Maize Transformation

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Abstract Plant genetic transformation technologies have brought fundamental changes to both plant biology laboratory research as well as to modern agricultural field practices. Once a recalcitrant plant for tissue culture and gene delivery, maize is becoming one of the most targeted cereal crops using genetic transformation for both basic and applied purposes. This chapter provides a brief review of the history of maize transformation technology development, but focuses extensively on technical aspects of the methodology, including DNA delivery systems, target tissues and genotypes, selectable markers for transformation, and various issues related to integration and expression of transgenes. Some recent observations and improvements from two maize transformation groups are discussed. It is anticipated that increasing genomics information will assist further enhancement of maize transformation technology leading to more rapid progress in understanding and improvement of this important crop.

1 Introduction

Transformation is an indispensable technology in both applied and basic studies in maize. In 2007, more than 70% of US maize fields were planted with genetically engineered (GE) varieties (http://www.ers.usda.gov/Data/BiotechCrops/), the largest acreage since GE crop commercialization in 1996. Given that the share of transgenic crops in the other two major cereals, rice and wheat, is close to zero, and that the

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commercial value of maize seed makes up approximately one half that of the global seed market, the impact of gene transfer technology is more significant for maize than for many other crops. Unlike conventional breeding in which introgression of beneficial traits through hybridization from one variety to another often leads to carry-over of undesired genetic materials, genetic transformation methods allow introduction of a defined DNA segment carrying well characterized genes and regulatory sequences into the plant genome.

For basic research, genetic transformation technology enables scientists to study gene function and regulation by altering or perturbing signaling or metabolic pathways. The scope of transformation experiments covers topics of ever growing complexity such as conducting experiments in over-expression, regulated expression, down-regulation or silencing of exogenous or endogenous genes, analyses of promoters or other regulatory elements, and complementation of mutations with genomic sequences.

Currently, particle bombardment and *Agrobacterium*-mediated methods are the two major options for gene delivery to maize. A number of maize genotypes can be transformed by both methods and these techniques are well developed in maize compared to other major crops such as wheat or soybean. We can proudly state that the basic setup for gene transfer in maize has been accomplished. However, there still are considerable limitations, especially to meet the large demand for transgenic plants in functional genomics. For example, compared to rice, the model cereal crop for genomic research, maize transformation techniques suffer low overall transformation frequency and high genotype dependency. Thus, we still need to achieve a long list of desired improvements.

In this chapter, technology for DNA delivery, maize target tissues and genotypes, selectable markers for transformation, and various issues related to integration of transgenes are discussed, with an emphasis on recent developments.

2 DNA Delivery Technologies

Considerable efforts to develop gene transfer technology in maize have been made since the mid 1980s, and both direct DNA transfer and *Agrobacterium*-mediated methods have been actively studied (Armstrong 1999; Torney et al. 2007). The first successful production of fertile transgenic maize was achieved by particle bombardment in the late 1980s (Gordon-Kamm et al. 1990). Initially, most transgenic maize was produced by direct DNA transfer techniques due to wide-spread skepticism as to whether *Agrobacterium* was suitable for monocotyledonous plant transformation. While some early efforts were made to transform maize using *Agrobacterium*, it was not until 1996 that the first convincing and efficient protocol for *Agrobacterium*-mediated maize transformation was reported (Ishida et al. 1996). Because of the quality of transgenic plants generated by this method (high frequency of single and low copy number transgene integration), *Agrobacterium*-mediated maize transformation is becoming the method of choice in maize research.

2.1 *Direct DNA Transfer Methods*

Numerous direct DNA transfer methods have been developed to deliver genetic material into plant cells. These methods include particle bombardment, electroporation, polyethylene glycol (PEG) incubation, silica carbide whiskers, microinjection, macroinjection, microlaser, liposome, pollen tube pathway, and electrophoresis (Potrykus and Spangenberg 1995; Armstrong 1999). However, only a few methods have produced transgenic maize plants. Currently, the most efficient and popular direct DNA transfer method is particle bombardment or biolistic gun-mediated transformation. The methods that have been successfully used for generating transgenic maize plants are discussed here.

