico who assisted me with the sterilization system development described in chapter 2. Anita Dutta is a high school teacher who did an internship under my direction and assisted me for the experiments in the assessment of seedling vigor. Jessica Zimmer is an undergraduate student at Iowa State and provided technical assistance for routine work derived from the transformation experiments described in chapter 3.

## References

- Anderson, E., W. L. Brown. 1952. Origin of Corn Belt maize and its genetic significance. *In* Heterosis. A Record of Researches Directed Toward Explaining and Utilizing the Vigor of Hybrids. J. W. Gowen (Ed). Iowa State College Press, Ames, IA. pp. 124–148.
- Armstrong, C.L., Parker, G.B., Pershing, J.C., Brown, S.M., Sanders, P.R., Duncan, D.R., Stone, T., Dean, D.A., Deboer, D.L., Hart, J., Howe, A.R., Morrish, F.M., Pajeau, M.E., Petersen, W.L., Reich, B.J., Rodriguez, R., Santino, C.G., Sato, S.J., S.Schuler, W., Sims, S.R., Stehling, S., Tarochione, L.J., Fromm, M.E. 1995. Field evaluation of European corn borer control in progeny of 173 transgenic corn events expressing an insecticidal protein from *Bacillus thuringiensis*. *Crop Sci* 35:550-557.
- Armstrong, C.L., Petersen, W.I., Buchholz, W.G., Bowen, B.A., Sulc, S.L. 1991. Factors affecting PEG-mediated stable transformation of maize protoplasts. *Plant Cell Rep* 9:335:339.
- Armstrong, C.L., Romero-Severson, J., Hodges, T.K. 1992. Improved tissue culture response of an elite maize inbred through backcross breeding, and identification of chromosomal regions important for regeneration by RFLP analysis. *Theor Appl Genet* 84:755-762.
- Armstrong, CL., Green, CE. 1985. Establishment and maintenance of friable, embryogenic maize callus and the involvement of l-proline. *Planta* 164:207–214.
- Austin, D. F., Lee, M., Veldboom, L.R. 2001. Genetic mapping in maize with hybrid progeny across testers and generations: plant height and flowering. *Theor. Appl. Genet.* 102: 163–176.
- Austin, D.F., Lee, M., Veldboom, L.R., Hallauer, A.R. 2000. Genetic mapping with hybrid progeny across testers and generations: grain yield and grain moisture. *Crop Sci.* 40:30-39.
- Barreiro, J. 1989. Indian corn of the Americas: Gift to the World. *Northeast Indian Quarterly*, Spring/Summer. 6: 56-58.
- Benchimol, L.L., De Souza Jr, C.L., Garcia, A.A.F., Kono, P.M.S., Mangolin, C.A., Barbosa, A.M.M., Coelho, A.S.G., De Souza, A.P. 2000. Genetic diversity in tropical maize inbred lines: heterotic group assignment and hybrid performance determined by RFLP markers. *Plant Breeding* 119:491-196.
- Bergvinson, D. 2002. Maize entomology. *In*: A briefing book. External review of CIMMYT maize program. Listman, M. (Ed). CIMMYT, Mexico. p.p. 23-27.
- Bevan, M.W., Flavell, R.B., Chilton, M.D. 1983. A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* 304:184-187.
- Brettschneider, R. Becker, D. Lorz, H. 1997. Efficient transformation of scutellar tissue of immature maize embryos. *Theor Appl Genet* 94:737–748.
- Brookes, G., Barfoot, P. 2006. GM crops: The first ten years, global socio-economic and environmental impacts. *In* ISAAA Briefs No. 36, Ithaca, NY.
- Bruce, W., Desbons, P., Crasta, O., Folkerts, O. 2001. Gene expression profiling of two related maize inbred lines with contrasting root-lodging traits. *Journal of Experimental Botany* 52:459-468.

- Bruce, W.B., Edmeades, G.O., Barker, T.C. 2002. Molecular and physiological approaches to maize improvement for maize drought tolerance. *Journal of Experimental Botany* 53:13-25.
- Burr, B., Burr, F.A., Thompson, K.H., Albertson, M.C., Stuber, C.W. 1988. Gene mapping with recombinant inbreds in maize. *Genetics* 118:519-526.
- Burr, B., F. A. Burr, K. H. Thompson, M. C. Albertson and C. W. Stuber.1988. Gene mapping with recombinant inbreds in maize. *Genetics* 118: 519–526.
- Carvalho, C.H.S., Bohorova, N., Bordallo, P., Abreu, L.L., Valicente, F.H., Bressan, W., Paiva, E. 1997. Type II callus production and plant regeneration in tropical maize genotypes. *Plant Cell Rep* 17:73–76.
- Casa, A.M., Brouwer, C., Nagel, A., Wang, L., Zhang, Q., Kresovich, S. and Wessler, S. 2000 The MITE family heartbreaker (Hbr): molecular markers in maize. *Proc Natl Acad Sci USA* 97:10083–10089.
- Castle, L.A., Wu, G., McElroy, D. 2006. Agricultural input traits: past, present and future. *Current Opinion in Biotechnology* 17:105.
- Chandra, A., Pental, D. 2003. Regeneration and genetic transformation of grain legumes: an overview. *Curr. Sci.* 84:381–387.
- Che, P., Love, T.M., Frame, B.R., Wang, K., Carriquiry, A.L., Howell, S.H. 2006. Gene expression patterns during somatic embryo development and germination in maize Hi II callus cultures. *Plant Mol Biol* 62(1-2):1-14.
- Chen, L., Zhang, S., Beachy, R.N., Fauquet, C.M. 1998. A protocol for consistent, large-scale production of fertile transgenic rice plants. *Plant Cell Rep* 18:25–31.
- Ching, A., Caldwell, K.S., Jung, M., Dolan, M., Smith, O.S., Tingey, S., Morgante, M., Rafalski, A.J. 2002. SNP frequency, haplotype structure and linkage disequilibrium in elite maize inbred lines. *BMC Genetics* 3:19.
- Coe, E.H., Sarkar, K.R. 1966. Preparation of nucleic acids and a genetic transformation attempt of maize. *Crop Sci* 6:432-435.
- Crosbie, T.M., Mock, J.J., Pearce, R. 1978. Inheritance of photosynthesis in a diallel among eight maize inbred lines from Iowa Stiff Stalk Synthetic. *Euphytica* 27:657-664.
- Crow, J.F. 2001. Plant breeding giants: Burbank, the artist; Vavilov, the scientist. *Genetic* 158:1901-1905.
- Dai, S., Zheng, P., Marmey, P., Zhang, S., Tian, W., Chen, S., Beachy, R.N., Fauquet, C. 2001. Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Mol Breed* 7:25–33.
- Dowswell, C. 1996. *Maize in the third world*. Westview Press, USA. pp. 156.
- Duvick, D.N. 2001. Biotechnology in the 1930s: the development of hybrid maize. *Perspectives, Nature reviews. Genetics* 2: 70-74.
- Edmeades, G.O., Bolaños, J., Eilings, A., Ribaut, J.M., Banziger, M., Westage, M. E. 2000. The role and regulation of anthesis silking interval in maize. *In Physiology and Modeling Kernel Set in Maize*, CSSA Special Publication 29. CSSA, Madison, Wi, p.p. 43-73.
- Edwards, M.D., Stuber, C.W., Wendel, J.F. 1987. Molecular marker-facilitated investigations of quantitative-trait loci in maize. *In Numbers, genomic distribution and types of gene action. Genetics* 116:113-125.

- Fehr, W., Fehr, E.L., Jessen, H.J. 1987. Principles of cultivar development. Iowa State University, Iowa, USA. pp. 526.
- Fowler, J.E., Freeling, M. 1996. Genetic analysis of mutations that alter cell fates in maize leaves: dominant liguleless mutations. *Dev Genet* 18:198-222.
- Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adam, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffman, N.L., Woo, S.C. 1983. Expression of bacterial genes in plant cells. *Proc Natl Acad Sci USA* 80:4803-4807.
- Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Wilson, H.M., Dunwell, J.M., Thopmson, J.A., Wang, K. 1994. Production of fertile transgenic maize plants by silicon carbide whiskers-mediated transformation. *The Plant J* 6:941-948.
- Frame, B.R., Shou, H., Chikwamba, R.K., Zhang, Z., Xiang, C., Fonger, T.M., Pegg, S.E.K., Li, B., Nettleton, D., Pei, D., Wang, K. 2002. *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol* 129:13-22
- Frame, B.R., Zhang, H., Cocciolone, S.M., Sidorenko, L.V., Dietrich, C.R., Pegg, S.E., Zhen, S., Schnable, P.S., Wang, K. 2000. Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cell Dev Biol Plant* 36:21-29.
- Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., Klein, T.M. 1990. Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Bio/Technology* 8:833-839.
- Fromm, M.E., Taylor, L.P., Walbot, V. 1986. Stable transformation of maize after gene transfer by electroporation. *Nature* 319:791-793.
- Fussell, B., 1992. The story of corn. Alfred Knopf, Inc, NY, USA. pp. 156-159.
- Galinat, W.C. 1978. The inheritance of some traits essential to maize and teosinte. *In* Walden, D.B. (Ed). *Maize breeding and genetics*. USA. pp 93-128.
- Gelvin, S.B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration *Annue Rev Plant Physiol Plant Mol Biol* 51:223-256.
- Golovkin, V.M., Abraham, M., Morockz, S., Bottka, S., Fefer, a., Dudits, D. 1993. Production of transgenic maize plants by direct DNA uptake into embryogenic protoplasts. *Plant Sci* 90:41-52.
- Gordon-Kamm, W.J. Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, W.R., Jr., Willets, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P., Lemaux, P.G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* 2:603-618.
- Gould, J., Devey, M., Hasegawa, O., Ulian, E.C., Peterson, G., Smith, R.H. 1991. Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiol* 95:426-434.
- Ha, C.D., Lemaux, P.G., Cho, M-J. 2001. Stable transformation of a recalcitrant Kentucky bluegrass (*Poa pratensis* L.) cultivar using mature seed-derived highly regenerative tissues. *In Vitro Cell Dev Biol Plant* 37:6-11.
- Henry, A., Damerval, C. 1997. High rates of polymorphism and recombination at *Opaque-2* locus in cultivated maize. *Mol Gen Genet* 256:147-157.

- Herrera-Estrella, L., Depicker, A., Van Montagu, M., Schell, J. 1983. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* 303:209-213.
- Ishida, Y., Saito, H., Hiei, Y., Komari, T. 2003. Improved protocol for transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Plant Biotechnol* 20(1):57-66.
- Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat Biotechnol* 14:745-750.
- Jahuar, P.P. 2006. Modern Biotechnology as an Integral Supplement to Conventional Plant Breeding: The Prospects and Challenges. *Crop Sci* 46:1841-1859.
- Jeffers, D., Cordoba, H., Vasal, S.K., Srinivasan, G., Beck, D., Barandiaran, M. 2000. Status in breeding for resistance to maize disease at CIMMYT. *In* Proceedings of the seventh Asian regional maize workshop. Vasal, S.K, Gonzalez, F., Ming, F.H. (Eds) PCARRD. Los Baños, Phillipines, pp 257-266.
- Johnson, L.A., 2000. Corn, the major cereal of the Americas. *In* Kulp K, Ponte J. (Eds). Handbook of cereal science and technology. DeJjer, New York, pp 31-80.
- Kaepler, H.F., Gu, W., Somers, D.A., Rines, H.W., Cockburn, A.F. 1992. Silicon carbide fiber-mediated stable transformation of plant cells. *Theor Appl Genet* 84:560-566.
- Kaepler, S.M., parke, J.L., Mueller, S.M., Senior, L., Stuber, C., Tracy, W.F. 2000. Variation among maize inbred lines and detection of quantitative trait loci for growth at low phosphorus and responsiveness to *Arbuscular Mycorrhizal* fungi. *Crop Sci* 40:358-364.
- Ke, S., Lee, C.W. 1996. Plant regeneration in Kentucky bluegrass (*Poa pratensis* L.) via coleoptile tissue cultures. *Plant Cell Rep* 15:882-887.
- Klein, T.M., Kornstein, L., Sanford, J.C., Fromm, M.E. 1989. Genetic transformation of maize cells by particle bombardment. *Plant Physiol* 91:440-444.
- Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327:70-73.
- Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M.R., Merline, E., Rhodes, R., Warren, G.W., Wright, M., Evola, S.V. 1993. Field performance of elite transgenic maize plants expressing and insecticidal protein derived from *Bacillus thuringiensis*. *Bio/Technology* 11:194-200.
- Lamkey, K.R. and M. Lee (Eds). 2006. *Plant Breeding: The Arnel R. Hallauer International Symposium*. Blackwell Publishing, Ames, Iowa. p.p. 379.
- Lee, M., Phillips, R.L., 1987. Genomic rearrangements in maize induced by tissue culture. *Genome* 29:122-128.
- Li, W., Masilamany, P., Kasha, K.J., Pauls, P.K. 2002. Developmental, tissue culture, and genotypic factors affecting plant regeneration from shoot apical meristems of germinated (*Zea mays* L.) seedlings. *In Vitro Cell Dev Biol Plant* 38:285-292.
- Liu, K., Goodman, M., Muse, S., Smith, J.S., Buckler, E., Doebley, J. 2003. Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. *Genetics* 165:2117-2128.

- Lowe, K., Bowen, B., Hoerster, G., Ross, M., Bond, D., Pierce, D.A., Gordon-Kamm, W. 1995. Germline transformation of maize following manipulation of chimeric shoot meristems. *Bio/Technology* 13:677-682.
- Ma, L., Sun, Q.Q., Zhou, Y.L., Wu, C.L., Zhang, C.Q. 2007. Study of the relationship between inheritance and immature embryo culturing capacity of maize inbreds. *Journal of Molecular Cell Biology*. 40(2):164-72.
- Maqbool, S., Zhong, H., E.L. Maghraby, Y., Wang, W., Ahmad, A., Chai, B., Sticklen, M. B. 2002. Competence of oat (*Avena sativa* L.) shoot apical meristems for integrative transformation, inherited expression and osmotic tolerance of transgenic lines containing *hva1*. *Theor. Appl. Genet.* 105:201–208.
- Martin, S.A., Darrah, L.L., Hibbard, B.E. 2004. Divergent selection for rind penetrometer resistance and its effects on European corn borer damage and stalk traits in corn. *Crop Sci* 44:711-717.
- Medford, J. I. 1992. Vegetative apical meristems. *Plant Cell* 4:1029–1039.
- Meng, E. and J. Ekboir, 2000. Current and Future Trend in Maize Production and Trade. CIMMYT World Maize Facts and Trends. p.p. 84.
- Mihm, J.A., Bergvinson, D. Kumar, H. 1997. Maize entomology. *In* Maize research in 1995-96 CIMMYT maize program special report. CIMMYT, Mexico, D.F., Mexico, pp. 42-46.
- Ming-Zhang, Y., mao, Y., Xie, C., Smith, H., Luo, L., Xu, S. 2005. Mapping quantitative trait loci using naturally occurring genetic variance among commercial inbred lines of maize (*Zea mays* L.). *Genetics* 169:2267-2275.
- Morocz, S., Donn, G., Nemeth, J., Dudits, D. 1990. An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture. *Theor Appl Genet* 80:721-726.
- Negrotto, D., Joleey, M., Beer, S., Wenck, A.R., Hansen, G. 2000. The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium transformation*. *Plant Cell Rep* 19:798-803.
- O'Connor-Sanchez, A., Cabrera-Ponce, J.L., Valdez-Melara, M., Tellez-Rodriguez, P., Pons-Hernandez, J.L., Herrera-Estrella, L. 2002. Transgenic maize plants of tropical and subtropical genotypes obtained from calluses containing organogenic and embryogenic-like structures derived from shoot tips. *Plant Cell Rep* 21:302-312.
- Omirulleh, S., Abraham, M., Golovkin, M., Stefanov, I., Karabaev, M., Mustardy, L., Morocz, S., Dudits, D. 1993. Activity of chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. *Plant Mol Biol* 21:415-428.
- Pareddy, D., Petolino, J., Skokut, T., Hopkins, N., Miller, M., Welter, M., Smih, K., Clayton, D., Pescitelli, S., Gould, A. 1997. Maize transformation via helium blasting. *Maydica* 42:143-153.
- Patterson, G.I., Kubo, K.M., Shroyer, T., Chandler, V.L. 1995. *Genetics* 140:1389-1406.
- Pingali, P. and S. Pandey, 2000. "Meeting World Maize Needs: Technological Opportunities and Priorities for the Public Sector." CIMMYT World Maize Facts and Trends.
- Poehlman, J. M., Sleper, D. A. 1995. *Breeding field crops*. Iowa: Iowa State University Press, 4th ed. 494 p.

- Poethig, R.S. 1988. Heterochronic mutations affecting shoot development in maize. *Genetics* 119: 6080-6084.
- Quarrie, S.A., Lanzic-Jancic, V., Kovacevic, D., Steed, A., Pekic, S. 1999. Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. *Journal of Experimental Botany*. 50 (337):1299-1306.
- Rashid, V.A. 2002. Induction of multiple shoots by thidiazuron from caryopsis cultures of minor millet (*Paspalum scrobiculatum* L.) and its effect on the regeneration of embryogenic callus cultures. *Plant Cell Rep* 21:9–13.
- Rashid, V.A. 2002. Induction of multiple shoots by thidiazuron from caryopsis cultures of minor millet (*Paspalum scrobiculatum* L.) and its effect on the regeneration of embryogenic callus cultures. *Plant Cell Rep* 21:9–13.
- Register, J.C. III, Peterson, D.J., Bell, P.J., Bullock, W.P., Evans, I.J., Frame, B., Greenland, A.J., Higgs, N.S., Jepsen, I., Jiao, S., Lewnau, C.J., Silick, J.M., Wilson, H.M. 1994. Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. *Plant Mol Biol* 25:951-961.
- Remington, D.L., Thornsberry, J.M., Matsuoka, Y., Wilson, L.M., Whitt, S.R. 2001. Structure of linkage disequilibrium and phenotypic associations in the maize genome. *Proc Natl Acad Sci USA* 98:11479-11484.
- Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D., Detmer, J.J. 1988. Genetically transformed maize plants from protoplasts. *Science* 2740:204-207.
- Ritchie, S.W., Liu, C.N., Sellmer, J.C., Kononowicz, H., Hodges, T.K., Gelvin, S.B. 1993. *Agrobacterium tumefaciens*-mediated expression of *gusA* in maize tissues. *Transgenic res* 2:252-265.
- Rogers, R. R., Owens, J. C., Tollefson, J. J., and Witkowski, J. F. 1975. Evaluation of commercial corn hybrids for tolerance to corn rootworms. *Environ Entomol.* 4:920–922.
- Rosielle, A.A., and J. Hamblin. 1981. Theoretical aspects of selection for yield in stress and non-stress environments. *Crop Sci* 21:943–946.
- Schlappi, M., Hohn, B. 1992. Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. *Plant cell* 4:7-16.
- Schouten, H.J., Krens, F.A., Jacobsen, E. 2006. Cisgenic plants are similar to traditional bred plants: International regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO reports* 7:750-753.
- Senior, M.L., Murphy, J.P., Goodman, M.M., Stuber, C.W. 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. *Crop Sci* 38:1088-1098.
- Shen, W.H., Escudero, J., Schlappi, M., Ramos, C., Hohn, B., Koukolikovia-Nicola, Z. 1993. T-DNA transfer to maize cells: histochemical investigation of  $\beta$ -glucuronidase activity in maize tissues. *Proc Natl Acad Sci USA* 90:1488-1492.
- Shull, G.H. 1909. The composition of a field maize. *Am Breed Assoc Rep* 4:296-301.
- Simmonds, J. A. 1997. Mitotic activity in wheat shoot apical meristems: effect of dissection to expose the apical dome. *Plant Sci.* 130:217–225.
- Smith, C., et al., 2004. *Corn: Origin, history, technology, and production*. Wiley Series in Crop Sciences.
- Smith, E.F., Townsend, C.O. 1907. A plant tumor of bacterial origin. *Science* 25:671-673.

- Smith, J.S.C., Chin, E.C.L., Shu, H., Smith, O.S., Wall, S.J. 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparison with data from RFLPS and pedigree. *Theor Appl Gen* 95:163-173
- Songstad, D.D., Armstrong, C.L., Petersen, W.L., Hairston, B., Hinchey, M.A.W. 1996. Production of transgenic maize plants and progeny by bombardment of Hi II immature embryos. *In Vitro Cell Dev Biol-Plant* 32:179-183.
- Sticklen, M.B., Oraby, H.F. 2005. Shoot apical meristem: a sustainable explant for genetic transformation of cereal crops. *In Vitro Cell Dev Biol-Plant* 41, 187-200.
- Tenaillon, M.I., Sawkins, M.C., Long, A.D., Gaut, R.L., Doebley, J.F. 2001. Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.). *Proc Natl Acad Sci USA* 98:9161-9166.
- Tisserat, B. 1985. Embryogenesis, organogenesis and plant regeneration. *In Plant Cell Culture, a practical approach*. Dixon, R.A. (Ed). IRL Press. Oxford, WA. p.p. 79-105.
- Torney, F., Frame, B.R., Wang, K. 2007. Maize. *In E.C Pua and M.R. Davey (Eds), Transgenic crops IV*. Springer, Berlin Heidelberg. p.p. 73-105.
- Troyer, A. F., 2001. Temperate corn, *In Specialty Corns*. A. Hallauer (Ed). CRC Press, Boca Raton, FL. pp. 393-466.
- Vasal, S.K., Riera-Lizarazu, O., Jauhar, P.P. 2006. Genetic enhancement of maize by cytogenetic manipulation, and breeding for yield, stress tolerant and high protein quality. *In Singh, R.J., and Jauhar, P.P. (Eds). Genetic resources. Chromosome engineering and crop improvement*. Boca Raton, Florida, pp 159-197.
- Vinocur, B., Altman, A. 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current Opinion in Biotechnology*. 16:123.
- Walters, D.A., Vetsch, C.S., Potts, D.E., Lundquist, R.C. 1992. Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. *Plant Mol Biol* 18:189-200.
- Wan, Y., Widholm, J.M., Lemaux, P.G. 1995. Type I callus as a bombardment target for generating fertile transgenic maize (*Zea mays* L.). *Planta* 196:7-14.
- Wang, K., Frame, B.R., Marcell, L. 2003. Maize genetic transformation. *In P. K. Jaiwal and R.P. Singh (Eds). Plant Genetic Engineering: Improvement of Food Crops*. Sci-Tech publication. Houston, Texas, USA. p.p. 175-217.
- Warburton, M., Xianchun, X., Ambriz, S., Diaz, L., Villordo, E., Hoisington, D. 2001. Use of molecular markers in maize diversity studies at CIMMYT. Seventh Eastern of Southern Africa Regional maize Conference. 11-15 February, 2001. pp 130-133.
- White, S.E., Doebley, J.F. 1999. The molecular evolution of *terminal ear1*, a regulatory gene in genus *Zea*. *Genetics* 153:1455-1462.
- Xia, X.C., Reif, J.C., Hoisington, D.A., Melchinger, A.E., Frisch, M., Warburton, M.L. 2004. Genetic diversity among CIMMYT maize inbred lines investigated with SSR markers: I. Lowland tropical maize. *Crop Sci* 44:2230-2237.
- Yu, J., Buckler Iv, E.S. 2006. Genetic association mapping and genome organization of maize. *Current Opinion in Biotechnology*. 17:155-160.
- Zhang, S., Williams-Carrier, R., Lemaux, P.G. 2002. Transformation of recalcitrant maize elite inbreds using in vitro shoot meristematic cultures induced from germinating seedlings. *Plant Cell Rep* 21:263-270.