2.1.1 Electroporation and PEG Methods

Electroporation is a method in which the DNA of interest is introduced into protoplasts by applying an electric pulse to a mixture of the two. The first transgenic maize plants were produced by this method (Rhodes et al. 1988). Protoplasts were isolated from embryogenic suspension cultures that originated from immature zygotic embryos (IEs) of the maize inbred line, A188. After electroporation, protoplasts were placed on plant media allowing callus formation and selection. Calli that survived kanamycin selection was then regenerated into plants. However, none of the transformed plants set seed.

Instead of using an electric pulse, PEG may be added to a mixture of protoplasts and the DNA of interest (PEG method). This chemical is not toxic to plants and has been used in other plant transformation protocols (Armstrong et al. 1990). Indeed, Golovkin et al. (1993) produced fertile transgenic plants of the maize germplasm He/89 using this method.

In spite of this success, methods using protoplasts are inherently difficult in maize. Unlike dicotyledonous plants, protoplasts that regenerate plants cannot be prepared from mesophyll cells in cereals. Regenerable maize protoplasts can be prepared only from embryogenic suspension or callus cultures. Only a limited number of tissue-culture adapted maize genotypes can produce the desired embryogenic cultures. Additionally, prolonged culture leads to loss of capacity for these protoplasts to regenerate plants after transformation and to maintain fertility for seed production. Due to these limitations, transformation techniques based on protoplasts have never been widely used.

To avoid using protoplasts as target material for transformation, electroporation methods were used to transform intact maize tissues. The targeted tissues are briefly treated with an enzyme that digests cell walls, after which DNA is introduced by an electric pulse. Using this approach, maize IEs and Type I callus (compact embryogenic callus, D'Halluin et al. 1992), and suspension cultures and Type II callus (friable embryogenic callus, Laursen et al. 1994; Pescitelli and Sukhapinda 1995) were transformed and transgenic plants were obtained. This method has not been widely

adopted mainly because of its low transformation frequency (TF, where transformation frequency is defined as the number of herbicide or antibiotic resistant events recovered per 100 targeted explants).

2.1.2 Particle Bombardment

Using this method, target cells or tissues are bombarded with fine metal particles coated with DNA. Because highly accelerated particles can easily penetrate cell walls, various types of tissues can be targeted. The earliest fertile transgenic maize lines generated by this method were from cell suspension cultures and Type II callus (Fromm et al. 1990; Gordon-Kamm et al. 1990; Walters et al. 1992; Vain et al. 1993). Then, Type I callus, IEs, shoot meristems, organogenic callus from shoot tips and shoot meristem cultures were successfully employed (Wan et al. 1995; Zhong et al. 1996; Zhang et al. 2002; Ahmadabadi et al. 2007). Using this technology, fertile transgenic plants have been efficiently produced in a wide range of maize genotypes and, to date, it has been the major transformation method used for producing genetically engineered commercial varieties of maize (http://www.agbios.com/).

2.1.3 Silicon Carbide Whiskers

Needlelike silicon carbide fibers, which are approximately 20 μm in length and referred to as "whiskers", penetrate the cell wall and plasma membrane of target cells to effect DNA uptake and subsequent transformation (Southgate et al. 1998). This technique is relatively simple and fast, and does not require expensive equipment and supplies. In maize, the whisker method was first used to transform non-regenerable Black Mexican Sweet suspension cells (Kaeppler et al. 1992). Production of fertile transgenic maize from suspension cultures and Type II callus have been reported (Frame et al. 1994; Petolino et al. 2000). In addition to low TF, the major limitation of this technology is that it can only deliver DNA to fine cell aggregates such as suspension cultures and friable callus cultures, and, as such, has not been widely employed in maize transformation.

2.2 *Agrobacterium-Mediated Transformation*

The soil pathogen *Agrobacterium tumefaciens* can genetically transform plant cells with DNA segments (T-DNA) from its tumor-inducing plasmid (Ti plasmid, Gelvin 2003). High frequency transformation protocols based on this naturally occurring machinery for the integration of foreign DNA into plant chromosomes have been continuously reported in many dicotyledonous plants since the early 1980s. Initially, it was assumed that this technology could not be extended to monocotyledonous plants because they are not natural hosts for *A. tumefaciens* (Hernalsteens et al. 1984).

Nevertheless, early attempts to infect maize and other cereals with *A. tumefaciens* laid the ground work for later successes using this biological vector to effectively transform maize.

2.2.1 Early Attempts

Graves and Goldman (1986) infected germinating seedlings of maize with a wild type *Agrobacterium* strain and detected the expression of an *Agrobacterium* opine gene in the inoculated plants two weeks after infection. Grimsely et al. (1987) inoculated apical meristems of maize plants with an *Agrobacterium* strain that carried T-DNA in which maize streak virus DNA had been inserted. The transformed maize displayed symptoms of systemic infection of the virus. Gould et al. (1991) then inoculated apical meristems of maize with *Agrobacterium* and confirmed by Southern blot analysis and the expression of the GUS reporter gene that some seeds from the resulting plants carried the introduced gene. While these early documentations did not offer reproducible results or a workable frequency for maize transformation, they were encouraging. Other reports also included evidence of *Agrobacterium* attachment to maize cell surfaces (Graves et al. 1988) and identification of substances inducing virulence (*vir*) genes by monocots, including maize (Primich-Zachwieja and Minocha 1991).

2.2.2 Development of Efficient Protocols

A highly efficient method of monocot transformation using *Agrobacterium* was finally reported in rice (Hiei et al. 1994), followed by successful reports of transformation in other important cereals such as maize (Ishida et al. 1996), wheat (Cheng et al. 1997), barley (Tingay et al. 1997) and sorghum (Zhao et al. 2000). Types of plant materials used for infection with *Agrobacterium*, choices of vectors and strains of *Agrobacterium*, and optimization of tissue culture techniques are among the key factors that contributed to these achievements.

Ishida et al. (1996) inoculated IEs of the maize inbred A188 with a modified *Agrobacterium* strain that carried a super-binary vector (in which extra copies of *vir* genes assisted DNA transfer) and obtained transgenic maize plants from 5% to 30% of the infected embryos. According to their study, it was necessary to use IEs at a specific stage of development from a specific germplasm of maize, A188. The IEs needed to be obtained from non-stressed healthy plants grown in a well-conditioned greenhouse. In addition, the marker genes, vectors, *Agrobacterium* strains, media composition and concentration of bacteria all had to be optimized. The complexity of multiple factors involved and the narrow ranges of optimal parameters in the process were probably the main reasons why efficient maize transformation by *Agrobacterium* had been difficult.

The benefits observed in rice when using the *Agrobacterium*-mediated transformation method – a relatively high transformation frequency accompanied by a high frequency

of single or low copy insertion events with few rearrangements – were also evident in maize. Initially, *Agrobacterium*-mediated gene transfer to maize was not rapidly and broadly adopted because very few genotypes, effectively only the agronomically poor inbred line, A188 and its derivatives, were transformable at a high frequency using the original protocol. Painstaking efforts have been gradually resolving this issue to expand transformable genotypes and to improve the efficiency of transformation (Zhao et al. 2001; Frame et al. 2002; Ishida et al. 2003; Quan et al. 2004; Danilova and Dolgikh 2005; Huang and Wei 2005; Frame et al. 2006a).

2.3 *Comparison of Particle Bombardment and* Agrobacterium*-Mediated Transformation*

Various methods for transformation of higher plants have been examined extensively in maize and other plants for the last two decades, and it is now quite clear that, for transforming maize, the relevant choice is between the particle bombardment and *Agrobacterium*-mediated methods. The major advantages and disadvantages of the two methods are compared in Table 1.