- Zhang, S., Williams-Carrier, R., Lemaux, P.G., 2002. Transformation of recalcitrant maize elite inbreds using in vitro shoot meristematic cultures induced from germinating seedlings. *Plant Cell Rep* 21:263-270.
- Zhao, Z.Y., Gu, W., Cai, T., Tagliani, L.A., Hondred, D.A., Bond, D., Krell, S., Rudert, M.L., Bruce, W.B., Pierce, D.A. 1998. Molecular analysis of T0 plants transformed by *Agrobacterium* and comparison of *Agrobacterium*-mediated transformation with bombardment transformation in maize. *Maize Genet Coop Newslett* 72: 34–37.
- Zhao, ZY, Gu, W., Cai T., Tagliani, L., Hondred, D., Bond, D., Shroeder, S., Rudert, M., Pierce, D. (2001). High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. *Mol Breed* 8:323–333.
- Zhong, H., Sun, B., Warkentin, D., Zhang, S., Wu, R., Sticklen, M.B. 1996. The competence of maize shoots meristems for integrative transformation and inherited expression of transgenes. *Plant Physiol* 110:1097-1107.
- Zhong, H., Sun, B., Warkentin, D., Zhang, S., Wu, R., Sticklen, M.B. 1996. The competence of maize shoots meristems for integrative transformation and inherited expression of transgenes. *Plant Physiol* 110:1097-1107.

## **CHAPTER 2. OPTIMIZATION OF STERILIZATION TECHNIQUES FOR MAIZE MATURE SEED USED FOR IN VITRO CULTURE AND TRANSFORMATION**

### **Abstract**

An efficient and reproducible method for seed sterilization was developed specifically for maize mature seeds harvested from field-grown plants. The method includes three major disinfection stages. First stage involves disinfecting the whole maize seed surface. The second stage involves disinfecting of seeds and dissection of mature embryos. The third stage involves disinfection of dissected embryos. With this method, vigor (measured by Seedling Growth Rate (SGR)), development of normal plants and callus initiation, is not compromised. Out of five inbred lines tested (B73, B104, H99, Mo17 and W22) genotype H99 showed the highest performance in vigor and callus initiation appearance. This system ensures minimum contamination for mature seed-derived explants used for in vitro tissue culture and genetic transformation.

### **2.1 Introduction**

In vitro culture techniques require a sterile environment and sterile initial material (Dixon, 1985). The common contaminants affecting in vitro-cultured tissues, are bacterial and fungi. The enrichment of the media used, make explants very susceptible to these microorganisms (Leifert and Waites, 1990). Despite of careful attention while applying sterilization techniques, contamination still may result in 100 % lost in field-grown material due to the different amount of microorganisms present in the explants (Skirvin et al, 1999) One of the first steps that have to be overcome during the process of genetic transformation in any plant is to establish a reliable system to acquire sterile material to initiate a tissue culture system. In maize there is a well established protocol to sterilize initial material when immature embryos are used (Frame et al, 2000). In the case of mature seeds there has been little attention in the improvement of the aseptic techniques and there is not a clear report about the problems that the sterilization techniques present for this type of material. Mature seeds harvested from field-grown plants present higher seed-borne pathogen rates than those harvested in greenhouse mainly because they are much more exposed to air-borne and soil-borne pathogens. In order to have sterile seeds to initiate maize in vitro cultures, researchers work on the basis of using large amounts of initial material to assure sterile seeds, in that

case, the contamination factor is not completely overcoming but simply the assurance of sterile material is fulfilled by increasing the chances of having low rate seed-borne pathogens in a bigger amount of material.

In the initial experiments of this work there was a tremendous amount of contamination in field-grown harvested seeds which restricted genetic transformation. The objective of this study was to develop a sterilization system specifically designed for maize mature seeds harvested from field-grown plants to assure the availability of sterile material to generate in vitro culture systems, regardless the amount used and the amount of seed-borne pathogens. We focused on seeds harvested in field since greenhouse seeds will create a supply-dependency. In this chapter we assessed three surface sterilization methods for maize mature seeds. These methods included essential oils, chlorine gas and liquid sterilization (ethanol and sodium hypochlorite at different concentrations). The latest method was modified and optimized for seed sterilization to establish the tissue culture systems for maize.

## **2.2 Materials and methods**

### **2.2.1 Plant material**

Five maize inbred lines were used as initial material including B73, B104, H99, Mo17, and W22. B73 genotype was developed in Iowa and selected for recurrent selection population (C5) from Iowa Stiff Stalk Synthetic (BSSS). Accession number PI 550473 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1445409>). B104 was developed in Iowa from BS13(S)C5, a strain from Iowa Stiff Stalk Synthetic after 12 cycles of recurrent selection. Accession number PI 594047 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1519249>). H99 was developed in Indiana. Accession number PI 587129 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1073895>). Mo17 was developed in Missouri, genotype resistant to leaf bright (*Helminthosporium turcicum*), pedigree CI 187-2/C103. Accession number PI 558532 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1453504>). W22 was developed in Wisconsin, from second cycle inbred line, plants susceptible to smut, excellent tolerance to stalk rooting organisms. Accession number NSL 30053 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1098978>).

Seeds of all genotypes were kindly provided by Tina Paque (Plant Transformation Facility, Iowa State University) and the North Regional PI Station in Ames, Iowa. B73 seeds harvested from field, all in 2006 used for optimization experiments were kindly provided by Paul Scott (Agronomy Department, Iowa State University).

## **2.2.2 Seed sterilization methods**

### *2.2.2.1 Plant-extracted essential oils sterilization method*

Two different plant-extracted essential oils were tested, oregano (supplied by Amrita™ Fairfield, IA) and cinnamon (supplied by Aromaland™ Sta. Fe, NM) at three different concentrations, 1 000, 10 000 and 500 000 ppm. Each treatment was applied to fifty seeds of the inbred line B73 harvested from field and provided by Tina Paque. Dilutions at the specific concentration were prepared using soybean oil as a solvent by adding the essential oil to a 15 ml falcon tube containing soybean oil and mixing. Dry seeds were placed in a plastic bag and the oil dilution added. Seeds were shaken until they were evenly coated with a thin layer of the dilution (Christian, Erik, personal communication). Seeds were kept in the plastic bag overnight to assure penetration of the oil and then 10 seeds were sowed in a Petri plate containing MS solid basal media. Plates were incubated at 28 °C and 16 h photoperiod. Contamination was scored after 3 days.

### *2.2.2.2 Chlorine gas sterilization method*

Chlorine gas sterilization was performed as described by Paz et al (2006) with modifications. Twenty or fifty maize seeds of B73 inbred line (provided by Tina Paque) were sterilized using chlorine gas at two different concentrations 3N (Jose Luis Cabrera, personal communication) or 0.42N (Paz et al, 2006) at different periods of time (Table 1). Seeds were put in a Petri dish and arranged in a single layer. The Petri dish was placed in a desiccator where the chlorine gas was released. Chlorine gas at 3N was generated by adding 30 ml of 12 N HCl to a beaker containing 120 mL of commercial bleach (6 % v/v) while the concentration 0.42 N was produced by mixing 3.5 mL of 12 N HCl to a beaker containing 100 mL of commercial bleach. After sterilization, seeds were vented in a flow bench for one hour and then sowed in a Petri dish containing MS basal media and incubated at 28 °C and 16 h photoperiod. Contamination was scored after 3 days.

### 2.2.2.3 Liquid sterilization method

The modified liquid sterilization methods tested in this work were based on the protocol described by Clemente *et al*, 2000. The original sterilization was as follows: Thirty to fifty seeds of inbred line B73 were first soaked in 80% ethanol solution for 2 min, then into 50 % commercial bleach (6% v/v) containing 0.04% of Tween®20 for 15 min. Seeds were subsequently rinsed six times with sterile water. Several modifications such as varying bleach immersion time, use of biocides such as plant preservative mixture (PPM ®) and silver nitrate, applying double sterilization, seed softening, mature embryo dissection and embryo sterilization were tested in order to achieve a reliable and efficient method to sterilize maize seeds.

### 2.2.3 Plant tissue culture media

In preliminary experiments only inbred line B73 was used. Subsequently more inbred lines were tested such as B104, H99, Mo17, and W22, all harvested from field in bulk. In preliminary experiments seeds of B73 inbred line were germinated in MS basal media after sterilization. In subsequent experiments seeds were germinated directly either in CSMD9B media (MS base salts, 2.0 mg/L BAP, 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 500 mg/L casein hydrolysate, 3% sucrose and 3 g/L gelrite, pH 5.8) to induce formation of shoot meristematic cultures (SMC's) (Zhong *et al*, 1996) or in MSVS34 media (MS base salts, 100 mg/L casein hydrolysate, 1.95 g/L MES [2-(N-morpholino) ethanesulfonic acid], 750 mg/L glutamine, 500 mg/L ascorbic acid, amended with 10 mg/L picloram and 3 mg/L BAP (6-benzylaminopurine) and 2.5 g/L gelrite at pH 5.8) to induce somatic embryogenesis (Sidorov *et al*, 2006).

### 2.2.4 Mature embryo dissection

To isolate mature embryos, surface sterilized seeds were dissected under sterile conditions and under a stereoscope. Two thirds of the primordial root was excised and the remnant part (including the plumule containing the shoot apical meristem (SAM)) was sterilized and placed into a Petri dish with the either media mentioned above. Plates were incubated in a growth chamber 16 h photoperiod and 28 °C. A hand-made strainer tool was used to facilitate embryo dissection (Figure 1). (See appendix 1 and 2 for details)

### **2.2.5 Seedling vigor test**

Twenty five maize seeds from each of four inbred lines were used for seedling vigor test: B73, B104, H99 and W22. Seeds were surface sterilized using the optimized method (Appendix 1). Mature embryos were isolated and germinated on MSVS34 media for eight days at 28 °C and 16 hours photoperiod. After eight days, number of dead and abnormal seedlings were recorded and then discarded. Roots and approximately 1 cm above the mesocotyl of normal plants were removed from all seedlings.

Leaves were used to conduct the seedling growth rate (SGR) test to assess seedling vigor. Leaves were placed into a coin envelope to be dried at 80 °C for 24 hours. The dried leaves were weighed to the nearest mg and recorded. The total dry weight of the normal seedlings per treatment and per inbred line is divided by the number of seedling included to arrive at a SGR of mg/seedling. Seedling growth rate test was based on the procedure developed by the International seed testing association (ISTA, 1999) although some modifications were made to adjust for in vitro conditions (Such as the germination in culture media instead of paper towels. The test was applied either under in vitro and non in vitro conditions.

### **2.2.6 In vitro callus induction**

For in vitro callus induction, SAM explants containing the mesocotyl and part of the coleoptile were excised from seedlings and placed onto MSW57 media (MS base salts, 500 mg/L thiamine, 500 mg/L casaminoacids, 3% sucrose, 1.38 g/L L-proline, 3.4 mg/L silver nitrate, 0.5 mg/L 2,4-D, 2.2 mg/L BAP, 2.5 g/L gelrite, pH 5.8) for callus induction. Plates were incubated under 16 h photoperiod and 28 °C. Callus induction was evaluated as the first callus appearance (number of days) and the number of callus-deriving lines (every seedling was considered as a line).

### **2.2.7 Statistic analysis**

The experimental design was a complete randomized design (CRD) with three treatments and three replications per treatment. Seedling vigor (seedling growth rate), contamination rate and in vitro callus induction were subjected to analysis of variance (GLM procedure, SAS institute) and treatment mean comparison was estimated using Duncan's test.

## **2.3 Results and Discussion**

Initial seed sterilization experiments with inbred line B73 harvested from greenhouse showed moderate contamination. However, seeds from field-grown plants resulted in 100% contamination. This problem was reported by other groups using field-grown material that were more exposed to air and soil borne pathogens (Skirvin, 1999). Therefore, three different approaches for seed sterilization were evaluated to obtain aseptic material suitable for in vitro culture and genetic transformation.

### **2.3.1 Evaluation of various seed sterilization methods**

#### *2.3.1.1 Plant extracted essential oils method*

Three concentrations of essential oil, 1 000, 10 000 and 500 000 ppm were tested for seed surface contamination. This method has been successfully used to disinfect seeds earmarked for sowing in field (Erik Christian, personal communication; Association of Official Seed Analysts (AOSA). 2002. Rules for Testing Seeds. AOSA, Las Cruces, NM). However, when tested for in vitro culture, full contamination was observed in all treatments even with the highest concentration used (data not shown), no further experiments were performed using this method.

#### *2.3.1.2 Chlorine gas method*

Chlorine gas has been used as a standard seed sterilization method for soybean in the Iowa State University Plant Transformation Facility (Paz et al, 2006). To adopt this method to corn seed sterilization, we have tested two different chlorine concentrations (3N HCl or 0.42N HCl) and four different treatment durations (5, 10 and 20 min for 3N HCl and 10 h for 0.42N HCl, (Table 1). Twenty to fifty seeds were used for each treatment. Seeds sterilized with the high concentration of chlorine gas (3N) for 5, 10 and 20 min showed full contaminated after treatment. Seeds exposed with low chlorine gas (0.42N) but longer time exposure (10 hr) showed no contamination on germination media. However, this treatment duration appeared to be detrimental to seed because only few seeds germinated and developed into abnormal seedlings. No further experiments were performed.

### *2.3.1.3 Liquid sterilization method*

Liquid sterilization using sodium hypochlorite and ethanol has been the common method to sterilize maize mature seeds (Huang et al, 2004; O'Connor-Sanchez et al, 2002; Sidorov et al, 2006) and for other plant seeds as soybean (Clemente et al, 2000). Our first attempt using liquid sterilization was as described by Clemente et al (2000). Although it was not completely successful, it worked better compared to the essential oil and chlorine gas treatments. Approximately 20% of axenic seed was obtained by this method (modification #1 in Figure 2). No abnormal growth from these seedlings was observed. This method was chosen for further optimization.

### **2.3.2 Improvement of liquid surface sterilization method for maize seed**

Because liquid surface sterilization method has given most encouraging results, we continued to modify the procedures to further reduce seed contamination rate. Initially, whole maize seeds were used for germination after surface sterilization as described by the original sterilization method (Clemente et al., 2002). However, we observed that such surface sterilization could not eliminate seed-borne pathogens that were in the interior of the seeds (underneath the pericarp and around the embryo).

A total of 18 different modifications based on the original liquid sterilization method (Clemente et al, 2002) were tested (Figure 2). These modifications included among others, varying bleach immersion time, use of biocides such as plant preservative mixture (PPM<sup>®</sup>, Caisson Labs. Inc.) and silver nitrate, applying double sterilization, seed softening, mature embryo dissection and embryo sterilization. Starting with the third modification experiment, we decided to sterilize the seeds in two steps: first remove the pathogens on seed surface, then dissect embryos and sterilize the dissected embryos to eliminate any pathogens present in the interior of the seeds.

As can be seen from Figure 2, the modifications tested in experiment 18 had reduced contamination rate drastically. The method developed includes three main sterilization stages. First sterilization was carried out using the seeds as they came from field to eliminate external pathogens, seeds were surface sterilized for 3 min ethanol 80%, then 2 x 15 min 50% commercial bleach (v/v) and rinsed 5 times with sterile water. Seeds were then soaked in water for 48 hours for softening and facilitate the embryo dissection. The second

sterilization was performed after those 48 hours of softening, to eliminate the pathogens that may remain on the surface of the seeds prior the embryo dissection and the procedure was the same as the first sterilization. The embryo dissection was conducted to expose the very inner-pathogens in the seeds to the antiseptic chemicals. Finally the third sterilization step was conducted to eliminate those pathogens exposed through the embryo dissection or embryo-borne. Embryos were surface sterilized 10 min with 15% commercial bleach (v/v) and rinsed 4 times with sterile water. This multiple step sterilization method is reliable, reproducible and efficient. This method is suitable for sterilization of mature maize seed, especially seeds harvested from field-grown plants, used for in vitro tissue culture establishment.

### **2.3.3 Further optimization of the liquid seed sterilization method**

Our improved multi-step seed sterilization method was further optimized by reducing the concentrations of disinfectants. This is because we wanted to minimize any damage on seed during the sterilization process.

Three optimizations (treatments) of the seed sterilization method developed were evaluated on 2006 field harvested B73 seeds (Table 2). The initially improved multi-step method was used as a control (Treatment 1). Three variation of treatments included change soaking seeds in water for 48 hr to placing seeds on wet paper towel for 24 hr in the step 1, reduced ethanol sterilization duration in the step 2, and reduced bleach concentrations and durations in the step 3 (Table 2).

Number of contaminated embryos was first scored two days after the treatments and then every day until the eighth day to finally obtain the total number of contaminated embryos per treatment. Number of abnormal seedlings was recorded 8 days after treatments. Some examples of normal and abnormal plantlets and their seedling vigor are shown in Figure 3. Replications were performed all the same day except for treatment 1 which involved 48 hours soaking in water.

Figure 4 summarizes the percentage of contamination and abnormal seedlings resulted from the four treatments. Contamination ratios among treatments were not significantly different; however the percentage of abnormal seedling of Treatments 1 and 2 were significantly higher than that of Treatments 3 and 4 (Figure 4). Treatments 3 and 4 are

not significantly different from each other (Significance at the 0.05 probability level  $P=0.01$ ). Treatment 4 was chosen for analysis of seedling vigor.

These data indicate that even though there is no significant difference in contamination ratios among treatments, decrease of strengths of ethanol and bleach treatments lead to better germination of normal plants. It seems that the strength of the disinfectant has negative effect on seedling vigor (reflected in abnormal seedlings).

### **2.3.4 Effect of seed sterilization treatments on seedling vigor and in vitro callus induction.**

Because Treatment 4 gave the best seedling germination and minimum contamination, we used this protocol for further analysis of seedling vigor and in vitro callus induction on sterilized seeds. Our intention was to assess to what extent these sterilization methods, the optimized method (Treatment 4) and the original developed method, were affecting the seedlings vigor and consequently the in vitro callus induction.

Three tests were designed. **Test C (Tc): Control.** No sterilization, no in vitro conditions. Seeds were softened 24 hours in moisturized sterile paper towels and then embryos were dissected and placed into sterile soil in magenta boxes. This test was only used for seedling vigor assessment. **Test A (Ta):** Full strength seed sterilization method (the initially improved method, Treatment 1 in Table 2 except seeds were soaked in water for 24 hours). Isolated embryos were placed into MSVS34 media. **Test B (Tb):** Optimized method (Treatment 4 in Table 2). Isolated embryos were placed into MSVS34 media.

#### *2.3.4.1 Contamination rate assessment*

Seeds of four inbred lines (B73, B104, H99, and W22) harvested from field were used. Figure 5A shows the percentages of contamination and abnormal plants resulted from the two tests (Ta and Tb) under in vitro conditions. Inbred B104 has a lower contamination rate with no significant difference between tests. Inbred H99 showed a higher contamination rate in Test A. Comparison among genotypes showed that H99 has a higher contamination rate followed by B73 and W22, B104 showed the least contamination rate. This may be an indication about the seed-borne pathogen content. In general, Test A seems to be the best treatment to sterilize seeds, however even if Tests B showed some contamination, it doesn't go beyond 11% which may be acceptable in tissue culture

#### 2.3.4.2 Seedling vigor assessment

Ten days after seed sterilization and incubation on germination media or soil, germinating plants from the three tests were removed from their containers. The meristematic part of each plantlet (located in the base of the coleoptile and the upper part of the mesocotyl) was removed (around 1 cm length) and placed into MSW57 media (Sidorov *et al*, 2006) for callus induction (see below 2.3.4.3).

The rest of each plant without root was used to assess seedling vigor through the Seedling Growth Rate (SGR) Test (ISTA, 1999) as described in Materials and Methods in all three tests for each inbred. As can be seen from Table 3, seedling vigor of control for both inbred lines B73 and B104 was significantly lower than either, the full strength method or the optimized method. For line W22, data of control was lost due to uncontrolled contamination; however the seedling vigor ratios of the full strength method and the optimized methods were no significantly different when compared. For inbred line H99, all tests were significantly different. In all inbred lines, except W22 the control (grown in soil) showed the lower SGR which indicates that *in vitro* culture conditions enhanced the vigor of the seedlings even if the seeds suffer for the sterilization methods used. In H99 also all treatments are significantly different, the control showed the lowest vigor and the optimized sterilization method the highest. The mean comparison among inbred lines showed that H99 has the highest performance in seedling vigor. Inbred lines B73, B104 and W22 showed no significant difference in vigor among them (Table 3, Figure 6). Number of abnormal seedlings was also recorded (Figure 5B). Number of abnormal seedlings was higher in genotype B73 in both full strength and optimized methods, followed by B104 which may indicate that these two genotypes are the most susceptible to both seed sterilization methods. Genotypes W22 and H99 showed the lower ratio of abnormal seedlings. In previous experiments B104 has showed a lower performance in tissue culture (data not showed) which may be due to the genetic background of the genotype itself. Inbred line H99 exhibited the lowest ratio of abnormal seedlings (Figure 5B).

#### 2.3.4.3 Callus induction assessment

Callus induction was evaluated as the first callus appearance (number of days) and the number of callus-deriving lines (every mature embryo was considered as a line). Callus

initiation was observed on line H99 after 21 days, in line W22 after 40 days and in lines B73 and B104 after 45 days. Line H99 showed the highest callus induction ratio in both test: full strength (Test A) and optimized (Test B). Lines B73, B104 and H99 did not show significant difference in callus induction among tests (Figure 6). Full strength sterilization and optimized were no significantly different in any of the inbred lines. Finally the correlation between seedling vigor and callus induction (Figure 6) showed that while the vigor increased (reflected in SGR), the callus induction increased as well. Line H99 showed the highest seedling vigor and callus induction. Inbred lines B73, B104 and W22 remained almost constant without significant difference. Both tests, full strength (Test a) and optimized (Tests b) have no significant effect on seedling vigor.

#### **2.4. Conclusions**

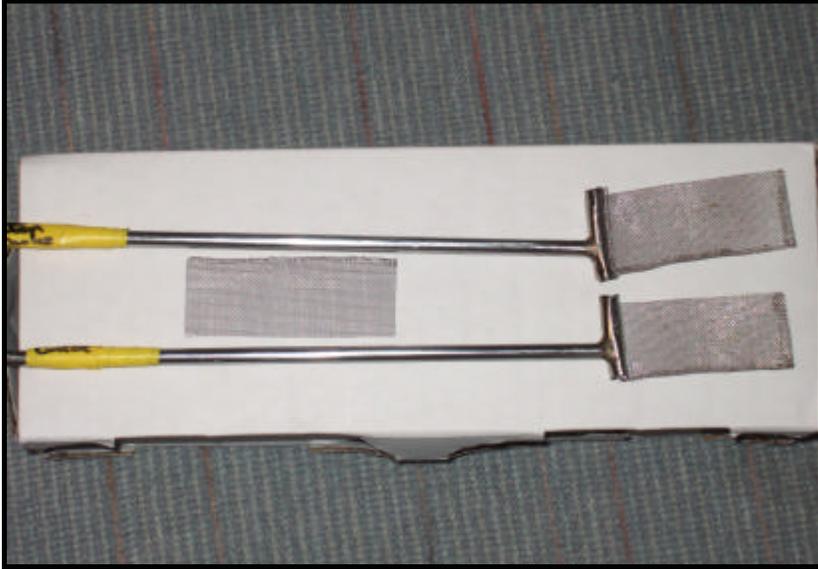
A reliable, repeatable and efficient seed sterilization method for maize, especially developed for field-grown mature seeds was achieved. (Appendix 1). The optimized seed sterilization method developed has a high efficiency in disinfecting seeds and at the same time the vigor is not compromised, which is reflected in a higher rate of plants germinated and higher rate of callus initiation in inbred line H99. Vigor assessed in terms of abnormal seedling rate, appears to be genotype dependant, however normal plants developed, seem to have a homogeneous SGR for the same genotype. Line H99 showed the best performance in seedling vigor and callus induction. Seedling vigor is positively correlated with callus initiation

#### **Acknowledgements**

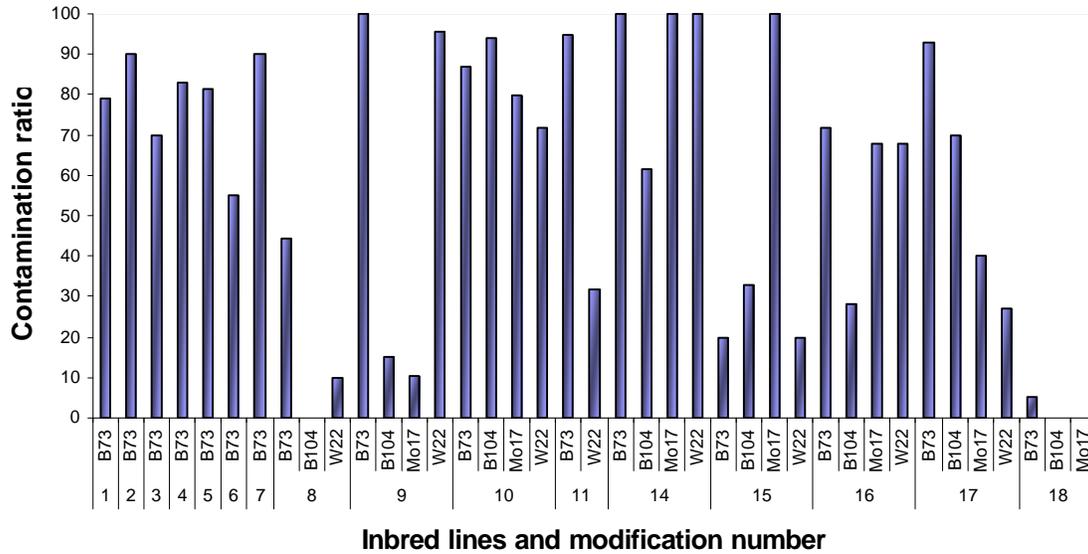
I'm very thankful to Bronwyn Frame, Dr. Kan Wang and Margie Paz for the scientific advices and helpful discussion. Dr. Susana Goggi for providing material, protocols and advice to conduct the seedling vigor assessment. Eric Christian for his guidance on the essential oil experiments, Tina Paque and the North Central Regional Plant Introduction Station for providing the germplasm used for the experiments. Javier Garcia and Jessica Zimmer for technical assistance. This project was funded by the National Science Foundation Plant Genome Program (DBI 0110023).

## References

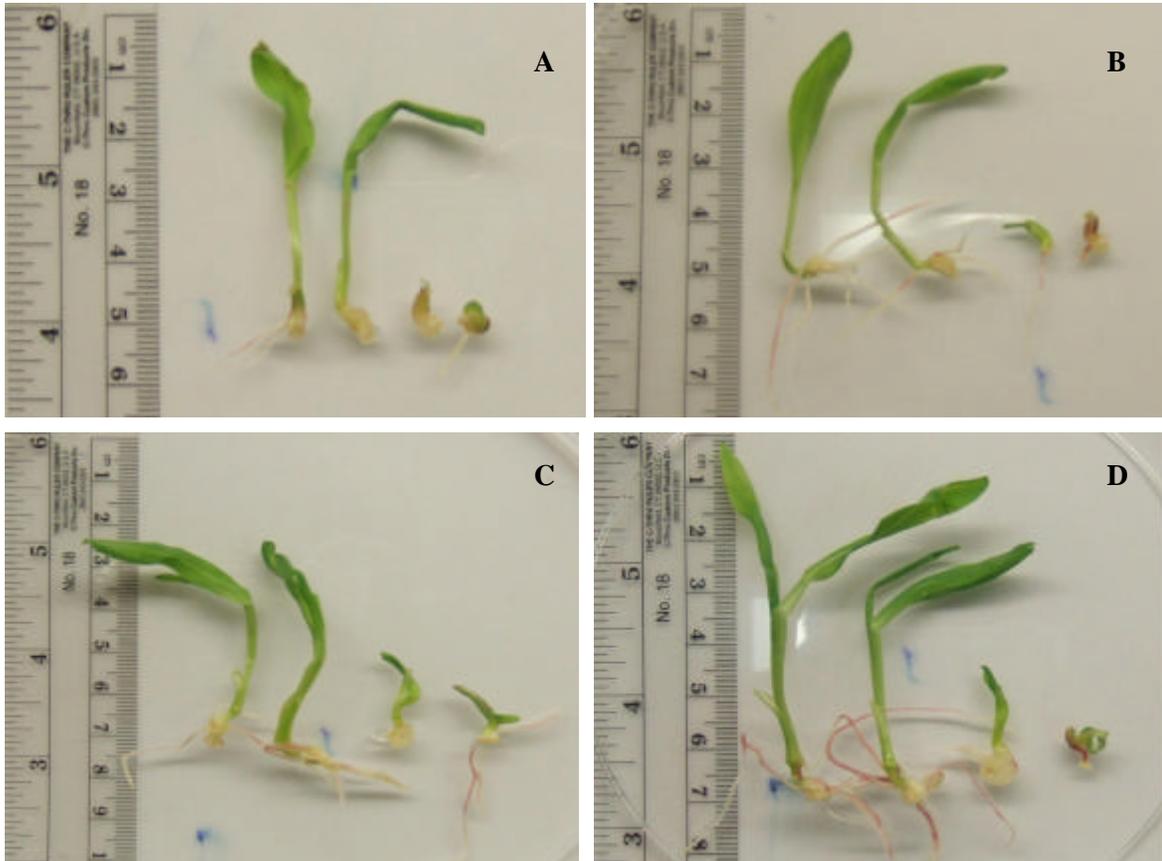
- Association of Official Seed Analysts (AOSA). 2002. Rules for testing seeds, AOSA, Las Cruces. NM.
- Clemente, T., B.J. LaValle, A.R. Howe, D.C. Ward, R.J. Rozman, P.E. Hunte, D.L. Broyles, D.S. Kasten and M.A. Hinchee. 2000. Progeny analysis of glyphosate selected transgenic soybeans derived from *Agrobacterium*-mediated transformation. *Crop Sci* 40:797-803.
- Dixon, R.A. 1985. Plant cell culture, a practical approach. R.A. Dixon (Ed). IRL Press. Oxford, WA.
- Frame, B.R., Zhang, H., Cocciolone, S.M., Sidorenko, L.V., Dietrich, C.R., Pegg, S.E., Zhen, S., Schnable, P.S. and Wang, K. 2000. Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cell Dev Biol Plant* 36:21-29.
- Huang, X.Q. and Wei, Z.M. 2004. High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays* L.). *Plant Cell Rep* 22:792-800.
- International Seed Testing Association (ISTA). 1999. International rules for seed testing. *Seed Sc. Technol.* 27(Suppl.):27-32.
- Leifert, C., Waites. W.M. 1990. Contaminants in plant tissue culture. *Int. Soc. Plant Tissue Cult. Newsletter* 60:2-13.
- O'Connor-Sanchez, A., Cabrera-Ponce, J.I., Valdez-Melara, m., Tellez-Rodriguez, P., Pons-Hernandez, J.L., Herrera-Estrella, L. 2002. Transgenic maize plants of tropical and subtropical genotypes obtained from calluses containing organogenic and embryogenic-like structures derived from shoot tips. *Plant Cell Rep* 21:302-312.
- Paz, M.M., Martinez, J.C., Kalvig, A.B., Fonger, T.M., Wang, K. 2006. Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. *Plant Cell Rep* 25:206-213.
- Sidorov, V., Gilbertson, L., Addae, P., Duncan, D. 2006. *Agrobacterium*-mediated transformation of seedling-derived maize callus. *Plant Cell Rep* 25:320-328.
- Skirvin, R.M., Motoike, S., Norton, M.A., Ozgur, M., Al-Juboory, K., McMeans, O.M. 1999. Workshop in micropropagation establishment of contaminant-free perennial plant in vitro. *In Vitro Cell and Devel Biol.* Vol 34 4:278-280.



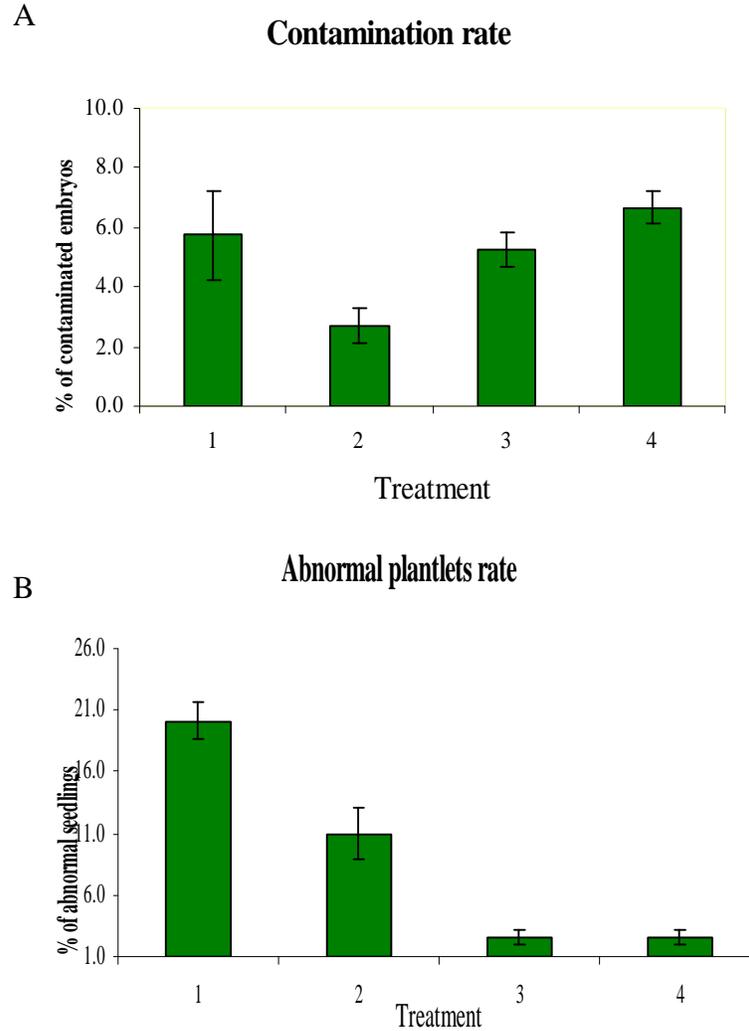
**Figure 1.** Hand-made strainers used for mature embryo sterilization



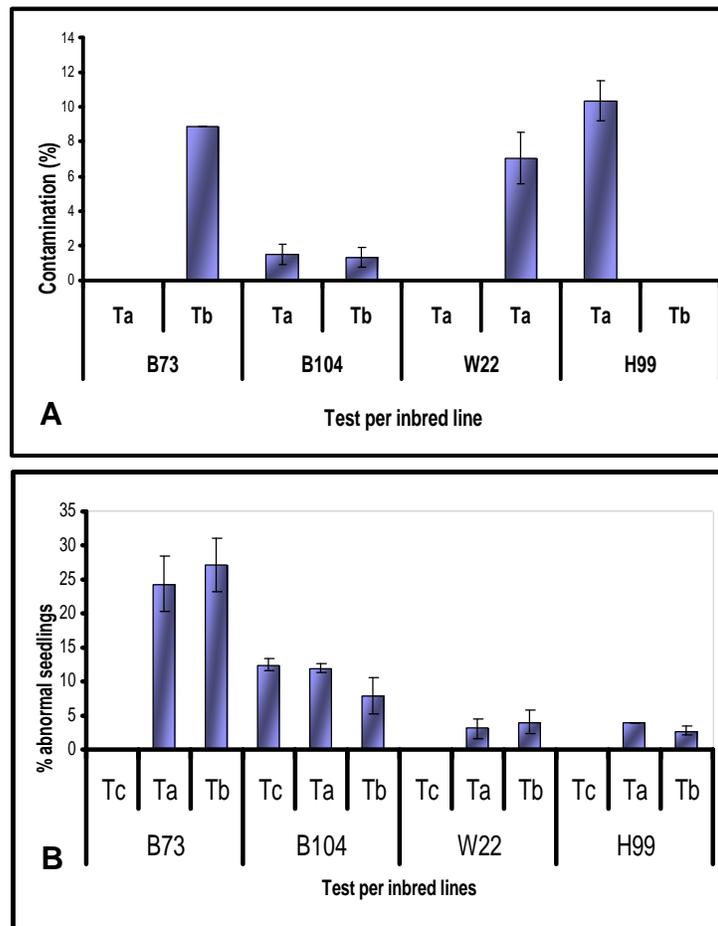
**Figure 2.** Contamination ratios of different modifications for liquid seed sterilization method. Data of modification 12 and 13 were completely lost due to external contamination. Contamination ratio was calculated as the number of sterile seeds obtained divided by the total number used (X 100). No replications were performed for these experiments.



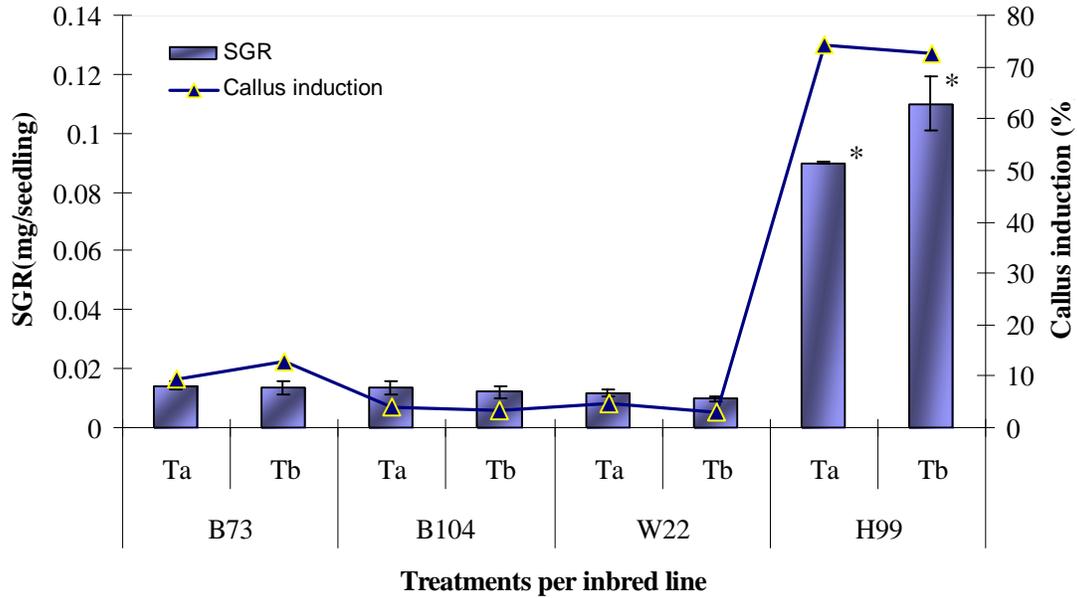
**Figure 3.** Best (two on the left in each picture) and worst (two on the right on each picture) B73 plantlets for each treatment of seed sterilization optimization experiment. **A.** Treatment 1 **B.** Treatment 2 **C.** Treatment 3 and **D.** treatment 4



**Figure 4** Contamination and abnormal plantlets rates for seed sterilization optimization experiment on B73. **A.** Contamination rate. No significant difference among treatments (Significant at the 0.05 probability level. ( $P= 0.01$ )). **B.** Abnormal plantlets ratio. Treatments 1 and 2 are significantly different from treatments 3 and 4 (Significant at the 0.05 probability level. ( $P= 0.01$ )). Three replications per treatment and 25 seeds per treatment.



**Figure 5.** Contamination and abnormal seedling rate in seedling vigor experiments **A.** Effects of 2 tests on contamination rate per genotype **B.** Effect of tests on the number of abnormal seedlings per genotype. T= Test. Significance at the 0.05 probability level. ( $P= 0.01$ ). Three replications per test and 25 seeds per test.



**Figure 6.** Correlation between SGR and callus induction. SGR of H99 is significantly different (\*) from the rest of inbred lines. Ta and Tb within inbred lines are no significantly different. (Significantly different at the 0.05 probability level ( $P=0.01$ )). Ta=Full strength sterilization. Tb=Optimized sterilization method. Callus induction frequency was estimated using the average number of seedling-deriving callus of each replication divided by the average number of embryos used ( $\times 100$ ).

<b>Treatment</b>	<b>Treatment description</b>	<b>No of seeds</b>
1	5 min, 3N CG	50
2	10 min, 3N CG	50
3	20 min, 3N CG	50
4	10 hours, 0.42N CG	20

N = Concentration expressed in normality

CG = Chlorine gas

**Table 1.** Treatment description for seed sterilization using chlorine gas

	Treatments			
	1	2	3	4
Step 1	3 min in ethanol, 15 min in bleach(50) 2x, 6x rinse. Soak in sterile water, 48 h	3 min in ethanol, 15 min in bleach(50) 2x,, 6x rinse. Softening on wet paper towel, 24 h	3 min in ethanol, 15 min in bleach(50) 2x,, 6x rinse. Softening on wet paper towel, 24 h	3 min in ethanol, 10 min in bleach(50) 2x, 6x rinse. Softening on wet paper towel, 24 h
Step 2**:	3 min in ethanol, 12 min in bleach(50), 6x rinse	1 min in ethanol, 5 min in bleach(50), 6x rinse	1 min in ethanol, 5 min in bleach(50), 6x rinse	2 min in bleach(50), 4x rinse
Step 3***:	10 min in bleach(15), 4x rinse	10 min in bleach(15), 4x rinse	5 min in bleach(15), 4x rinse	5 min in bleach(15), 4x rinse

Step 2\*\*, Ethanol: 80%; Bleach(50): 50% commercial bleach + 0.1% Tween 20.

Step 3\*\*\*, Bleach(15): 15% commercial bleach + 0.1% Tween 20.

**Table 2.** Steps used on different treatments for optimization of the seed sterilization method

Genotype	Test	SGR (mg/seedling)	
		Mean	Std error
B73	c control*	0.00171	0.00064
	a full strength	0.01436	0.00133
	b optimized	0.01339	0.0023
B104	c control*	0.00514	0.00059
	a full strength	0.0134	0.00237
	b optimized	0.01217	0.00216
W22	c control	N/A	N/A
	a full strength	0.01187	0.00141
	b optimized	0.00983	0.00079
H99**	c control*	0.07399	0.00012
	a full strength*	0.08999	0.00037
	b optimized*	0.10998	0.00924

\* Significantly different between treatments for each inbred line at the 0.05 probability level. (P= 0.01)

\*\* Significantly different between inbred lines at the 0.05 probability level. (P= 0.01)

**Table 3.** Effects of two different seed liquid sterilization techniques (Tests a and b, and no sterilization (control), on seedling vigor (SGR).

## CHAPTER 3. ESTABLISHMENT OF TISSUE CULTURE SYSTEMS AND GENETIC TRANSFORMATION FOR INBRED LINES USING MATURE SEEDS AS STARTING MATERIAL

### Abstract

Two mature seed derived maize tissue culture systems, shoot meristem culture (SMC) and somatic embryogenic culture (SEC), were developed for five inbred lines, B73, B104, H99, Mo17 and W22. Using published protocol, SMC of these inbred lines could be established. A construct carrying a selectable marker *bar* gene and a screenable marker *gus* gene was introduced into the SMC cultures using either biolistic gun or *Agrobacterium*-mediated method. While transient GUS expression could be detected from all callus materials tested, no transgenic plant was recovered from the SMC transformation experiments. On the other hand, inbred H99 responded with high frequency on SEC induction medium, whereas the other four inbreds responded poorly on the same medium. Bombardment of the H99 SEC has resulted in fertile transgenic maize plants. Progeny analysis indicated that both *gus* and *bar* transgenes had transmitted to the next generation. Transformation frequency for H99 SEC tissue ranged from 24.1% to 52.7%. This work indicates that transgenic plants can be produced from inbred H99 using mature seeds as starting material using the biolistic gun method.