Particle bombardment is purely a physical process for delivery of DNA into the plant genome. Genes of interest (GOI) can be cloned into popular high copy cloning vectors such as pUC, and pBlueScript. Multiple transgenes can be delivered either by bombarding one plasmid carrying multiple genes or co-bombarding several plasmids that carry the genes separately. To avoid introducing non-target DNA such as vector backbones, the target-only DNA can be purified from the plasmid using restriction enzyme digestion and gel purification prior to the bombardment. A typical DNA fragment used for transformation is less than 15 kb. Genomic DNA fragments as large as 100 kb were used for bombardment to examine the function of a GOI less than 15 kb contained in this fragment (V. Chandler, personal communication). Any maize genotype that can produce Type I or Type II callus responses from IEs with adequate frequency is likely to be transformed using this method. Particle bombardment is also a powerful tool in the analysis of transient expression of transgenes in intact and fully developed tissues. However, relatively large percentages of transgenic plants generated by this method have high copy number integration and complex transgene rearrangement, thus, high incidences of low, unstable or silenced transgene expression were reported (Pawlowski and Somers 1996, 1998; Shou et al. 2004).

Advantages of *Agrobacterium*-mediated transformation include high TF, integration of low numbers of T-DNA copies into chromosomes, and the ability to transfer relatively large T-DNA segments with defined ends and little rearrangement. However, because *Agrobacterium*-mediated transformation intrinsically depends on the complex biological interaction between bacterial and plant cells, specific vectors with components that facilitate the transfer of DNA through this interaction are needed, and manipulation of complicated biological systems is required for optimization of the experimental protocols. Specific binary vectors designed for *Agrobacterium*-mediated transformation must be used for cloning of GOI. One disadvantage of this method is that a certain percentage of *Agrobacterium*-derived transgenic plants may carry various DNA fragments from the binary vector backbones (Shou et al. 2004). The strategy to overcome this problem will be discussed under Subheading 5.

Therefore, both methods have their advantages and limitations. There are a very small number of studies directly comparing these two methods in maize (Zhao et al. 1998; Shou et al. 2004). Because these authors prioritized patterns of integration and stable expression of transgenes, they favored *Agrobacterium*-mediated transformation. In general, the decision for choosing a method for maize transformation depends not only on the nature of the project and long term objectives but also on laboratory infrastructure and researcher skills.

Table 1 Advantages and disadvantages of two transformation methods

Factors	Particle bombardment	*Agrobacterium*-mediated transformation
Average transformation frequencies*	5–40%	5–50%
Copy number and rearrangement of transgenes	High (<10% single or low copy**)	Low (10–80% single or low copy**)
Biological complication in transformation	None	Complex plant-bacteria interaction
Vector configuration	Requires any high-copy cloning vector such as pUC, pBlueScript, etc.	Requires gene of interest cassette to be cloned into *Agrobacterium* binary vectors
Vector construction	Straightforward	Need to handle large, low-copy plasmids and to transfer plasmids from *Escherichia coli* to *Agrobacterium*
Size of DNA to be transferred	Any size, but DNA larger than 15 kb is less efficient and can be fragmented	Typical sizes of T-DNA are 5–30 kb
Maize genotype requirement	Adequate frequency of Type I or Type II callus response and regenerability	Compatible with *Agrobacterium* interaction, and adequate frequency of Type I or Type II callus response and regenerability
Most successful target tissue for stable transformation	Immature embryos, Type I or Type II callus, suspension cells	Immature embryos, Type I or Type II callus
Transfer of non-target DNA	Target-only DNA can be obtained by DNA restriction digestion and purification, and used to bombard plant cells	Certain percentage of transgenic plants may carry DNA fragments from vector backbone in addition to T-DNA
Transient expression in mature tissues	Well-established for various tissues	Limited success only

*Transformation frequency: number of herbicide or antibiotics resistant events recovered per 100 transformed explants. The range of frequencies reasonably expected from experiments using immature embryos of Hi-II or A188 conducted by scientists with varying skills is shown.