### 3.1 Introduction

The efficiency in genetic transformation depends upon the establishment of a robust and reproducible plant tissue culture system to develop target tissue as well as an efficient delivery system for transformation. There are two important aspects to be considered when selecting a tissue to be suitable for genetic transformation: competence and regenerability. Competence reflects the ability of a cell/tissue to be transformed by receiving DNA and then regenerate in a full fertile germ-line plant suitable to inherit the trait from which it was transformed to the descendants (Torney et al, 2007). In general, the desirable target tissue would be the one from which genetic transformation can be achieved on the germ-line cells and also that it can maintained for a shorter period on tissue culture conditions since an

extended period is associated with an increase of somaclonal variation (Lee and Phillips, 1987).

For current maize transformation system, immature embryos are routinely used as starting material for *Agrobacterium* infection or particle bombardment. The major problem for using the immature embryos is that it requires maize plants to be grown in the greenhouse year-round to meet the research demands. This practice requires large greenhouse spaces, quality growth conditions and experienced supporting staff. One other problem with the current maize transformation system is that only Hi II, a hybrid germplasm bred specifically for tissue culture purposes, is amendable for transformation.

For the maize community, inbred lines are the most desirable target for transformation due to their genetic background and agronomic importance in crop improvement. To date maize inbred line transformation using mature seeds has been achieved by using either shoot meristem culture (SMC) or somatic embryogenic culture (SEC) (Torney et al, 2007). In most cases, the biolistic gun was used as the delivery system (O'Connor-Sanchez et al., 2002; Wang et al, 2003). Most recently *Agrobacterium*-mediated mature seed transformation has been reported (Sidorov et al, 2006). The objective of this work was to establish/improve tissue culture and development of transformation systems using maize mature seeds as starting material for inbred lines B73, B104, H99, Mo17 and W22.

## **3.2 Materials and Methods**

### **3.2.1 Plant material**

Mature seeds of five elite inbred lines were used as initial material: B73, B104, H99, Mo17, and W22. B73 genotype was developed in Iowa and selected for recurrent selection population (C5) from Iowa Stiff Stalk Synthetic (BSSS). Accession number PI 550473 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1445409>). B104 was developed in Iowa from BS13(S)C5, a strain from Iowa Stiff Stalk Synthetic after 12 cycles of recurrent selection. Accession number PI 594047 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1519249>). H99 was developed in Indiana. Accession number PI 587129 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1073895>). Mo17 was

developed in Missouri, genotype resistant to leaf blight (*Helminthosporium turcicum*), pedigree CI 187-2/C103. Accession number PI 558532 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1453504>). W22 was developed in Wisconsin, from second cycle inbred line, plants susceptible to smut, excellent tolerance to stalk rooting organisms. Accession number NSL 30053 (<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1098978>). Seeds of all genotypes were kindly provided by Tina Paque (Plant Transformation Facility, Iowa State University) and the North Regional PI Station in Ames, Iowa.

### **3.2.2 Seed sterilization**

Mature maize seeds were disinfected by a multi-step sterilization method developed in this laboratory (see Appendix 1 of Chapter 2). Sterilization consisted of three major steps: 1) Sterilization of mature seeds (80% ethanol, 3 min; 50% commercial bleach, 15 min x 2; 2) Seed softening in water for 24 hours and second sterilization once again as described in step 1, and 3) Embryo dissection followed by embryo sterilization (15% commercial bleach, 5 min).

### **3.2.3 Shoot meristem culture (SMC) establishment and regeneration**

Mature embryos of B73 and H99 maize inbred lines were germinated directly on SMC induction medium CSMD9B (MS base salts, 2.0 mg/L BAP, 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 500 mg/L casein hydrolysate, 3% sucrose and 3 g/L gelrite, pH 5.8) (Zhong *et al*, 1996) under 28 °C, 16 h photoperiod, for two weeks. After two weeks, leaves were removed and nodal portions containing the apical and adventitious meristems were transferred to a fresh CSMD9B media. Primordial SMCs were transferred and propagated every two weeks to fresh CSM9B media. After four to five weeks, SMC pieces were transferred to regeneration media (MS basal salts, 3% sucrose, 3 g/L gelrite, no hormones, pH 5.8). After 10 to 15 days, a “hedgehog”-type callus will form. This callus could regenerate to plants after 10 to 15 days. Conditions of incubation were the same in all transfers, 28 °C and 16 h photoperiod. Plantlets were then transferred to soil for acclimation and seven to ten days later transferred to a greenhouse and grown until maturation.

### 3.2.4 Somatic embryogenic culture (SEC) establishment

Sterilized embryos were germinated on MSVS34 (MS base salts, 100 mg/L casein hydrolysate, 1.95 g/L MES [2-(N-morpholino) ethanesulfonic acid], 750 mg/L glutamine, 500 mg/L ascorbic acid, amended with 10 mg/L picloram and 3 mg/L BAP (6-benzylaminopurine) and 2.5 g/L gelrite at pH 5.8) (Sidorov et al, 2006) plates were incubated at 28 °C and 16 h photoperiod for 7 days. Nodal section was removed in all seedlings (approximately 1.5 cm long sections of the mesocotyl-coleoptile) containing the apical and adventitious meristems and split longitudinally, then placed cut side down on MSW57 media (MS base salts, 500 mg/L thiamine, 500 mg/L casaminoacids, 3% sucrose, 1.38 g/L L-proline, 3.4 mg/L silver nitrate, 0.5 mg/L 2,4-D, 2.2 mg/L BAP, 2.5 g/L gelrite and pH was adjusted to 5.8) for callus induction. Plates were incubated at 28 °C and 16 h photoperiod for two weeks and transfers to fresh MSW57 media were made every two weeks, until primordial embryogenic callus appeared. Primordial embryogenic callus was transferred to fresh MSW57 and incubated at 28 °C in darkness. Embryogenic callus was propagated by making transfers to a fresh MSW57 media every two weeks.

### 3.2.5 Particle bombardment

#### 3.2.5.1 DNA construct

Plasmid pBU-B35S.IG (Figure 1, CSIRO Plant Industry) DNA was isolated from *E. coli* DH5a strain and purified using the Quiagen kit®. The selectable marker gene of this plasmid is the CaMV 35S-bar (phosphinothricin acetyltransferase) and the screenable marker gene is the CaMV 35S-gus ( $\beta$ -glucuronidase).

#### 3.2.5.2 Plant culture preparation

Four hours prior to bombardment, SMC explants from B73 inbred line were sliced and placed directly into resting media (CSMD9B medium amended with 4.25 mg/L silver nitrate). Thirty pieces of SMCs (5 mm in size) were placed in each bombarded plate directly onto the media.

For embryogenic callus bombardment, healthy pieces of embryogenic callus derived from mature seeds of different inbred lines were dissected and used as a target explants. About 19 to 29 pieces (3-5 mm in size) were plated directly on osmotic media (same

formulation as MSW57 but supplemented with 3.64 % mannitol and sorbitol (Vain et al, 1993)) 4 hours prior to bombardment.

### 3.2.5.3 Particle bombardment conditions

Biolistic transformation of either SMC tissue or somatic embryogenic callus culture followed the established protocol published by Frame et al (2000). Before bombardment a 1X aliquot of sterile gold particles (3 mg) was thawed and sonicated for 15 second. Plasmid DNA at the concentration of 0.1  $\mu\text{g}$  was added to the 1X gold tube. While the tube was mixed with a vortexer, 50  $\mu\text{L}$  of 2.5 M  $\text{CaCl}_2$  were added followed by 20  $\mu\text{l}$  spermidine (0.1 M). The gold was allowed to settle and the tube was centrifuged at 5000 rpm for 15 sec. Supernatant was discarded and pellet completely resuspended in 250  $\mu\text{l}$  of 100% chilled ethanol. The gold was allowed to settle down one more time and then the tube was centrifuged again at the same conditions. Supernatant was discarded and 120  $\mu\text{l}$  of chilled ethanol added.

Ten  $\mu\text{l}$  of the DNA-coated gold was pipetted on top of each macrocarrier while the suspension was continuously shaken to avoid gold settle down. Once loaded, macrocarriers were maintained in a container with of Drierite (Fisher) (Frame et al, 2000). Particle bombardment transformation was conducted using the PDS 1000/He biolistic gun (BioRad, Hercules CA) and the following parameters: 650 psi rupture disk pressure; 6 cm target distance, 6 mm gap, 1.2 cm from macrocarrier to stopping plate and 28 torr vacuum at rupture, also a 150  $\mu\text{m}$  screen (McMaster-Carr, Elmhurst, IL) was inserted between the target tissue and the launch assembly (Gordon-Kamm et al, 1990; Wilson et al, 1995). All supplies for the biolistic gun were obtained from BioRad. After bombardment, plates with SMC's were maintained in resting media for 16h and then transferred to fresh resting media for 7 days. Incubation conditions were 28  $^{\circ}\text{C}$  and 16 h photoperiod in all cases. Bombarded embryogenic callus were maintained in osmotic media for 16 hours at 28  $^{\circ}\text{C}$ , in darkness, and then transferred to fresh MSW57 media for resting during 7 days.

## 3.2.6 Agrobacterium-mediated transformation

### 3.2.6.1 Agrobacterium culture preparation

*Agrobacterium* strain EHA-101 hosting the binary vector pBU-B35S.IG (Figure 1, CSIRO Plant Industry) was used. *Agrobacterium* was refreshed from long term storage -80 $^{\circ}\text{C}$

glycerol stock and by streaking a scoop (using a bacterial loop) onto a plate containing solid YEP media (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl<sub>2</sub>, 15 g/L Bacto-agar, pH adjusted to 6.8 with NaOH) amended with 50 mg/L rifampicine and 50 mg/L spectinomycine. Plate was incubated at 28 °C for 36h. A single scoop of the bacteria culture obtained in the YEP plate was inoculated in a flask containing 250 ml Yep amended with antibiotics (same as mentioned above) and incubated for 36 hours (250 rpm, 28 °C) or until OD<sub>650</sub>=1.0 was reached.

### 3.2.6.2 *Agrobacterium* infection

For SMC's, bacteria cultures were resuspended in infection media (MS salts, 3% sucrose, 0.05% casein hydrolysate, 20 mM MES [2-(N-morpholino) ethanesulfonic acid], amended with 2 mg/L BAP (6-benzylaminopurine), 0.5 mg/L 2,4-D and 200 mM AS (acetosyringone), pH 5.4). The OD<sub>650</sub> was adjusted to 0.6. The infection media was based on the formulation used by Paz et al (2006) for soybean transformation and the formulation of the CSMD9B media (Zhong et al, 1996). Sliced SMC pieces were immersed into the infection media for 30 minutes under vacuum infiltration (20 In Hg), then blotted in sterile filter paper to remove the excess of infection media and placed directly in co-cultivation media with the cut-side down (MS salts, 3% sucrose, 500 mg/L casein hydrolysate, amended with 2 mg/L BAP, 0.5 mg/L 2,4-D, 300 mg/L cysteine and 100 mM AS and 3 mg/L gelrite). Plates were incubated at 24 °C and 16 hours light for three days. After co-cultivation, excess *Agrobacterium* was removed by washing the explants with CSMD9B media amended with 500 mg/L carbenicillin and 100 mg/L vancomycin and then transferred to resting media (CSMD9B media amended with 500 mg/L carbenicillin) for 7 days an incubated under the same conditions.

For embryogenic callus, bacteria cultures were resuspended into half strength MS salts media enriched with 1 mM L-proline, 3.6% glucose. 6.85% sucrose and 200 µM AS, pH 5.2. The OD<sub>650</sub> was adjusted to 1.0 (Sidorov et al, 2006). Sliced embryogenic callus pieces were immersed into the resuspension media for 30 minutes. After infection explants were blotted on sterile filter paper and placed into a Petri dish on top of a double layer of filter paper. Plates were sealed with parafilm and maintained at room temperature (~24 °C) in dark for two days (Sidorov *et al*, 2006).

### **3.2.7 Selection and regeneration for biolistic gun-mediated transformation on embryogenic callus**

After the resting period, embryogenic callus were transferred to selection media I (same formulation as MSW57 but containing 3 mg/L Bialaphos (Duchefa biochemicals)) for two weeks and then transferred to selection II (same formulation as in MSW57 amended with 5 mg/L Bialaphos). Transfers to a fresh selection II media were made every two weeks. After obtaining enough callus material, some pieces were transferred to regeneration media I (MS basal salts, 3% sucrose, 100 mg/L myo-inositol, 0.25 mg/L 2,4-D, 0.3% gelrite, pH 5.8, 5 mg/L Bialaphos) and incubated at 28 °C in darkness. After two weeks, callus were transferred to regeneration II media (MS basal salts, 6% sucrose, 100 mg/L myo-isnositol, 0.3 % gelrite, pH 5.8, 6 mg/L glufosinate, no hormones). Plates were incubated at 28 °C in darkness. After two more weeks, callus were transferred to regeneration III media (MS basal salts, 3% sucrose, 0.3% gelrite, pH 5.8) and incubated at 28 °C and 16 h photoperiod for two more weeks (Frame et al, 2006). After regeneration, plantlets were transferred to soil for acclimation, screened for glufosinate resistance and grown in a greenhouse until maturation.

### **3.2.8 Histochemical GUS assay and herbicide resistance test**

GUS transient expression assay was carried out on plant cultures 48 hours after the transformation experiments according to Jefferson (1987). Explants were immersed in X-GLUC solution (2 mM 5-bromo-4-chloro-3-indoyl- $\beta$ -D-glucuronic acid, 0.05 potassium ferricyanide, 0.05 mM potassium ferrocyanide, 0.1 % mM Triton X-100 (v/v), 0.1 M sodium phosphate buffer pH 7) and incubated for 2 to 24 hrs. Typically three samples of each inbred line were assayed and the histochemical data were scored 2 hrs to one day after the GUS incubation.

One week after plant acclimation in the soil, putative transgenic events were sprayed twice with 250 mg/L Liberty® (Bayer Crop-Science, USA) (glufosinate as active ingredient) with one day interval between herbicide application (Brettschneider et al, 1997). Resistant clones were scored after 3 days from the last application.

Bialaphos resistant callus cultures or leaves were analyzed for stable GUS expression using the histochemical GUS assay analysis (Jefferson, 1987). Samples of each putative transformed plant were taken from leaves using a punch-hole tool and collected in a 24-well

plate. Plates were placed in a vacuum infiltration chamber for 20 min to facilitate penetration of X-GLUC solution into the tissue and then incubated 2 to 24 hrs at 37 °C.

### **3.2.9 Progeny analysis**

Putatively transformed events were analyzed by transgene segregation in the progeny. To accelerate the progeny analysis, immature kernels from T1 ears (17-day after pollination) were harvested. Kernels were surfaced sterilized for 20 min with 50% commercial bleach and rinsed 3 times with sterile water. Immature embryos were dissected and allowed for germination on full strength MS medium incubated under 28 °C, 16 hr photoperiod. Seven days after germination, plantlets were moved to soil for further growth and then sprayed twice with 250 mg/L Liberty with one day interval between each application. Herbicide resistant plants were scored 3 days after the second spray.

### **3.2.10 Statistical analysis**

Segregation of the transgenes inheritance in the progeny was statistically analyzed for goodness-of-fit to simple Mendelian ratio by Chi-square ( $\chi^2$ ) test.

## **3.3 Results and Discussion**

To date maize inbred line transformation using mature seeds have been achieved by using either shoot meristem culture or embryogenesis culture (Torney et al, 2007). However few works have been reported and there is still a lack in transformation for some elite and very important inbred lines, since it is known that the performance of different maize types in tissue culture is genotype dependent. The first approach of this work was to develop a robust tissue culture system and then perform genetic transformation. Two tissue culture systems were explored SMC's and somatic embryogenic callus.

### **3.3.1 Shoot meristems culture (SMC) transformation**

The first approach to establish a tissue culture system suitable to be used for genetic transformation was through shoot meristems. The basis of this system is to induce the development of clumps of multiple meristematic shoots called shoot meristematic cultures (SMC's) (Zhang et al, 2002) using mature seeds as starting material.

### 3.3.1.1 SMC establishment

This system was the most successful in terms of tissue culture system development for both inbred lines B73 and H99. A whole system started from mature seeds to adult plants was established (Figure 2). Some modifications on the original system were made that resulted in the reduction of the time to obtain SMC's, from 3 to 5 weeks (Zhong et al, 1996) to 2 to 4 weeks.

In the original protocol, seeds were germinated first and then the portion containing the shoot tip was excised and placed on SMC induction media (CSMD9B) for 4 weeks under dark conditions. In our modified system, we placed disinfected mature embryos with the root excised directly on CSMD9B under light conditions. Initial SMC's could be observed after 2-4 weeks with some organogenic structures (Figure 2B). Primordial SMC's showed a faster growth and well developed into SMC structures after 4 weeks (Figure 2C). These structures could be induced for plant regeneration. During the time that SMC remained on regeneration media, a "hedgehog" type callus was observed (Figure 2E). This structure carried a large number of independent shoot-meristematic tips in which every single one was able to either develop into a whole plant if continue to be placed onto regeneration medium (Figures 2F, 2G and 2H) or into more SMC's if placed back onto CSMD9B medium. With this system, it was also possible to generate embryogenic callus but only with the inbred line H99 (Fig. 2D). Regenerated plants showed a high level of somaclonal variation with feminized tassels (Figure 3) and reduced amount and length of the ear stigmata.

Although no specific percentage of SMC callus induction rate from the B73 and H99 embryos was recorded, majority of the embryos produced SMC callus. The same procedure was also tested on inbred lines B104, Mo17 and W22. Each inbred gave different responses on the SMC induction CSMD9B media (Figure 4). Among 5 inbred lines tested, B73, H99 and W22 were able to respond well on CSMD9B medium at different degrees, with B73 produced most SMC and H99 produced the least. Mo17 and B104 produced minimum SMC (< 5%).

### 3.3.1.2 Biolistic transformation

We proceeded with genetic transformation on B73 SMC cultures using the biolistic gun. A total of 330 pieces of B73 SMC were bombarded in two different experiments. About

30 pieces of bombarded callus were assayed for transient GUS expression (Figure 5A). The remaining callus were transferred to resting medium for 7 days and further cultured in selection media (CSMD9B media containing 5 and 8 mg/L Bialaphos). A relatively high intrinsic resistance to herbicide Bialaphos was observed from B73 SMC, because it could grow on 5 or 8 mg/L Bialaphos media for as long as 10 or 6 weeks, respectively, in non transformed plants.

While strong transient GUS expression was obtained from these experiments, we recovered only one Bialaphos resistant cluster after 14 months of selection. This callus started to develop organogenic structures. When we subjected it for GUS assay, it appeared to be blue (Figure 5B). No plant was regenerated from this cluster.

Our result confirms the low transformation frequency as it was reported by Zhang et al, (2005) where they obtained 1.3% transformation frequency for B73 using mature seed derived SMC and particle bombardment. In our case, we only recovered one stable transgenic callus clump (out of 330 bombarded SMC pieces) after several cycles of selection for the inbred line B73 generated from the biolistic transformation.

#### *3.3.1.3 Agrobacterium-mediated transformation.*

SMC's of 4 inbred lines (B73, B104, Mo17 and W22) were used for *Agrobacterium*-mediated transformation. A total of Number of 583, 80, 32 and 24 explants, respectively, were infected (Table 1). Number of explants used for transformation varied depending on the availability of SMCs of each inbred line which depended also on the response for SMC induction. Transient GUS expression was assayed for each inbred line three days after infection (Table 1, Figure 6). GUS expression on explants assayed was strong in B73 but weak in the rest of inbred lines (Figure 6). Remaining infected explants were transferred to a fresh selection media (CSMD9B containing 5 mg/L first and then 8 mg/L Bialaphos) every two to three weeks. No *Agrobacterium* carry over contamination occurred during the experiments. Even though transient GUS expression was detected on some tissue sampled, we did not recover any stable transformant after 8 weeks of selection.

Our attempts to recover transgenic plants from either system in SMC's were not successful. However we improved a tissue culture system to obtain SMC for all five inbred lines tested. In addition we decreased the time of initial SMC establishment by 1-2 weeks.

Despite the unsuccessful attempts for transformation, SMC may still have the potential to be used as target for transformation since they have continuous growth of the shoot and reproductive organs, which are desirable traits for efficient genetic transformation (Sticklen and Oraby, 2005).

### **3.3.2 Somatic embryogenic callus (SEC) transformation**

In 2006, Sidorov et al reported success in transforming somatic embryogenic callus culture from maize mature seed using *Agrobacterium*-mediated method. Callus cultures were initiated from seedlings, derived from mature seeds of five inbred lines using the system described by Sidorov et al (2006) with some modifications. In this work we tested different inbred lines from those reported in this previous work. Particle bombardment was used as alternative delivery method for genetic transformation. Another difference is that we isolated embryos from mature seeds to develop seedlings instead of germinating the whole seed.

#### *3.3.2.1 Embryogenic callus initiation frequency*

SEC callus initiation frequencies of five inbred lines were assessed and are summarized in Table 2. Embryogenic callus initiation varied between genotypes. From five inbred lines tested H99 showed the higher frequency (179 out of 253 embryos responded, 70.8%) and the fastest growth, followed by W22 (6.02%), B73 (4.46%) and B104 (2.1%). Just one embryo (out of 403 embryos) developed embryogenic callus from Mo17 (0.25%). However this unique Mo17 line showed faster growth in subsequent subcultures. SEC could be initiated from inbred H99 around 21 days after placed on the induction medium, whereas it took twice amount of time (40 – 45 days) to initiate SEC from the other four inbred lines (Table 2). Our SEC induction frequency for H99 (70.8%. a total of 253 embryos tested) was higher when compared to what reported in Sidorov et al (2006) where 18.5% SEC induction (a total of 92 embryos tested) was achieved for the same genotype.

It was also observed that after consecutive subcultures, B73 and B104 callus had tendency to become degenerated and non-embryogenic. The SEC morphology of H99 was similar to that initiated from mature embryos reported by Sidorov et al (2006), whereas B104 SEC appeared to be similar to callus cultures obtained from immature embryos with the same genotype (Frame et al, 2006). Genotypes B73, Mo17 and W22 developed a similar pattern of

embryogenic type I callus (Figure 7) as reported for inbred line A188 (Armstrong and Green, 1985).

Because there is a well established protocol for callus development for hybrid Hi II genotype using immature embryos (Songstad et al, 1996; Pareddy et al, 1997; Zhao et al, 2001, Frame et al 2000 and 2002), we also tested SEC initiation from mature seed of this genotype. Hi II genotype developed well defined, friable, fast growing type II callus (Figure 7F). No callus induction frequency was evaluated for HiII genotype.

Embryogenic callus of all inbred lines were maintained and propagated until we create a stock to perform genetic transformation.