**Low copy: 1–3 transgene copies.

2.4 *Critical Factors in Particle Bombardment and* Agrobacterium*-Mediated Transformation*

No matter which method is chosen, transformation protocols must be optimized for particular experimental requirements. Apparently, more factors need to be considered in *Agrobacterium*-mediated transformation because the system involves complex interactions between bacteria and plant cells. Many of the parameters have been adjusted on the basis of transient expression of reporter genes, which is most often a GUS gene that has an intron in the coding sequence (*intron-gus*, Vancanneyt et al. 1990). The *intron-gus* gene is better expressed in maize cells than the ordinary *gus* gene (Ueki et al. 2004). It is especially important to use the *intron-gus* construct to optimize *Agrobacterium*-mediated transformation experiments. Because only eukaryotic cells can process an intron placed in the coding sequence of a gene, use of the *intron-gus* construct allows one to distinguish whether the GUS foci detected on the infected embryos or callus derives from transformed plant cells or simply from the presence of remnant *Agrobacterium* cells (Ohta et al. 1990).

Stable transformants have never been efficiently obtained under the conditions that yield only limited transient expression. However, it should be noted that such adjustments are less straightforward in maize. It has proved relatively easy to find conditions for high-level transient expression but stable transformants were obtained in only a few instances (Ishida et al. 1996). The main hurdles in transformation are not the delivery of DNA fragments to the plant cells but, rather, the recovery of the cells that have integrated the foreign DNA in their chromosomes via tissue culture systems.

2.4.1 Particle Bombardment

Critical factors for transformation success using particle bombardment have been comprehensively reviewed (Morrish et al. 1993; Potrykus et al. 1998; Kikkert et al. 2005). These factors can be grouped into two categories: physical parameters, which are related to preparation and acceleration of the particles, and biological parameters, which are related to target tissues/cells. Some relevant physical parameters include: the particles types (material, density, size), volume of the particles, particle velocity, the method used to accelerate particles, amount of DNA precipitated onto the surface of the particles, procedures for the precipitation of DNA, degree of vacuum in the sample chamber, and aperture characteristics of the stopping plates.

Three different types of particle bombardment devices based on driving forces and the type of macrocarriers used have been reported. The BioRad PDS-1000/He biolistic gun is a gas (helium) pressure-driven device using plastic film as a macrocarrier (Klein et al. 1987). ACCELL™ is an electrical discharge particle bombardment device using a metalized sheet as a macrocarrier (McCabe and Christou 1993). The Particle Inflow Gun (PIG) is a gas-stream driven device using no macrocarrier (Finer et al. 1992). All three biolistic devices can be used to effectively deliver DNA into cells. However, the BioRad PDS-1000/He is currently the most widely used device in maize transformation, therefore it is the focus of our biolistic gun discussion in this review.

Gold and tungsten particles between 0.6 and 1.2 µm in diameter are effective for transforming maize although, depending on the nature of the targeted explant, choice of particle size can improve TF. For example, reducing the gold particle size used to bombard Type II callus from 1.0 µm to 0.6 µm resulted in significantly higher TF (Frame et al. 2000). Magnetic particles were also shown to be used for plant transformation, in which targeted cells could be magnetically collected after being hit by a particle projectile (Horikawa et al. 1997). In most protocols, DNA is usually applied to the particles by precipitating with 2.5 M $CaCl_2$ and 0.1 M spermidine, even though details of handling may vary. Velocity of the particles, which is controlled by pressure of the gas, and parameters related to sample chambers and stopping plates, vary with tissues targeted.