### 3.3.2.2 Genetic transformation

Genetic transformation was performed in all inbred SEC in two experiments, except for H99 in which we performed a total of 5 experiments. Transient *gus* expression was observed in all inbreds, with strong blue stains seen on W22 and H99 SEC (Figure 8).

After 10 weeks of selection on 5 mg/L Bialaphos containing medium, Bialaphos resistant callus pieces from H99 could be identified. However, no resistant calli were obtained from inbred B73, B104, Mo17 and W22. This could be due to two reasons. One was that we did not bombard enough callus materials (we only bombarded between 15 to 30 pieces callus each inbred due to slow culture growth) to generate transgenic materials, the other one could be due to the lower growth rate of these inbred callus culture. It has been reported that a fast growth rate is an important parameter for successful genetic transformation (O'Connor-Sanchez et al, 2002). Cell lines that show a higher mitotic index are more responsive for transformation (Hazel et al, 1998)

When compared to H99, the rest of the four genotypes showed a slower callus initiation rate and low frequency (Table 2). Inbred lines B73 and B104 are also highly recalcitrant, and degenerate into a non-embryogenic callus after consecutive subcultures. Mo17 and W22 did not degenerate after subculturing, but no transgenic callus was obtained. All these four inbred were reported to be transformable with low frequencies using immature embryos or immature embryos derived callus cultures. B73 was transformed with the biolistic gun with low frequency (0.7%, Wang et al., 2003). B104 was transformed using *Agrobacterium*-mediated method (Frame et al, 2006) at a frequency of 6.8%. Mo17 was

transformed using *Agrobacterium* method at frequency of 2.6% (Huang and Wei, 2005) and W22 could be transformed using the biolistic gun at frequency of 1% (B. Frame, personal communication).

Bialaphos resistant callus frequency (BRCF) of inbred line H99 is showed in Table 3. Here we use BRCF instead of transformation frequency (TF %) because we did not know whether all Bialaphos resistant calli were true transformants. BRCF was calculated as the number of independent resistant calli grown on 5 mg/L Bialaphos media, recovered after 10 weeks on selection and produced from the total number of calli bombarded (responding and non responding) (X 100). Ten Bialaphos resistant calli from experiment 1 and 7 resistant calli from experiment 2 were recovered, respectively. Three more experiments were conducted using H99 (Experiments 3, 4 and 5) (Table 3). Stable transformation recovered after eight weeks on selection was assessed. BRCF for these three experiments were measured based on stable *gus* gene expression showed by the embryogenic callus (Table 3, Figure 9).

After 10 weeks on selection media, transgenic callus pieces from each independent event of experiments 1 and 2 of H99 inbred line were transferred to regeneration media. Two Bialaphos resistant and GUS expressing callus of experiment 2 did not yield any plant in spite best effort in regeneration (regeneration was attempted twice with these events). It is possible that the insertion and/or location of the transgenes may affect the regeneration process. It is also possible that the bar gene may be non-functional in these transformants due to gene silencing or lost of fragment because Bialaphos was included in the regeneration medium.

Approximately 1.5 months after placing on regeneration media, regenerated plantlets were transferred to soil and sprayed with the herbicide for phenotypic selection (glufosinate resistance). All plantlets were resistant to 250 mg/L Liberty and transferred to big pots and grown in the greenhouse (Figure 10).

### 3.3.2.3 Transgenic plant analysis

Stable transformation in all experiments on inbred line H99 were assessed through the expression of the *gus* gene at different stages. Leaf samples from each independent event (T<sub>0</sub>) were assayed for *gus* gene expression (Figure 11A). Phenotypic expression of the *bar* gene

(after herbicide spraying) corresponded to the *gus* gene expression because all T<sub>0</sub> plantlets were herbicide resistant plants were also positive for the *gus* gene.

T<sub>0</sub> plants were grown to maturation in a greenhouse. All plants were female fertile, however different degrees of somaclonal variation in tassels (feminization) were observed. The different levels of somaclonal variation in the tassels were classified as normal, moderate variation and severe variation (Figure 12). From 86 plants representing 15 different Bialaphos resistant events, the overall frequency (%) of normal plants was 55.3%, of moderate variation 44.2% and severe variation 1.16%. Pollen collected from plants with moderate variation was fertile and used for pollination. Most of the plants were pollinated by non-transgenic Hi II pollen.

Transmission and segregation of the transgenes were analyzed in T<sub>1</sub> progeny. To accelerate progeny analysis, immature embryos were rescued from some events for in vitro germination. Table 4 summarized the transgenes expression and segregation ratio from plants progeny of 3 events (out of a total of 15 events) analyzed up to date. Analysis of  $\chi^2$  for the *bar* and *gus* genes in progeny of H99 indicates that the transgenes were inherited in nine events assayed and except for events 7 and 10, all segregated in the expected ratio (1:1 or 3:1) for single locus integration. It is not clear why the transgenes did not behave as expected on events 7 and 10. To verify, more seeds should be tested for transgene expression. Molecular genotyping (PCR, Southern) should also be used to verify whether the transgenes are present in the progeny. Such abnormal segregation pattern for transgene has been observed previously (Frame et al, 2006), in which out of 6 events for inbred line B104, two did not fit into the expected Mendelian ratio. Transgene silencing or other epigenetic suppression may be the reasons behind such phenomenon.

Phenotypes of all T<sub>1</sub> plants recovered are normal and fertile.

### 3.4 Conclusions

Using published protocols, we have tested two mature seed derived tissue culture systems, shoot meristem culture (SMC) and somatic embryogenic culture (SEC), in five maize inbred lines, B73, B104, H99, Mo17 and W22.

We have improved the SMC system by shortening the SMC initiation duration by 1-2 weeks. SMC's performance in five inbred lines was genotype-independent to certain extent

since regardless the genotype background, all inbred lines developed SMC in our system at different levels.

While no transgenic plants were recovered from SMC transformation experiments, the SMC tissue culture responses results were encouraging. More experiments and optimization should be carried out for further development of SMC as target tissue for genetic transformation.

We achieved genetic transformation on mature seed derived SEC for H99 using the biolistic gun method. The average Bialaphos resistant callus frequencies of 5 bombardment experiments were 50.7 %, ranging from 24.1% to 74%. Progeny analysis indicated that the transgenes were transmitted to next generation and segregated as Mendelian fashion. Further research is needed to increase SEC response and transformation on more inbred lines.

### **Acknowledgements**

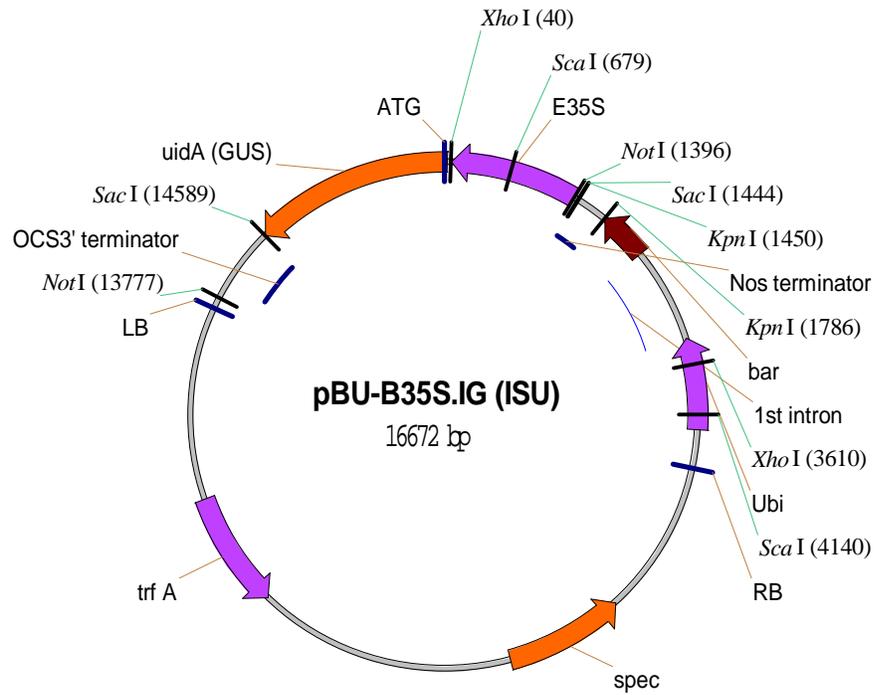
I'm very thankful to Bronwyn Frame, Dr. Kan Wang and Margie Paz for their scientific contribution on this work. Dr. Upadhyaya from CSIRO Plant industry for providing us with the plasmid used for this work. Jose Luis C, Francois Torney and Lorena Moeller for helpful scientific discussion, Tina Paque for providing material for pollination and Javier Garcia and Jessica Zimmer for technical assistance. This project was funded by the National Science Foundation Plant Genome Program (DBI 0110023).

### **References**

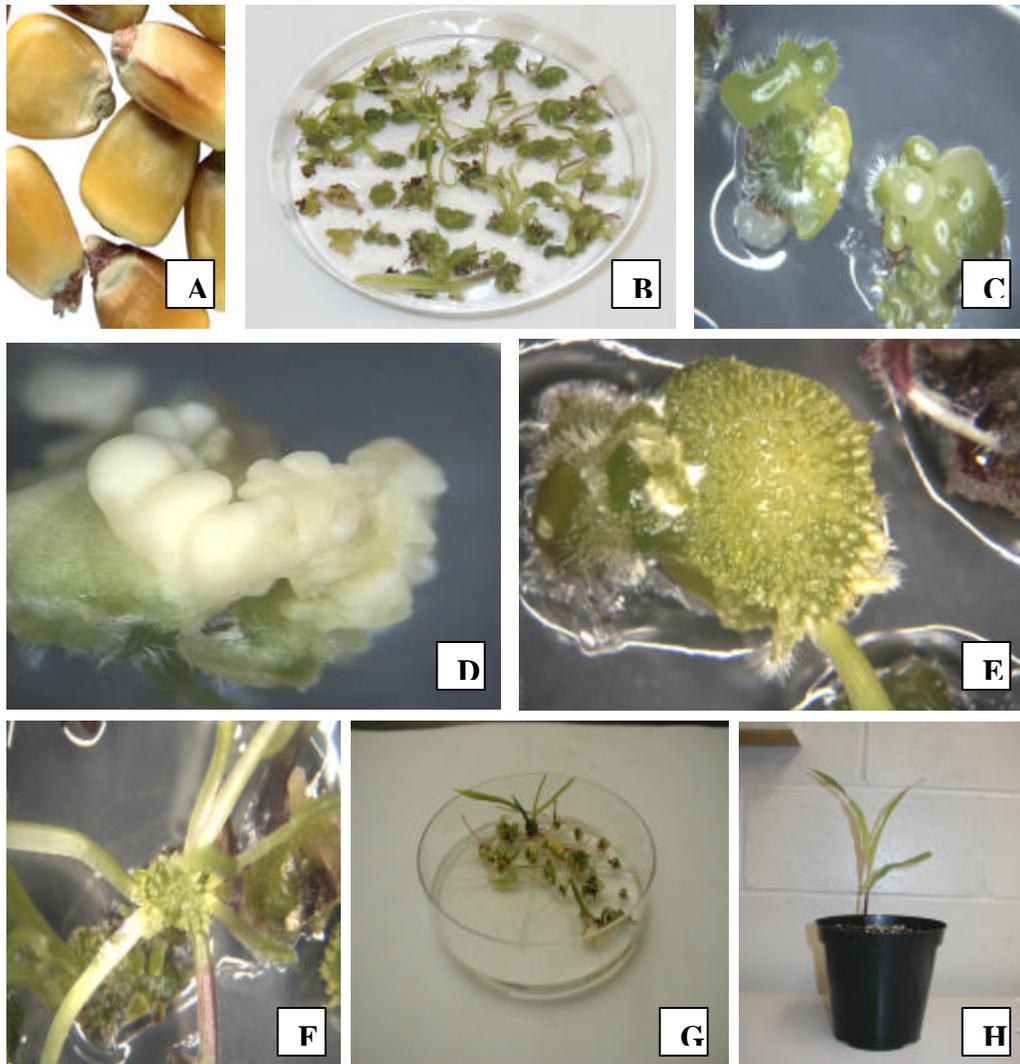
- Armstrong, C.L., Green, C.E. 1985. Establishment and maintenance of friable, embryogenic maize callus and the involvement of l-proline. *Planta* 164:207–214.
- Brettschneider R, Becker D, Lorz H. (1997). Efficient transformation of scutellar tissue of immature maize embryos. *Theor Appl Genet* 94:737–748.
- Frame, B.R., MacMurray, J.M., Fonger, T.M., Main, M.L., Taylor, K.W., Torney, F.J., Paz, M.M., Wang, K. 2006. Improved *Agrobacterium*-mediated transformation of three maize inbred lines using MS salts. *Plant Cell Rep* 25:10024-1034.
- Frame, B.R., Shou, H., Chikwamba, R.K., Zhang, Z., Xiang, C., Fonger, T.M., Pegg, S.E., Li, B., Nettleton, D., Pei, D., Wang, K. (2002). *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol* 129:13–22
- Frame, B.R., Zhang, H., Cocciolone, S.M., Sidorenko, L.V., Dietrich, C.R., Pegg, S.E., Zhen, S., Schnable, P.S. and Wang, K. 2000. Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cell Dev Biol Plant* 36:21-29.

- Gordon-Kamm, W.J. Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, W.R., Jr., Willets, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P., Lemaux, P.G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* 2:603-618.
- Hazel, C.B., Klein, T.M., Anis, M., Wilde, H.D., Parrot, W.A. 1998. Growth characteristics and transformability of soybean embryogenic cultures. *Plant Cell Rep* 17:765-772
- Huang, X., Wei, Z. 2005. Successful *Agrobacterium*-mediated genetic transformation of maize elite inbred lines. *Plant Cell Tiss Org* 83:187-200.
- Jefferson RA. 1987 Assaying chimeric genes in plants. The *gus* gene fusion system. *Plant Mol Biol Rep* 5:287-405.
- Lee, M., Phillips, R.L. 1987. Genomic rearrangements in maize induced by tissue culture. *Genome* 29:122-128.
- O'Connor-Sanchez, A., Cabrera-Ponce, J.I., Valdez-Melara, m., Tellez-Rodriguez, P., Pons-Hernandez, J.L., Herrera-Estrella, L. 2002. Transgenic maize plants of tropical and subtropical genotypes obtained from calluses containing organogenic and embryogenic-like structures derived from shoot tips. *Plant Cell Rep* 21:302-312.
- Pareddy, D., Petolino, J., Skokut, T., Hopkins, N., Miller, M., Welter, M., Smih, K., Clayton, D., Pescitelli, S., Gould, A. 1997. Maize transformation via helium blasting. *Maydica* 42:143-153.
- Paz, M.M., Martinez, J.C., Kalvig, A.B., Fonger, T.M., Wang, K. 2006. Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. *Plant Cell Rep* 25:206-213.
- Sidorov, V., Gilbertson, L., Addae, P., Duncan, D. 2006. *Agrobacterium*-mediated transformation of seedling-derived maize callus. *Plant Cell Rep* 25:320-328.
- Songstad, DD, Armstrong, CL, Petersen, WL, Hairston, B, Hinchee, M.A.W. 1996. Production of transgenic maize plants and progeny by bombardment of Hi II immature embryos. *In Vitro Cell Dev Biol-Plant* 32:179-183.
- Sticklen, M.B., Oraby, H.F. 2005. Shoot apical meristem: a sustainable explant for genetic transformation of cereal crops. *In Vitro Cell Dev Biol-Plant* 41, 187-200.
- Torney, F., Frame, B.R., Wang, K. 2007. Maize. *In* E.C Pua and M.R. Davey (Eds), *Transgenic crops IV*. Springer, Berlin Heidelberg, pp. 73-105.
- Vain, P., McMullen.M.D., Finer, J.J. 1993. Osmotic treatments enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Rep*. 12:84-88.
- Wang, K., Frame, B.R., Marcell, L. 2003. Maize genetic transformation. *In* P. K. Jaiwal and R.P. Singh (Eds). *Plant Genetic Engineering: Improvement of Food Crops*. Sci-Tech publication. Houston, Texas, USA. Pp. 175-217.
- Wilson, H.M., Bullock, W.P., Dunwell, J.M., Ellis, J.R., Frame, B., Register, III, J.C., Thompson, J.A., Maize, *In*: Wang, K., Herrera-Estrella, A. Van Montagu, M., eds. 1995. Transformation of plants and soil microorganisms. Cambridge: Cambridge University Press. p.p. 65-80.
- Zhang, S., Williams-Carrier, R., Lemaux, P.G., 2002. Transformation of recalcitrant maize elite inbreds using in vitro shoot meristematic cultures induced from germinating seedlings. *Plant Cell Rep* 21:263-270.

- Zhang, Y., Yin, X., Yang, A., Li, G., Zhang, J. 2005. Stability of inheritance of transgenes in maize (*Zea mays* L.) lines produced using different transformation methods. *Euphytica* 144:11-22.
- Zhao, ZY, Gu, W., Cai T., Tagliani, L., Hondred, D., Bond, D., Shroeder, S., Rudert, M., Pierce, D. 2001. High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. *Mol Breed* 8:323–333.
- Zhong, H., Sun, B., Warkentin, D., Zhang, S., Wu, R., Sticklen, M.B. 1996. The competence of maize shoots meristems for integrative transformation and inherited expression of transgenes. *Plant Physiol* 110:1097-1107.



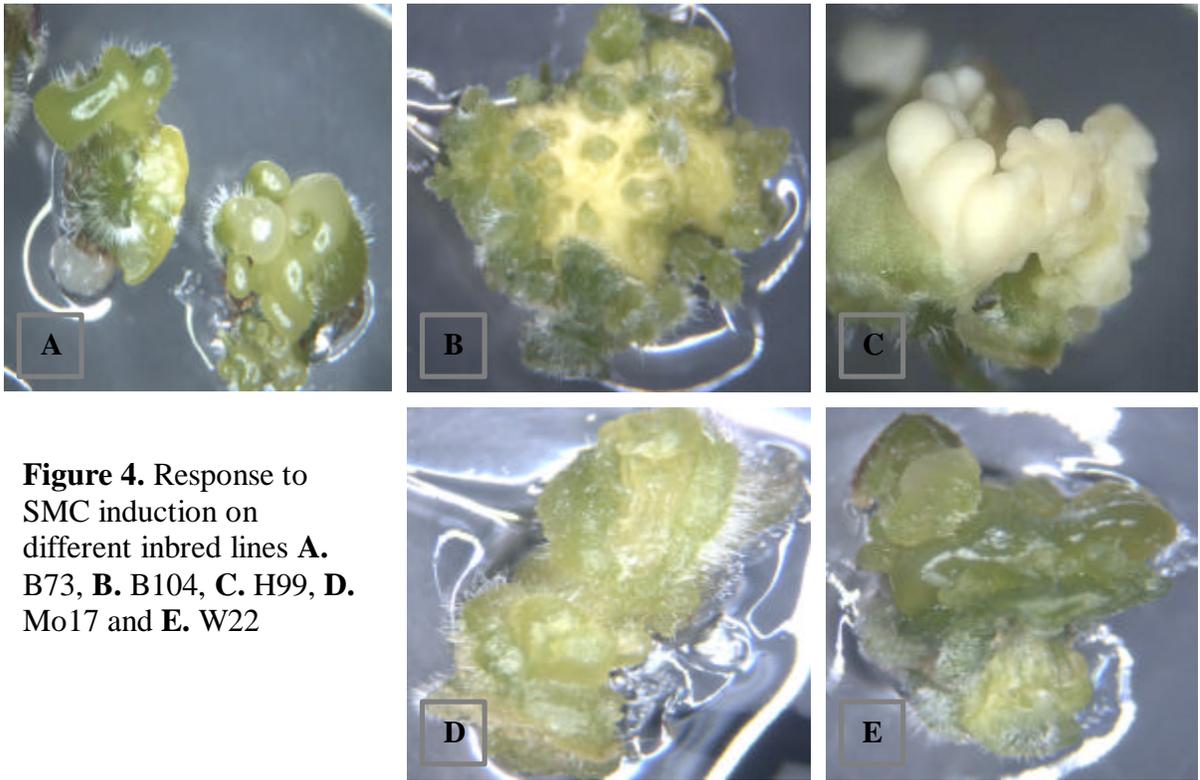
**Figure 1.** Plasmid pBU-B35S.IG used for genetic transformation with the screenable marker gene CaMV 35S-gus and the selectable marker CaMV 35S-bar



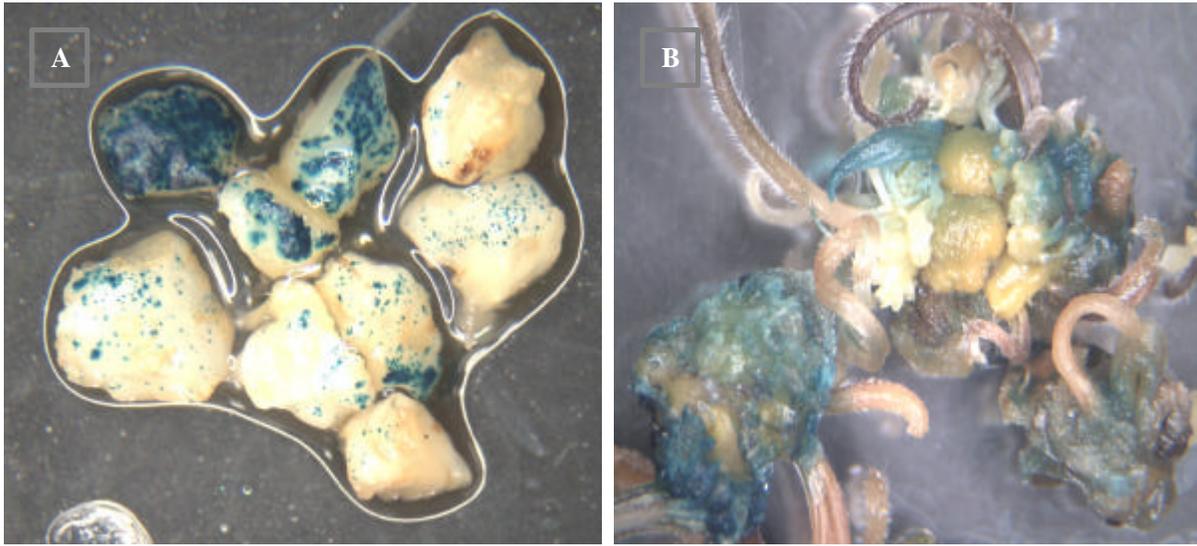
**Figure 2.** Tissue culture system for SMC's. **A.** Mature seeds. **B.** Embryos developing SMC. **C.** SMC development after 30 days. **D.** Embryogenic Tissue from genotype H99 **E.** SMC of B73 and H99, each small shoot can develop a plant or new clumps of SMC. Hereby called "hedgehog" stage **F.** Regeneration through either somatic embryogenesis or SMC **G.** Regenerated plants **H.** Plants in greenhouse



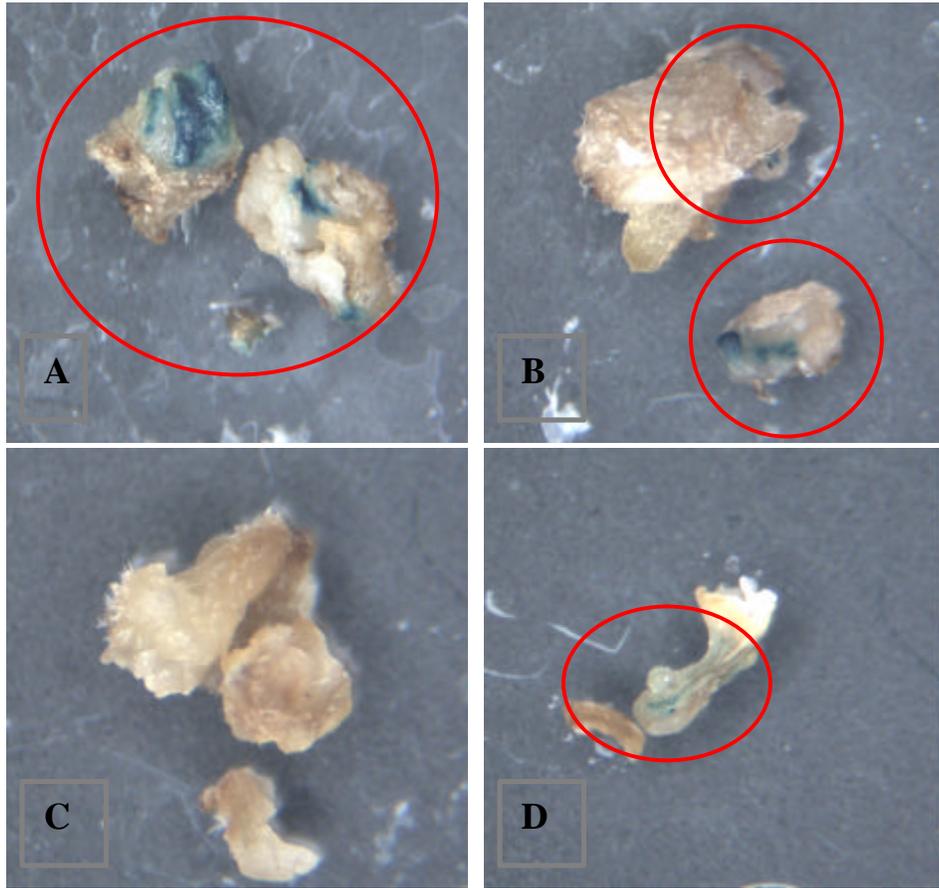
**Figure 3.** Somaclonal variation in B73 inbred line after regeneration from SMC in non-transformed plants



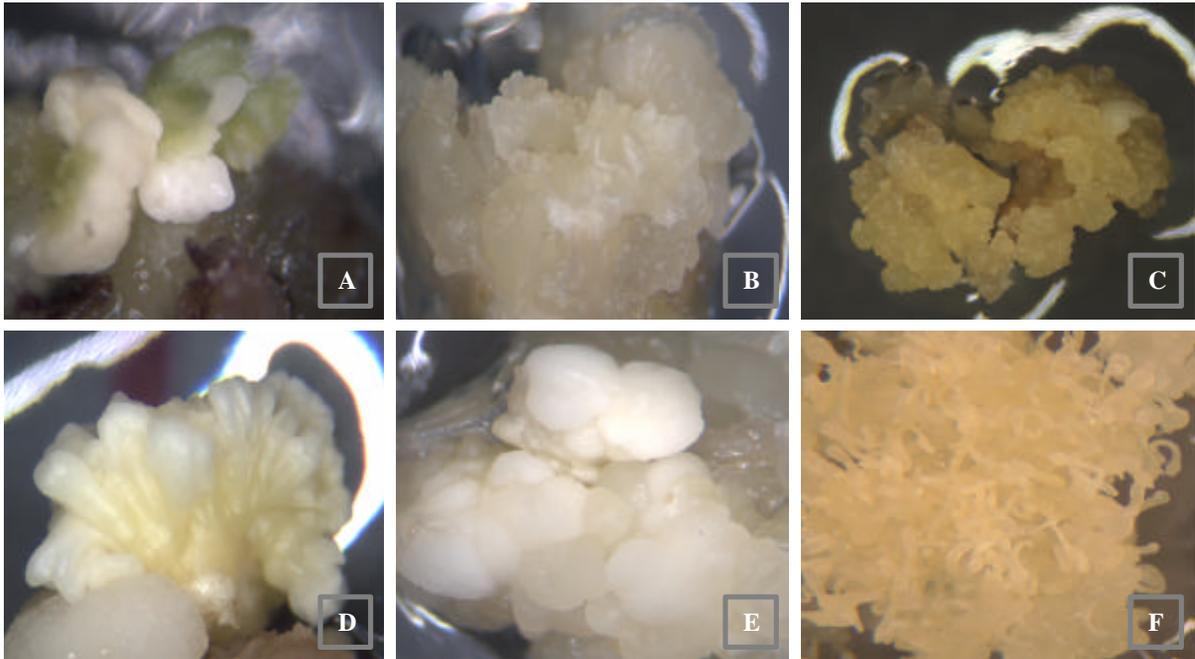
**Figure 4.** Response to SMC induction on different inbred lines **A.** B73, **B.** B104, **C.** H99, **D.** Mo17 and **E.** W22



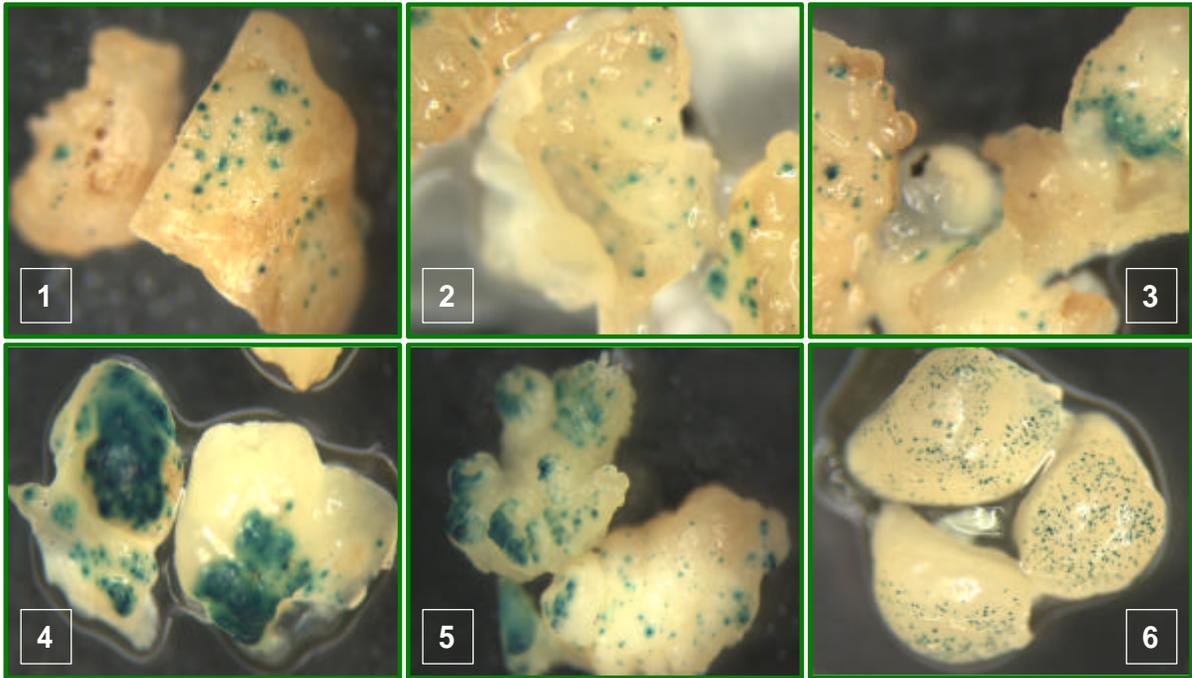
**Figure 5.** GUS gene expression for bombardment experiments. **A.** Transient GUS expression of B73 inbred line after 2 days SMC bombardment, **B.** Stable GUS expression after 14 months on selection



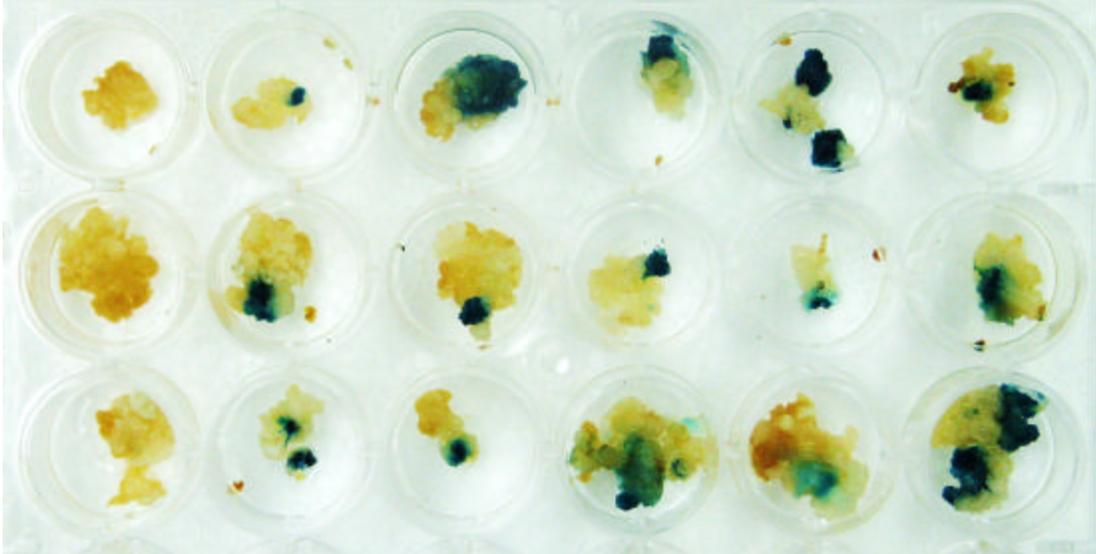
**Figure 6.** Transient GUS expression in SMC's after 3 days infection with *Agrobacterium*. **A.** B73, **B.** B104, **C.** Mo17 (No transient expression), **D.** W22



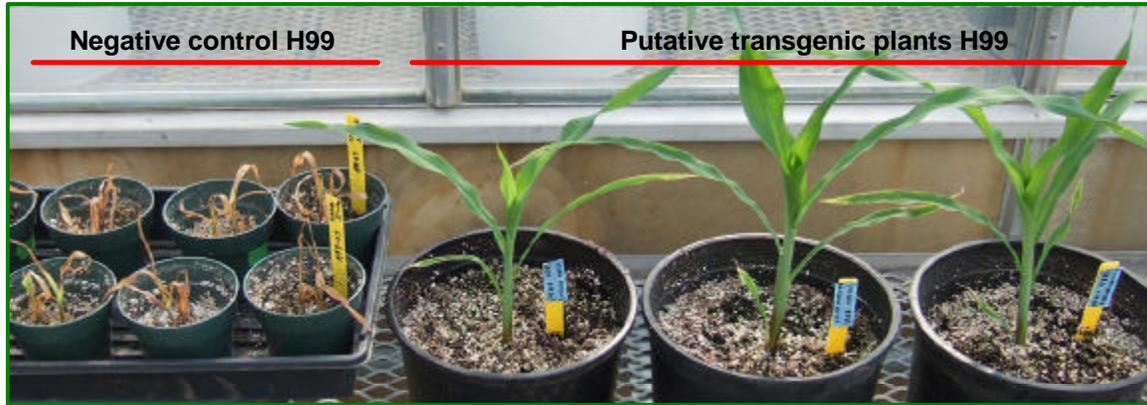
**Figure 7.** Morphology of embryogenic callus initiated from mature seeds of different inbred lines and hybrid Hi II. **A.** B73, **B.** B104, **C.** H99, **D.** Mo17 (non embryogenic), **E.** W22, **F.** Hi II (Type II callus)



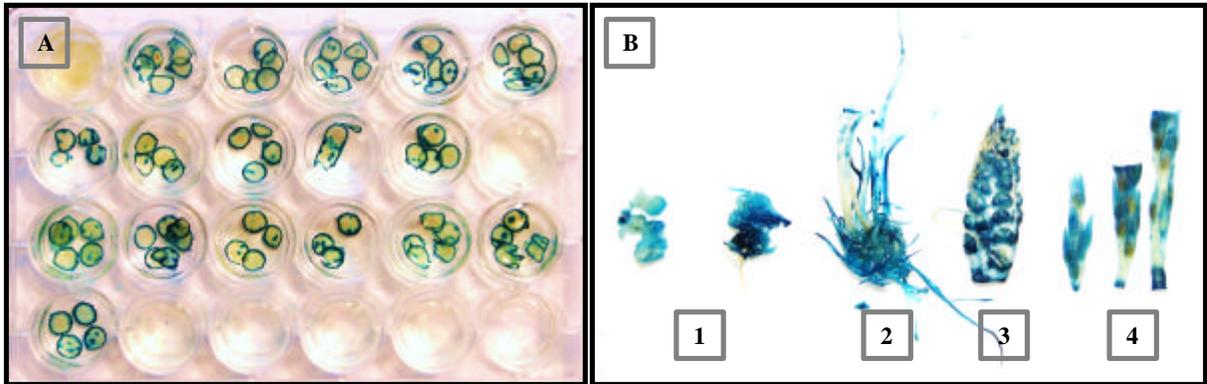
**Figure 8.** Transient GUS expression of embryogenic bombarded callus of different inbred lines. **1.** B73, **2.** B104, **3.** Mo17, **4.** W22, **5.** H99 and **6.** Positive control Hi II zygotic embryos bombarded with the same construct



**Figure 9.** Stable *gus* gene expression on embryogenic callus of H99 inbred line after 8 weeks selection for biolistic experiments number 4 (top row), 5 (middle row) and 6 (bottom row). The first well on the left is the negative control.



**Figure 10.** Phenotypic expression of the *bar* gene after herbicide spraying. All negative control plants died (left). All putative transgenic plants (T0) were resistant (right).



**Figure 11.** Stable *gus* gene expression for T0 plants of inbred line H99. **A.** Gus gene expression on leaf sectors (non-stained sectors are negative controls) for the first two experiments on H99. First well is negative control, followed by the 10 events recovered from experiment 1 and the 7 recovered from experiment 2. **B.** Stable *gus* expression on different plant tissue: **B1.** Embryogenic callus, **B2.** Plantlets, **B3.** Immature ear, **B4.** Tassels



**Figure 12.** Levels of somaclonal variation in tassels of T0 plants for the inbred line H99. **A.** Normal tassel, **B.** Moderate variation, **C.** Severe variation (female fertile)

<b>Genotype</b>	<b>Total # of explants infected</b>	<b>Transient GUS expression (No.positive/No. explants assayed)</b>
<b>B73</b>	583	4/58
<b>B104</b>	80	2/10
<b>Mo17</b>	32	0/3
<b>W22</b>	24	1/3

The total numbers of B73 include 4 different experiments

**Table 1.** Transient expression after transformation of different inbred maize genotypes using *Agrobacterium*-mediated and SMCs

<b>Genotype</b>	<b># embryos producing callus/ total # embryos cultured (%)</b>	<b># days to first appearance of callus**</b>
B73	23/516 4.46%	45
B104	11/523 2.10%	45
H99	179/253 70.80%	21
Mo17	1/403 0.25%	42
W22	31/515 6.02%	40

\* The frequency was calculated after callus initiation for each inbred line. Numbers were combined from different experiments

\*\* Including the germination period

**Table 2.** Callus initiation frequency for different inbred lines\*

Genotype	Experiment	# callus pieces bombarded	GUS expression analysis (No. positive/No. explants assayed)		Bialaphos resistant callus frequency (BRCF)	
			Transient	Stable	# recovered	%
B73	1	21	3/3	0	0	0
	2	15	2/2	0	0	0
B104	1	26	3/3	0	0	0
	2	22	2/2	0	0	0
H99	1	25	3/3	0	10	40
	2	29	3/3	0	7	24.14
	3	76	9/9	39/70	N/A	55.7
	4	74	9/9	36/60	N/A	60
	5	75	9/9	43/58	N/A	74
Mo17	1	29	4/4	0	0	0
	2	29	3/3	0	0	0
W22	1	28	3/3	0	0	0
	2	30	3/3	0	0	0

**Table 3.** Bialaphos resistant callus frequency (BRCF) of bombarded embryogenic callus of different inbred lines. BRCF was calculated as the number of independent resistant calli grown on 5 mg/L Bialaphos media, recovered after 10 weeks on selection and produced from the total number of calli bombarded (responding and non responding) (X 100)

Event #	Line	Segregation ratio				
		Herbicide			GUS	
		Res <sup>a</sup>	Sen <sup>b</sup>	$\chi^2$	Pos <sup>c</sup>	Neg <sup>d</sup>
8*	7	23	12	3.44	17	12
9*	7	1	2	0.33	1	8
5*	18	4	4	0	12	12
6*	18	1	5	2.66	1	5
7*	18	1	12	10.28	1	12
10*	18	3	11	4.57	3	11
11*	18	11	13	0.16	11	13
14**	18	6	3	0.47	6	3
19*	18	6	5	0.09	6	5

Transgenic plants were crossed as the female with pollen of non-transformed plants(\*) or self crossed (\*\*)

<sup>a</sup>Res, resistant to glufosinate spray (*bar*-expresser)

<sup>b</sup>Sen, sensitive to glufosinate spray (*bar* non-expresser)

<sup>c</sup>Pos, GUS assay positive (*gus*-expresser)

<sup>d</sup>Neg, GUS assay negative (*gus* non-expresser)

$\chi^2 = 3.8$  (0.05, 1df)

**Table 4.** Segregation analysis for *bar* and *gus* gene expression on T1 progeny plants. Two events of line 7 and 7 from line 18 were analyzed. Only two events (7 and 10 from line 18) did not fit the expected Mendelian ratio

## CHAPTER 4. GENERAL CONCLUSIONS

This work explored the possibility of performing genetic transformation using maize mature seeds as starting material in different elite inbred lines using two different approaches: Shoot meristem cultures (SMCs) and Somatic embryogenic callus (SEC) through *Agrobacterium*-mediated and the biolistic gun methods. This work also aimed to the development of a sterilization method able to provide sterile material to initiate either tissue culture system without losing material due to high contamination rates and also without compromising seedling vigor and SMC or SEC initiation.

In chapter 2, we developed an efficient and reliable optimized method for mature seeds sterilization. This method was especially developed for material coming from field-grown plants and has a high efficiency to provide with sterile, material without compromising the vigor. In this chapter it was observed that inbred line H99 has a higher potential to be transformed since it presented the higher vigor, expressed as the seedling growth rate (SGR) and callus induction rate, which are desirable traits when performing genetic transformation for plants. In general vigor and callus induction of the different inbred lines used for this work showed a genotype dependency. Seedling vigor is also positively correlated to callus initiation.

In chapter 3, we explored two different approaches to generate a tissue culture system for maize mature seeds and inbred lines, suitable to perform genetic transformation with the two most widely used methods for genetic transformation: *Agrobacterium*-mediated and particle bombardment. We demonstrated, based on published protocols that SMCs can be obtained for elite inbred lines B73, B104, H99, Mo17 and W22. We also observed that the performance of these five inbred lines was genotype-independent to a certain extent with this system, since regardless the genotype background, all inbred lines were able to develop SMC at different levels. We also improved the SMC system by shortening the time of initiation of this type of culture (by 1-2 weeks) compared to the original protocol already published. SEC was also explored and it was observed that all five inbred lines behave differently with this system, meaning that this response in tissue culture is genotype dependent and that inbred line H99 was the most responsive. Compared to the already published system, we improved the rate of callus initiation by 4 fold for this inbred line. We also successfully transformed

this inbred line but only using the biolistic gun with an average Bialaphos resistant callus frequency (BRCF) ranging from 24.1 % to 74 %. Analysis of progeny was also conducted and most of the plants segregated and transmitted the transgenes to the next generation in Mendelian fashion. No plants were recovered for the rest of inbred lines (B73, B104, Mo17 and W22).

For both systems, SMCs and SEC, more research is needed to explore further genetic transformation using SMC in these and other inbred lines and also improvement is needed to enhance the SEC system to increase the callus induction frequency of different inbred lines to ultimately expand the possibilities of successful genetic transformation.

Appendix 1 shows the optimized protocol developed to sterilize maize mature seeds harvested from field-grown plants. The protocol and the supplies needed are described in detail.

Appendix 2 is a manuscript on soybean transformation in which I made contributions. This work is an improvement of the soybean cotyledonary node transformation system by using half mature seeds through *Agrobacterium*-mediated transformation.

The work presented in this thesis led to the attainment of a seed sterilization system for mature seeds with high contents of seed-borne pathogens which can be used for all types of corn regardless the source from which they are coming from. We also demonstrated the induction of SMC and SEC in different inbred lines that can be used for genetic transformation. Finally based on SEC system and biolistic gun, we stably transformed the inbred line H99.

**APPENDIX I****OPTIMIZED SEED STERILIZATION AND EMBRYO DISSECTION FOR  
FIELD-HARVESTED SEEDS****MATERIALS**

- ✍ Fifty seeds of each maize inbred line (genotype)
- ✍ One 150 ml beaker per genotype
- ✍ One stir bar per beaker
- ✍ One stirrer plate per beaker
- ✍ Ethanol 80 % (300 ml) enough for 200 seeds

Ethanol 100%	240 ml
Millipore water	60 ml

- ✍ Bleach 50 % v/v (900ml) enough for 200 seeds

Commercial bleach	450 ml
Millipore water	450 ml
Tween-20	2 drops

- ✍ One 60X20 Petri dish per genotype
- ✍ Sterile Millipore water
- ✍ One forceps per genotype
- ✍ One scalpel per genotype
- ✍ Two or more home made strainers (see picture)
- ✍ Bleach 15 % (v/v) (100 ml) enough for 200 dissected embryos

Commercial bleach	15 ml
Millipore sterile water	85 ml
Tween-20	1 drop

- ✍ Aluminum foil
- ✍ Marker
- ✍ Shaker plate
- ✍ One parafilm strip per Petri dish
- ✍ Paper towels
- ✍ Half of one 100X150 Petri dish for embryo dissection per genotype
- ✍ Stereoscope
- ✍ Ethanol 70 % in a spray bottle
- ✍ Big container for liquid waste
- ✍ Medium size container for liquid waste
- ✍ Timer
- ✍ Micropore tape

## METHODS

### **First seed sterilization**

1. Before starting make sure to turn on the oven-sterilizer placed into the flow bench at least half an hour in advance. Put the forceps inside and keep them there.
2. Place 50 seeds of each genotype in different beakers along with a stir bar and cover it with a piece of aluminum foil, label the surface of the aluminum foil as well as on the wall of the beaker with the name of genotype, year of harvesting and the date.
3. Add ~ 75 ml of ethanol 80 % to each beaker, cover with the aluminum foil and take them (with the tray) to a stirrer plate, stir under medium speed for 3 minutes. (Use a timer to take all the periods of time)
4. Take the beakers back to the flow bench and pour the ethanol into a liquid-waste container, use the sterile forceps to retain seeds and avoid throwing away any to the waste container, do this any time you pour away liquid waste and re-sterilize the forceps
5. Add the same amount (as above) of 50 % bleach+tween, cover with the aluminum foil and take the beakers again to the stirrer plate and stir for 15 minutes on medium speed
6. Take beakers back to the flow bench and pour the bleach in the liquid-waste container,

repeat step 5

7. Take the beakers to the flow bench and pour the bleach away
8. Rinse 5 times with sterile Millipore water
9. Keep seeds in sterile water, cover with aluminum foil and keep the beakers inside the flow bench for 24 hours (seeds also can be soften by keeping them in moisturized paper towels for 24 hours)

#### **Second seed sterilization**

10. After 24 hours, sterilize seeds again. Add 50 % bleach + tween to the beakers and cover with the aluminum foil and take the beakers again to the stirrer plate and stir for 2 minutes on medium speed. Repeat steps 7 and 8.

#### **Embryo dissection**

11. Clean the work space in the flow bench you will use for the dissection spraying ethanol 70% and drying up with paper towels. Put the forceps and scalpels without blades into the hot sterilizer at least 5 minutes, take them out and let them cool, once they are cool enough set a new blade in each scalpel.
12. Set everything aside before starting dissection, put the scope on the flow bench and clean it with 70 % ethanol as well. Prepare 4, 60X20 Petri dishes and label them on both lids, pour around 10 ml of Millipore sterile water into each. Take two whole 100X150 plates and place them into the flow bench. Have enough MSVS34 media (germination) to plate the embryos after the last sterilization (~20 plates).
13. Take half of one 100X150 plate and place it on the platform of the stereoscope, turn the light on. Take one of the small plates with water next to the stereoscope and open it
14. Start with the first genotype, take the stir bar out of the beaker with sterile forceps and leave it in a paper towel, make sure you do not touch the paper towel with the forceps. Take seed by seed from the beaker to the half plate with forceps, count 25 or as you wish.
15. Start dissecting with forceps and scalpel taking the seed from the middle and narrowest part, cut the flanks away, cut the base of the seed and two thirds of the root (tip), then gently take the embryo out using the scalpel and place it in the small plate with water.

16. Repeat step 15 until you dissect all the seeds. Place the rest for dissection in the same half of the big plate. Once you are done, trash the plate with the garbage (rests of the kernels) and close the small Petri dish with the embryos, reserve.
17. Repeat steps 13 to 16 with the remainder genotypes.

### **Embryo sterilization**

18. Put the hand-made strainers into the oven-sterilizer, after ~3 minutes take them out and get them cool. Take the small Petri dishes with the embryos and place them on a large paper towel (do everything inside the flow bench) open the plates and pour away the water into the medium size waste-container using the strainer to retain the embryos, repeat this with all the genotypes. Re-sterilize the strainers every time you change genotype by spraying ethanol 70% on the mesh and putting those 30 seconds into the hot oven-sterilizer (the re-sterilization has to be made every time the strainers are used).
19. Add ~ 15 ml of 15% bleach to each Petri plate for 5 minutes, start the timer, cover with the lid and wrap tightly with one strip of parafilm around the plate (to avoid contamination, assuming that the shaker is out of the flow bench). Take the plates to the shaker and shake on medium-low speed for the rest of the time.
20. Take the plates back to the flow bench, unwrap and pour the bleach away (remember to re-sterilize the strainer each time you pour a liquid).
21. Rinse the embryos 4 times with Millipore sterile water.
22. Pour away the water of the last rinse, reserve the embryos. Re-sterilize the forceps for around 1 minute, let them cool.
23. Place ten embryos per plate of MSVS34 media using a different forceps for each genotype. The root-side has to be in contact with the media but not deeply.
24. Seal the plates with micropore tape and incubate embryos for germination at 27 C, for 7 to 10 days
25. Check the plates after 2, 3 and 4 days for possible contamination, if one embryo seems to be contaminated, move out the rest of the embryos to a fresh plate. Do this OUTSIDE the flow bench (the sink or a place where air blows are not present is recommended).

## APPENDIX II

Plant Cell Rep (2006) 25: 206–213  
 DOI 10.1007/s00299-005-0048-7

## GENETIC TRANSFORMATION AND HYBRIDIZATION

Margie M. Paz · Juan Carlos Martinez ·  
 Andrea B. Kalvig · Tina M. Fonger · Kan Wang

## Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation

Received: 17 May 2005 / Revised: 18 July 2005 / Accepted: 22 July 2005 / Published online: 25 October 2005  
 © Springer-Verlag 2005

**Abstract** The utility of transformation for soybean improvement requires an efficient system for production of stable transgenic lines. We describe here an improved cotyledonary node method using an alternative explant for *Agrobacterium tumefaciens*-mediated soybean transformation. We use the term “half-seed” to refer to this alternative cotyledonary explant that is derived from mature seed of soybean following an overnight imbibition and to distinguish it from cotyledonary node derived from 5–7-day-old seedlings. Transformation efficiencies using half-seed explants ranged between 1.4 and 8.7% with an overall efficiency of 3.8% based on the number of transformed events that have been confirmed in the  $T_1$  generation by phenotypic assay using the herbicide Liberty® (active ingredient glufosinate) and by Southern analysis. This efficiency is 1.5-fold higher than the cotyledonary node method used in our laboratory. Significantly, the half-seed system is simple and does not require deliberate wounding of explants, which is a critical and technically demanding step in the cotyledonary node method.

**Keywords** *Agrobacterium tumefaciens* · Cotyledonary node · Glufosinate · Half-seed · Soybean · Transformation

### Introduction

The utilization of genetic transformation techniques to introduce useful or novel gene(s) into soybean [*Glycine max* (L.) Merr.] requires an efficient method of transgene integration and regeneration of transformed plants. There are two modes of DNA delivery that are currently

utilized by most researchers to transform soybean. One method utilizes particle bombardment of embryogenic tissue with DNA-coated carrier particles of inert materials (Hadi et al. 1996; Santarem and Finer 1999; Droste et al. 2002). This technique often requires a prolonged tissue culture period to prepare target tissue. The other method involves *Agrobacterium*-mediated transformation of plant tissue such as embryonic axis, immature cotyledons or cotyledonary tissue from germinated seedlings (Hinchee et al. 1988; Parrott et al. 1989; Somers et al. 2003; Paz et al. 2004). Parrott et al. (1989) used immature seeds to obtain cotyledonary tissues that were macerated on nylon or steel mesh, infected with *Agrobacterium* and placed on culture medium to generate somatic embryos. Earlier Hinchee et al. (1988) reported *Agrobacterium* infection of the cotyledonary node (CN) area to produce transgenic soybean. Improvements to this CN protocol have been actively pursued to increase efficiency (Clemente et al. 2000; Olhoft et al. 2001; Olhoft et al. 2003; Liu et al. 2004; Paz et al. 2004; Zeng et al. 2004). The CN system involves wounding of explants derived from 5–7-day-old seedlings by making accurate incisions on the adaxial side using a surgical blade. This wounding procedure requires precise cutting of the explant prior to infection. Discrepancies in transformation efficiency via the *Agrobacterium* method have been partially attributed to non-reproducibility of CN wounding procedures among operators (M. Paz, unpublished). Other soybean transformation approaches involving some forms of deliberate wounding on the explant have also been explored resulting in different transformation efficiencies. For example, sonication was used to assist *Agrobacterium*-mediated transformation (SAAT) of both CN explants (Meurer et al. 1998) and immature cotyledons (Santarem et al. 1998; Finer and Finer 2000). Biotic treatment was used to wound embryogenic tissue derived from cotyledonary explants followed by *Agrobacterium* inoculation of the tissue (Droste et al. 2000). Explant wounding of excised embryonic axes from immature seeds using a multi-needle wounding prong (Ko et al. 2003) and cotyledons using forceps (Yan et al. 2000) have also been reported. Although some of these wounding treatments

Communicated by J. C. Register

M. M. Paz · J. C. Martinez · A. B. Kalvig · T. M. Fonger ·  
 K. Wang (✉)  
 Department of Agronomy, Iowa State University,  
 Ames, IA 50011-1010  
 e-mail: kanwang@iastate.edu  
 Tel.: +515-294-4429  
 Fax: +515-294-2299

resulted in enhanced transient expression of marker genes, they did not imply the improvement of stable transgenic plant recovery, which has been corroborated using other plant systems (Wroblewski et al. 2005).

In this study we describe an alternative cotyledonary explant derived from mature soybean seed for *Agrobacterium*-mediated transformation. We use the term "half-seed" to refer to cotyledonary tissue obtained from mature seed after overnight imbibition to distinguish it from CN explants derived from 5–7-day-old seedlings. In contrast to the CN technique that requires making precise cuts on the explant for effective transformation and regeneration, the half-seed approach does not involve deliberate manual wounding. The objective of this study was to develop an efficient soybean transformation method using the half-seed explant as a target tissue and demonstrate its efficacy in producing transgenic plants.

## Materials and methods

### Seed materials

Mature soybean seeds of cultivar Thorne, Williams, Williams79 and Williams82, were utilized in *Agrobacterium*-mediated transformation experiments. Soybean seeds were surface sterilized for 16 h using chlorine gas produced by mixing 3.5 ml of 12 N HCl and 100 ml commercial bleach (5.25% sodium hypochlorite, Di et al. 1996) in a tightly sealed desiccator.

### Transformation vector and *Agrobacterium* strain

The vectors pTF102 (Frame et al. 2002) and pTF101.1 (Paz et al. 2004) were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986). pTF102 was derived from base vector pTF101.1 by inserting a *Hind* III fragment containing a *gus*-intron cassette driven by a CaMV 35S promoter (Vancanneyt et al. 1990) into pTF101.1. The *gus*-intron cassette prevents background GUS activity derived from contaminating *Agrobacterium* in plant tissue culture. pTF101.1 has the following key elements: (1) double CaMV 35S promoter (Odell et al. 1985); (2) tobacco etch virus translational enhancer (Carrington and Freed 1990) at the 5' end of the *bar* gene; (3) soybean vegetative storage protein terminator (Mason et al. 1993) cloned to the 3' end of the *bar* gene; (4) a selectable marker gene (*bar* gene for resistance to herbicide phosphinothricin, White et al. 1990) for selection during plant transformation; and (5) a multiple cloning site (MCS) to facilitate subcloning of a gene of interest (GOI) between the T-DNA right border region and the *bar* gene. The base vector pTF101.1 is a derivative of the pPZP binary vector (Hajdukiewicz et al. 1994) and contains a selectable marker gene (*aadA* gene for resistance to antibiotics spectinomycin and streptomycin) for bacterium, origin of replication for *E. coli*, and right and left border fragments of *A. tumefaciens* T-DNA. Constructs ST42 and ST43 were derived from vector pTF101.1 by inserting a GOI in the MCS.

### *Agrobacterium* culture and infection medium

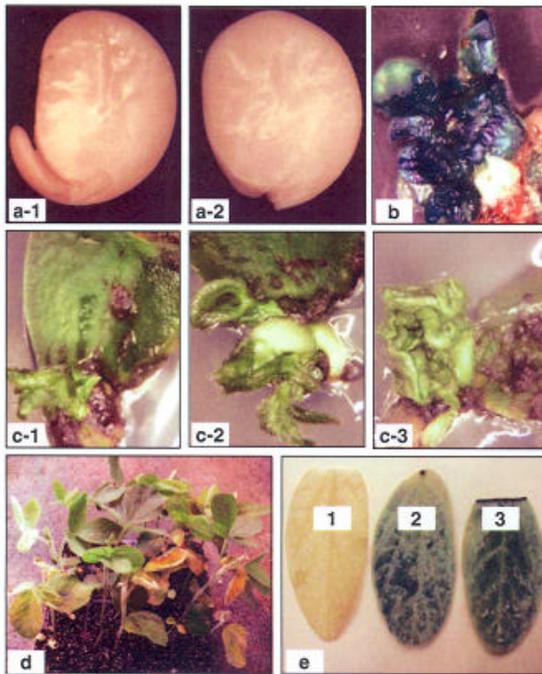
Construct EHA101(pTF102, pTF101.1, ST42 or ST43) was grown on YEP (An et al. 1988) containing 50 mg l<sup>-1</sup> kanamycin, 100 mg l<sup>-1</sup> spectinomycin and 25 mg l<sup>-1</sup> chloramphenicol at 28°C for 2 days. Single colonies of *Agrobacterium* were obtained from the plate and inoculated into 2-ml liquid YEP containing antibiotics (starter culture) for 8 h at 28°C, 250 rpm. Subsequently, 300 µl of the 2-ml starter culture was transferred to a 250-ml YEP culture, and grown overnight to OD<sub>650</sub> = 0.7 to 1.0 at 28°C, 250 rpm using a shaker incubator. On the day of infection, a bacterial pellet was obtained by spinning the overnight culture at 3500 rpm for 10 min and resuspended in infection medium containing 1/10 Gamborg's B5 medium (Gamborg et al. 1968) with 7.5 µM 6-benzylaminopurine (BAP), 0.7 µM gibberellic acid (GA<sub>3</sub>), 20 mM MES [2-(*N*-morpholino)ethanesulfonic acid], 3% sucrose and 200 µM acetosyringone, pH 5.4. Bacteria cell density was adjusted to OD<sub>650</sub> = 0.7 to 0.8 using a spectrophotometer before infection of half-seed explants.

### Explant preparation and infection

Disinfected seeds were soaked in sterile distilled water overnight for about 16 h (100 seeds in a 100×25 mm Petri dish). A longitudinal cut along the hilum was made to separate the cotyledons, and the seed coat was removed. The embryonic axis found at the junctions of the hypocotyl and the cotyledon was excised to obtain the half-seed explant (Fig. 1, panel a-1 & a-2). About 60–100 half-seed explants were immersed in a 100×20 Petri dish containing *Agrobacterium* suspension culture. Half-seed explants were inoculated with *Agrobacterium* for 30 min, at room temperature (RT). After inoculation, explants were placed adaxial side (flat side) down on cocultivation medium lined with filter paper (six explants per plate). The cocultivation medium contained 1/10 B5 salts, B5 vitamins, 7.5 µM BAP, 0.7 µM GA<sub>3</sub>, 20 mM MES, 3% sucrose, 200 µM acetosyringone, 100–400 mg l<sup>-1</sup> cysteine and 154 mg l<sup>-1</sup> dithiothreitol, pH 5.4 with 0.425% Noble agar (Fisher Scientific, USA). Cocultivation was continued for 5 days at 24°C under 18 h photoperiod (140 µmoles s<sup>-1</sup> m<sup>-2</sup>).

### Culture conditions and regeneration

After cocultivation half-seed explants were washed in liquid shoot induction (SI) medium (B5 salts, B5 vitamins, MSIII iron stock, 3% sucrose, 3 mM MES, 5.0 µM BAP, 50 mg l<sup>-1</sup> timentin, 200 mg l<sup>-1</sup> cefotaxime and 50 mg l<sup>-1</sup> vancomycin, pH 5.7). The explants were subsequently cultured (flat side up) on SI medium solidified with 0.7% agar. The SI medium contained 0–6 mg l<sup>-1</sup> glufosinate (glufosinate-ammonium Pestanal®, Sigma-Aldrich). The base of the explant (i.e., the part of the explant from where the embryonic axis was removed) was embedded in the medium. Shoot induction was carried out at 24°C with 18 h



**Fig. 1** *Agrobacterium*-mediated transformation of half-seed explants and regeneration. (a) Half seed explant, 1-with embryonic axis, 2-after embryonic axis was removed; (b) GUS positive shoots during regeneration stage; (c) shoot formation, c-1 minimal, c-2 moderate; c-3 good; (d) One week after Liberty® spraying  $T_1$  plants were scored: green plants were herbicide resistant; yellow (dead) plants were sensitive; (e) GUS assay of  $T_1$  plants prior to herbicide spraying showed 1-herbicide sensitive plant was negative, 2 and 3- resistant plants were GUS positive

photoperiod ( $140 \mu\text{moles s}^{-1} \text{m}^{-2}$ ) in a Percival Biological Incubator. After 14 days, explants were transferred to fresh SI medium containing  $6 \text{ mg l}^{-1}$  glufosinate for *bar* selection. After 4 weeks of culture on SI medium, explants were transferred to shoot elongation (SE) medium containing MS basal salts (Murashige and Skoog 1962), MSIII iron stock, 3% sucrose, 3 mM MES, B5 vitamins,  $50 \text{ mg l}^{-1}$  asparagine,  $100 \text{ mg l}^{-1}$  L-pyroglyutamic acid,  $0.1 \text{ mg l}^{-1}$  IAA,  $0.5 \text{ mg l}^{-1}$  GA<sub>3</sub>,  $1 \text{ mg l}^{-1}$  zeatin riboside,  $50 \text{ mg l}^{-1}$  timentin,  $200 \text{ mg l}^{-1}$  cefotaxime,  $50 \text{ mg l}^{-1}$  vancomycin, pH 5.7, and solidified with 0.7% agar. Selection during the shoot elongation stage was  $6 \text{ mg l}^{-1}$  glufosinate. Starting at 2 weeks after transfer of half-seed explants to SE medium, elongated shoots ( $>2.5 \text{ cm}$ ) were obtained. Each individual shoot was dipped in sterile IBA ( $1 \text{ mg l}^{-1}$ ) then transferred to rooting medium (MS salts, MSIII iron stock, B5 vitamins, 3 mM MES, 2% sucrose, pH 5.6 and 0.7% agar) without glufosinate. After 2 weeks, rooted plantlets were rinsed with water to wash off the agar medium and then, transplanted to soil (Redi-Earth Peat-Lite Mix, Scotts-Sierra Horticultural Products Company) in jiffy pots. The plants ( $T_0$ ) were grown at  $24^\circ\text{C}$ , 18 h photoperiod for 1–2 weeks then, transferred to the greenhouse.

#### Phenotypic screening of transgenic plants

$T_0$  plants with at least two trifoliates were screened in the greenhouse using an herbicide paint assay to identify putative transformants that expressed the *bar* gene. The upper surface of a leaf was treated with  $150 \text{ mg l}^{-1}$  glufosinate (herbicide Liberty®, Bayer CropScience, USA) along the midrib using a cotton bud (Q-tip). The Liberty®-treated leaf tissue was scored for tolerance to glufosinate at 3–5 days after herbicide application. Glufosinate-resistant  $T_0$  plants were grown in the greenhouse until maturity and seeds were harvested. Fourteen seeds from  $T_0$  plants were sown together with seeds from a *bar* gene expressing transgenic line as positive control and non-transgenic soybean as negative control. The progeny tests were done by spraying 2-week-old seedlings with  $250 \text{ mg l}^{-1}$  Liberty® to confirm the presence of the *bar* transgene in  $T_1$  progeny. When the  $T_0$  plant produced fewer than 14 seeds, all available seeds were sown.  $T_1$  plants were scored for resistance or sensitivity to Liberty® 1 week after spraying.

#### Molecular analysis

Southern blot analyses of  $T_0$  and  $T_1$  generations of transgenic events were carried out. Total genomic DNA was extracted using the CTAB method based on the method by Doyle and Doyle (1987). Ten micrograms of genomic DNA was digested with *Xho* I or *Hind* III, the restriction products were separated on a 0.8% agarose gel and transferred to a Magnacharge nylon membrane (Osmonics Inc.). The membranes were hybridized with <sup>32</sup>P-labeled *gus* fragment for 18 h at  $65^\circ\text{C}$ . After hybridization, the membranes were washed in solutions of SDS (sodium dodecyl sulfate) detergent and SSC (sodium chloride and sodium citrate solution) to remove excess probe from the membranes. The Southern blots were then exposed to X-ray film (Fujifilm Medical Systems) at  $-80^\circ\text{C}$  for 2–3 days and subsequently developed.

#### Statistical analysis

Completely random design (CRD) was used to analyze the effect of BAP (cytokinin treatments) on regeneration. Data were scored using nonparametric values based on shoot formation. Each soybean explant (half-seed) was considered as an experimental unit and eight levels of BAP were tested. Regeneration data were analyzed with ANOVA and Duncan's multiple range test ( $p=0.05$ ) using the Statistical Analysis System package (SAS Institute v. 9.1).

## Results and discussion

#### Transformation efficiency using half-seed explants

Primary regenerants ( $T_0$  plants) produced from *in vitro* culture of half-seed explants infected with *Agrobacterium*

**Table 1** Efficiency of *Agrobacterium*-mediated transformation using half-seed explants of cultivar Thorne and vector pTF102

Experiment no	No. of explants infected	No. of glufosinate-resistant events in $T_1$ generation <sup>a</sup>	Final transformation efficiency (%) <sup>b</sup>
1	65	3	4.6
2	115	10	8.7
3	228	11	4.8
4	250	8	3.2
5	210	3	1.4
<sup>a</sup> Resistant to 250 mg l <sup>-1</sup> Liberty <sup>®</sup>	6	100	4.0
	7	151	2.6
<sup>b</sup> Final transformation efficiency = (No. of glufosinate-resistant $T_1$ events/No. of explants infected) × 100	8	60	3.3
		Mean transformation efficiency	4.1
		Standard error	0.8

and selected on medium containing 6 mg l<sup>-1</sup> glufosinate, were screened by leaf painting with Liberty<sup>®</sup> herbicide (active ingredient glufosinate). Subsequent evaluation of  $T_1$  progeny by spraying with 250 mg l<sup>-1</sup> Liberty<sup>®</sup> was also carried out. Final transformation efficiency based on the number of Liberty<sup>®</sup>-resistant events in the  $T_1$  generation per number of explants infected ranged between 1.4 and 8.7% (Table 1). Forty-five independent transformation events were confirmed based on their resistance to the herbicide, giving an overall efficiency of 3.8% (45/1179). Chi-square analysis for goodness of fit for a 3:1 segregation ratio of glufosinate resistance was carried out for 30 of the 45 events. Segregation ratios showed the expected Mendelian inheritance in the  $T_1$  progeny consistent for segregation of a single locus conferring glufosinate resistance in 26 of 30 events while four events indicated abnormal segregation ratios (data not shown). Deviation from the Mendelian segregation of 3:1 could be due to multiple loci, or due to chimerism of the  $T_0$  transformant. The generation of chimeric soybean plants by *Agrobacterium*-mediated transformation has been reported previously by Clemente et al. (2000) and Olhoft et al. (2003).

Transgene inheritance was verified in the  $T_1$  generation using a histochemical GUS assay (Fig. 1e) and by molecular analysis. Southern blot analysis of Liberty<sup>®</sup>-resistant  $T_1$  plants was carried out for 32 of 45 events. One glufosinate-resistant  $T_1$  plant was randomly selected from each event and leaf tissues were collected for DNA extraction. Total genomic DNA was digested with *Xho* I and the Southern blot was hybridized with a *gus* probe. Since there is a single restriction site of *Xho* I in the T-DNA region of pTF102 vector (Fig. 2a), the presence of one hybridization band suggests a single transgene integration site while multiple bands suggest multiple integration loci. All 32 plants analyzed contained DNA fragments that hybridized to *gus*, confirming meiotic transmission of the transgene (Fig. 2b). The majority of these events (20/32) had a single copy of the transgene based on the presence of a single *gus* band. Several plants showed more than one hybridization band which may indicate multiple transgene integrations and could reflect the approximate transgene copy number. Southern blot analysis was also carried out using *Hind* III digestion of total genomic DNA in additional progeny of events pTF102-56, -62 and -105, and all genotypes exhib-

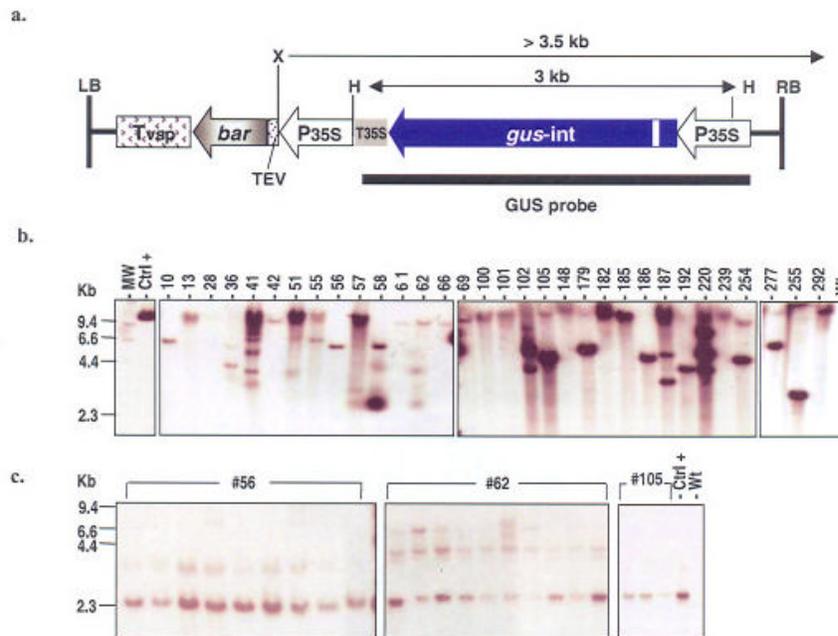
ited the presence of the *gus* fragment after hybridizing with *gus* probe (Fig. 2c).

#### Tissue culture condition and cultivar comparison

##### *Alternative parameters tested in the half-seed treatment*

Explants were prepared by splitting the seed and removing the embryonic axis, causing a break on the cotyledonary tissue. This break may provide an entry point for *Agrobacterium* during infection. In an effort to enhance *Agrobacterium* infection of half-seed explants in the absence of making incisions on the tissue, we examined the effect of a sucrose plasmolyzing pretreatment on transformation efficiency. Plasmolysis of plant cells has been employed to promote gene transfer via electroporation (Wu and Feng 1999; Koscianska and Wypijewski 2001). In our study, half-seed explants were placed in 1/10 MS liquid medium (Murashige and Skoog 1962) with 1.0 M sucrose (from this point forward 1/10 MS liquid medium with 1.0 M sucrose will be referred to as 1.0 M sucrose) for varying periods of pretreatment (0, 30, or 60 min at RT) prior to infection with *Agrobacterium*.

Our results show that 1.0 M sucrose had negative effect on transformation. Final transformation efficiency based on the number of glufosinate-resistant  $T_1$  plants per number of explants infected, decreased from 7.0% to from 0.6 to 2.4% following sucrose pretreatment (Table 2). Moreover, shoot regeneration declined following sucrose pretreatment. Regeneration frequency, which was based on the number of explants producing shoots per number of explants infected, decreased from 59% in the absence of sucrose pretreatment, to 45 and 31% following 30 and 60 min pretreatments, respectively (Table 2). These results suggest that sucrose pretreatment may have caused permanent injury to plant cells that reduced their susceptibility to *Agrobacterium* or that may have reduced the ability of plant cells to attain their organogenic potential. We also examined the effect of vacuum infiltration on T-DNA delivery and shoot regeneration. Vacuum (24 in of Hg) was applied for 15–45 min during *Agrobacterium* infection of half-seed explants after which explants were placed on cocultivation medium for 5



**Fig. 2** pTF102 vector and Southern analysis of pTF102 transformants ( $T_1$  progeny). (a) T-DNA region of binary vector pTF102. LB, left border; RB, right border; Tvsp, soybean vegetative storage protein terminator; *bar*, phosphinothricin acetyltransferase gene; TEV, tobacco etch virus translational enhancer; P35S, CaMV 35S promoter; T35S, CaMV 35S terminator; *gus-int*,  $\beta$ -glucuronidase gene containing an intron. H, *Hind* III; X, *Xho* I. Ten micrograms of genomic DNA were digested with (b) *Xho* I enzyme that cuts once in

the T-DNA region of the plasmid, or (c) *Hind* III that cuts at two restriction sites in pTF102 to generate a drop out fragment. A DNA fragment from the *gus* gene was used as a probe. Ctrl + is a positive control in which 10  $\mu$ g of non-transgenic soybean genomic DNA spiked with 63 pg of pTF102 plasmid DNA was loaded. Wt is a non-transformed Thorne plant.  $T_1$  refers to the second generation of transgenic soybean plants

days. The number of explants expressing GUS transient activity (blue spots) declined when vacuum was applied. No regeneration from vacuum-treated explants was obtained. Overall, these results demonstrate that neither sucrose pretreatment nor vacuum infiltration increases transformation frequencies from *Agrobacterium*-mediated transformation of half-seed explants.

#### Cultivar response

Half-seed explants of Thorne, Williams, Williams79 and Williams82 were infected with *Agrobacterium* and cultured on selective regeneration medium containing 6 mg l<sup>-1</sup>

glufosinate. Transformed  $T_0$  events showing resistance to Liberty® were obtained from all four cultivars (Table 3). Transgenic events of Thorne, Williams, Williams79 and Williams82 have been confirmed in the  $T_1$  generation after Liberty spraying (data not shown). Our results demonstrate the applicability of the half-seed technique to several soybean cultivars.

#### Effect of BAP on regeneration

We examined the effect of varying levels of BAP present in the SI medium on shoot regeneration. Thorne half-seed explants were infected with EHA101(pTF102) and were

**Table 2** Transformation efficiency and shoot regeneration on half-seed explants of Thorne transformed with pTF102 at different durations of sucrose pretreatment before infection

Duration of sucrose pretreatment (min)	No. of explants infected	Regeneration frequency (%) <sup>a</sup>	No. of glufosinate® resistant $T_1$ events	Final transformation efficiency (%) <sup>b</sup>
0	302	59.3	21	7.0
30	293	45.1	7	2.4
60	170	31.2	1	0.6

<sup>a</sup>Regeneration frequency = (No. of explants with one or more shoots/No. of explants infected) × 100

<sup>b</sup>Final transformation efficiency = (No. of glufosinate®-resistant  $T_1$  events/No. of explants infected) × 100

Data were based on five experiments.  $T_1$  refers to the second generation of transgenic soybean plants

**Table 3** Shoot regeneration and transformation efficiency in  $T_0$  generation of different soybean cultivars transformed with *Agrobacterium*

Cultivar	No. of explants infected	Regeneration frequency (%) <sup>a</sup>	No. of glufosinate-resistant $T_0$ plants	Transformation efficiency (%) <sup>b</sup>
Thorne	413	60	4	1.0
Williams	357	46	13	3.6
Williams79	390	37	7	1.8
Williams82	375	56	17	4.5

<sup>a</sup>Regeneration frequency = (No. of explants with one or more shoots/No. of explants infected) × 100

<sup>b</sup>Transformation efficiency = (No. of glufosinate-resistant  $T_0$  events/No. of explants infected) × 100

Constructs ST43 and pTF102 were used; data based on six experiments

$T_0$  refers to the first generation of transgenic soybean plants

cultured on SI medium without BAP or with different BAP concentrations (2.5–100  $\mu$ M). Use of 5  $\mu$ M BAP significantly increased the regeneration frequency (number of explants forming shoots per number of explants infected × 100) ( $P=0.05$ ; Table 4). Similar observations were reported by Dan and Reichert (1998) and Saka et al. (1980) where use of 5.0  $\mu$ M BAP improved regeneration of hypocotyl explants and stem node segments of soybean, respectively. Wright et al. (1986) and Hinchee et al. (1988) also obtained shoot regeneration on cotyledonary explants at 5  $\mu$ M BAP. Use of 7.5  $\mu$ M BAP (as routinely used in the CN protocol; Olhoft et al. 2003; Paz et al. 2004) gave the lowest regeneration rate (29%) when applied to the half-seed system. This observation suggests that culture conditions optimized in the CN system were not immediately applicable to the half-seed protocol. By providing an optimum hormonal environment to the explant *in vitro*, the ability of transformed cells to regenerate into plants may be improved.

In our study the capacity of half-seed explants for shoot induction was based on a visual evaluation of the number of shoots formed (i.e., degree of shoot formation: 0 – no shoot; 1 – minimal, Fig. 1c-1; 2 – moderate, Fig. 1c-2; 3 – good, Fig. 1c-3). The mean degree of shoot formation was calculated using all explants with or without shoots. Because regeneration frequencies were generally low, a lot of the explants were given a score of “0” and this resulted in the mean degree of shoot formation being low for all

treatments. When only explants with shoots were scored, the mean degree of shoot formation ranged from 1.8 to 2.8 (data not shown). Shoot formation was enhanced by treatment with BAP (except 7.5  $\mu$ M treatment) (Table 4). Buising et al. (1994) observed a similar trend on embryonic axes of soybean where the number of shoots per axis increased with higher BAP level. A high concentration of BAP disrupts DNA replication and initially slows down cell division resulting in a break down of apical dominance (Wright et al. 1986) thereby triggering profuse shoot formation.

#### Half-seed versus cotyledonary node

To compare transformation efficiencies using the half-seed or CN protocols, four independent experiments using different genotypes and constructs were carried out (Table 5). In experiment 1, using pTF102 and cultivar Thorne, a 5.7% transformation rate was obtained using half-seed explants while the CN method did not produce any transformed plants. A similar trend was obtained in experiment 2 using vector pTF102 and cultivar Williams82 wherein the half-seed method generated transgenic events (2.5% transformation efficiency) while the CN protocol produced no transgenic events. In experiment 3, transgenic plants of Williams were generated from both half-seed (5.6%) and CN (5.3%) explants using vector pTF101.1. When

**Table 4** Shoot formation on half-seed explants of Thorne transformed with pTF102 at different levels of BAP present in shoot induction medium

BAP concentration ( $\mu$ M)	No. of explants infected	Regeneration frequency (%) <sup>a</sup>	Mean degree of shoot formation <sup>b</sup>
0	113	47 <sup>bc</sup>	0.58 <sup>bc</sup>
2.5	55	52 <sup>bc</sup>	0.74 <sup>b</sup>
5	111	80 <sup>a</sup>	1.06 <sup>a</sup>
7.5	102	29 <sup>c</sup>	0.37 <sup>c</sup>
15	111	65 <sup>ab</sup>	1.03 <sup>a</sup>
30	108	32 <sup>c</sup>	0.60 <sup>bc</sup>
50	105	32 <sup>c</sup>	0.61 <sup>bc</sup>
100	109	59 <sup>ab</sup>	1.06 <sup>a</sup>

<sup>a</sup>Regeneration frequency = (No. of explants with one or more shoots/No. of explants infected) × 100

<sup>b</sup>Degree of shoot formation: 0 – No. of shoot, 1 – minimal (Fig. 1c-1), 2 – moderate (Fig. 1c-2), 3 – good (Fig. 1c-3)

Regeneration frequency or mean degree of shoot formation not followed by the same letter differ significantly at the 5% level

**Table 5** Transformation efficiency using half-seed or cotyledonary mode explants of soybean that were transformed with *Agrobacterium*

Experiment no	Construct	Cultivar	Type of explant	No. of explants infected	No. of glufosinate-resistant events in $T_0$ generation <sup>a</sup>	No. of glufosinate-resistant events in $T_1$ generation <sup>b</sup>	Final transformation efficiency (%) <sup>c</sup>
1	pTF102	Thorne	CN	70	0	0	0
			Half-seed	70	4	4	5.7
2	pTF102	Williams82	CN	80	5	0	0
			Half-seed	119	4	3	2.5
3	pTF101.1	Williams	CN	150	15	8	5.3
			Half-seed	89	5	5	5.6
4	ST42	Williams	CN	150	2	1	0.7
			Half-seed	98	3	1	1

<sup>a</sup>Resistant to 150 mg l<sup>-1</sup> Liberty®

<sup>b</sup>Resistant to 250 mg l<sup>-1</sup> Liberty®

<sup>c</sup>Final transformation efficiency = (No. of glufosinate-resistant  $T_1$  events/No. of explants infected) × 100

$T_0$  and  $T_1$  refer to the first and second generation of transgenic soybean plants, respectively

construct ST42 and Williams were used in experiment 4 transformation efficiencies were 1.0 and 0.7% utilizing half-seed and CN, respectively. Overall, more consistent transgenic plant recovery was obtained using the half-seed protocol. We also observed fewer escapes among putative transformants derived from half-seed explants in experiments 2 and 3. For example, in experiment 3 all 5 putative  $T_0$  events derived from half-seeds were confirmed in the  $T_1$  generation. Meanwhile, eight transgenics were identified out of 15 putative events generated by the CN protocol. These results suggest that *bar* selection is more effective with the half-seed than the CN protocol. A reduced number of escapes, i.e. non-transgenic shoots that survived glufosinate selection *in vitro* and  $T_0$  Liberty® painting, among half-seed regenerants contributed to an efficient transformation system and efficient use of laboratory resources.

It is not clear why the half-seed and CN methods produced different results in recovering transgenic soybean plants. One possible explanation might be a more effective selection for the *bar* gene in half-seed than CN due to the differences in the age of explants (i.e., cotyledonary tissue from mature half-seed versus CN tissue from 5 to 7-day-old germinating seedlings). Another explanation might be the wounding effect. Precise wounding at the CN area of 5–7-day-old germinating seedlings was believed to facilitate *Agrobacterium* infection by (1) providing entry site for *Agrobacterium* and (2) destroying primary shoots and promoting secondary shoot formation (Townsend and Thomas 1993; Meurer et al. 1998). This wounding on the CN tissues, however, may also trigger plant defense mechanisms such as the production of polyphenol oxidases that results in browning of wounded tissue, which may weaken the *Agrobacterium*-mediated transformation process (Olhoft et al. 2001). Since deliberate wounding was not performed on half-seed explants, it was possible that there were less plant oxidases produced, thereby improving the interaction between *Agrobacterium* and plants cells. Olhoft et al. (2001) described the positive effect of thiol compounds as protective agents against oxidases produced by wounding of CN explants. In our study, we did not observe any

significant difference on stable GUS expression in shoots regenerated from half-seeds cocultivated in the presence or absence of cysteine or DTT, i.e. GUS blue shoots were obtained with or without cysteine or DTT (M. Paz, unpublished).

In this study we describe an improved cotyledonary node transformation method using a half-seed explant derived from mature soybean seeds. We demonstrate that it is a robust system when the *bar* gene/bialaphos selection regime is used. Further experiments are needed to investigate the application of this method when using different selectable markers.

**Acknowledgements** The authors thank Joanna Harbaugh, Amanda Ehrler and Francois Torney for their technical assistance; Mike Webber, Bayer CropScience, USA for graciously giving us Liberty® herbicide; and Bronwyn Frame and John Pesek for critical review of the manuscript and valuable suggestions. This work was partially supported by the Iowa Soybean Promotion Board and the North Central Soybean Research Program.

## References

- An G, Evert PR, Mitra A, Ha SB (1988) Binary vectors. In: Gelvin SB, Schilperoort RA (eds.) Plant molecular biology manual A3. Kluwer Academic Publishers, Dordrecht, pp 1–19
- Buising CM, Shoemaker RC, Benbow RM (1994) Early events of multiple bud formation and shoot development in soybean embryonic axes treated with the cytokinin, 6-benzylaminopurine. *Am J Bot* 81:1435–1448
- Carrington JC, Freed DD (1990) Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J Virol* 64:1590–1597
- Clemente T, LaVallee BJ, Howe AR, Ward DC, Rozman RJ, Hunter PE, Broyles DL, Kasten DS, Hinchey MA (2000) Progeny analysis of glyphosate selected transgenic soybeans derived from *Agrobacterium*-mediated transformation. *Crop Sci* 40:797–803
- Dan Y, Reichert NA (1998) Organogenic regeneration of soybean from hypocotyl explants. *In Vitro Cell Dev Biol-Plant* 34:14–21
- Di R, Purcell V, Collins GB, Ghabril SA (1996) Production of transgenic soybean lines expressing the bean pod mottle virus coat protein precursor gene. *Plant Cell Rep* 15:746–750
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15

- Droste A, Pasquali G, Bodanese-Zanettini MH (2000) Integrated bombardment and *Agrobacterium* transformation system: an alternative method for soybean transformation. *Plant Mol Biol Rep* 18:51–59
- Droste A, Pasquali G, Bodanese-Zanettini MH (2002) Transgenic fertile plants of soybean [*Glycine max* (L.) Merrill] obtained from bombarded embryogenic tissue. *Euphytica* 127:367–376
- Finer KR, Finer JJ (2000) Use of *Agrobacterium* expressing green fluorescent protein to evaluate colonization of sonication-assisted *Agrobacterium*-mediated transformation-treated soybean cotyledons. *Lett Appl Microbiol* 30:406–410
- Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SEK, Li B, Nettleton DS, Pei D, Wang K (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol* 129:13–22
- Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50:151–158
- Hadi MZ, McMullen MD, Finer JJ (1996) Transformation of 12 different plasmids into soybean via particle bombardment. *Plant Cell Rep* 15:500–505
- Hajdukiewicz P, Svab Z, Maliga P (1994) The small versatile pZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25:989–994
- Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Biotechnology* 6:915–922
- Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J Bacteriol* 168:1291–1301
- Ko T, Lee S, Krasnyanski S, Korban SS (2003) Two critical factors are required for efficient transformation of multiple soybean cultivars: *Agrobacterium* strain and orientation of immature cotyledonary explant. *Theor Appl Genet* 107:439–447
- Koscianska E, Wypijewski K (2001) Electroporated intact BY-2 tobacco culture cells as a model of transient expression study. *Acta Biochim Pol* 48:657–661
- Liu H, Yang C, Wei Z (2004) Efficient *Agrobacterium tumefaciens*-mediated transformation of soybeans using an embryonic tip regeneration system. *Planta* 219:1042–1049
- Mason HS, DeWald D, Mullet JE (1993) Identification of a methyl jasmonate-responsive domain in the soybean vspB promoter. *Plant Cell* 5:241–251
- Meurer CA, Dinkins RD, Collins GB (1998) Factors affecting soybean cotyledonary node transformation. *Plant Cell Rep* 18:180–186
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–479
- Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 6:810–812
- Olhoft PM, Fligel LE, Donovan CM, Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta* 216:723–735
- Olhoft PM, Lin K, Galbraith J, Nielsen NC, Somers DA (2001) The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells. *Plant Cell Rep* 20:731–737
- Parrott WA, Hoffman LM, Hildebrand DF, Williams EG, Collins GB (1989) Recovery of primary transformants of soybean. *Plant Cell Rep* 7:615–617
- Paz MM, Shou H, Guo Z, Zhang Z, Banerjee AK, Wang K (2004) Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. *Euphytica* 136:167–179
- Saka H, Voqui-Dinh TH, Cheng T (1980) Stimulation of multiple shoot formation on soybean stem nodes in culture. *Plant Sci Lett* 19:193–201
- Santarem ER, Finer JJ (1999) Transformation of soybean [*Glycine max* (L.) Merrill] using proliferative embryogenic tissue maintained on semi-solid medium. *In Vitro Cell Dev Biol Plant* 35:451–455
- Santarem ER, Trick HN, Essig JS, Finer JJ (1998) Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. *Plant Cell Rep* 17:752–759
- Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. *Plant Physiol* 131:892–899
- Townsend JA, Thomas LA (1993) An improved method of *Agrobacterium*-mediated transformation of cultured soybean cells. *US Patent WO 94/02620*
- Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol Gen Genet* 220:245–250
- White J, Chang SP, Bibb MJ, Bibb MJ (1990) A cassette containing the bar gene of *Streptomyces hygroscopicus*: a selectable marker for plant transformation. *Nucl Acids Res* 18:1062
- Wright MS, Koehler SM, Hinchee MA, Carnes MG (1986) Plant regeneration by organogenesis in *Glycine max*. *Plant Cell Rep* 5:150–154
- Wroblewski T, Tomczak A, Michelmore R (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. *Plant Biotechnol J* 3:259–273
- Wu FS, Feng TY (1999) Delivery of plasmid DNA into intact plant cells by electroporation of plasmolyzed cells. *Plant Cell Rep* 18:381–386
- Yan B, Reddy MSS, Collins GB, Dinkins RD (2000) *Agrobacterium tumefaciens*-mediated transformation of soybean [*Glycine max* (L.) Merrill] using immature zygotic cotyledon explants. *Plant Cell Rep* 19:1090–1097
- Zeng P, Vadnais DA, Zhang Z, Polacco JC (2004) Refined glufosinate selection in *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill]. *Plant Cell Rep* 22:478–482