Two important biological parameters influencing the effectiveness of using particle bombardment to transform maize are osmotic treatments (Vain et al. 1993) and duration of IE pre-culture prior to bombardment (Songstad et al. 1996). To increase TF, subjecting targeted cells to concentrations of 0.35 to 0.4 M sorbitol, mannitol, sorbitol and mannitol, or sucrose before and/or after bombardment have been used (Vain et al. 1993; Dunder et al. 1995). This treatment is assumed to limit cell damage at bombardment by plasmolyzing targeted cells. Secondly, by reducing IE pre-culture duration after dissection and before bombardment from 3 to 2 days, average TF increased from 15±2.4 % to 21±1.8 % (L. Marcell and B. Frame, unpublished results). A robust maize transformation protocol using the BioRad PDS-1000/He gun can be found in Frame et al., 2000, and http://www.agron.iastate.edu/ptf/index.aspx#.

2.4.2 *Agrobacterium*-Mediated Transformation

The *Agrobacterium*-mediated transformation system involves two biological organisms: a disarmed but pathogenic bacterium, and a plant cell. Fine tuning the system requires considerations of both parties to minimize the negative impact of the bacteria on plant cells and maximize the recovery of transformants. Many alterations have been made to the infection and co-cultivation stages with the aim of enhancing interaction between these two organisms. Techniques such as sonication of IEs in a bacterial suspension (Trick and Finer 1997), addition of a surfactant to the bacterial infection culture (Huang and Wei 2005), and vacuum infiltration of embryogenic callus with the bacterial suspension (Danilova and Dolgikh 2005) could be helpful. In addition, using the optimal concentration of bacterial culture (between 0.5 x 10^9 and 1.0 x 10^9 cells per ml) for IE infection (Ishida et al. 1996; Zhao et al. 2001), adjusting co-cultivation media pH to ~ 5.8 (Ishida et al. 1996), addition of 100 µM acetosyringone (Ishida et al. 1996), 5 µM silver nitrate (Zhao et al. 2001), 5 µM $CuSO_4$ (Ishida et al. 2007) or between 2.5 and 3.3 mM L-cysteine (Frame et al. 2002) to the co-cultivation media, and conducting co-cultivation at temperatures between 20 °C and 25 °C (Frame et al. 2002) all improved TF in independent studies.

After co-cultivation, a resting culture in which no selective pressure is applied may be used to increase TF (Zhao et al. 2001). For removal of bacteria from the culture, use of the antibiotic carbenicillin instead of cefotaxime also resulted in higher TF (Zhao et al. 2001; Ishida et al. 2003). MS-based media (Murashige

and Skoog 1962) was reported to improve TF of three inbred lines when compared with N6-based media (Chu et al. 1975; Frame et al. 2006a). Addition of 10 μM $CuSO_4$ to medium also improved frequency of regeneration and vigor of regenerated plants (Ishida et al. 2007).

Certain IE treatments prior to infection can also improve TF. Hiei et al. (2006) demonstrated that TF in both maize and rice was improved by treating IE with heat and centrifugation before infection with *Agrobacterium*. This effect was detected both at the level of transient expression and rate of stable transformants recovered. For maize, the optimal conditions were 46°C for 3 min followed by centrifugation at 20,000 x g for 10 min. The effect of heat was greater than that of centrifugation in maize and the combination of the two was the most effective treatment. For example, TF of A188 IEs was increased three-fold by pretreatment with heat and centrifuging from 10% to 30% and transgenic plants were generated from two previously non-transformable genotypes (inbred A634 and a hybrid of A634 x Oh43). Transformation with a less efficient vector (non-superbinary vector) was also enhanced (Hiei et al. 2006).

Several disarmed strains of *Agrobacterium* have been employed in plant transformation and differ in effectiveness. Disarmed strains derived from A281, a hypervirulent L,L-succinamopine type strain (Hood et al. 1986), Ach5, an octopine type strain (Ooms et al. 1982), and C58, a nopaline type strain (Koncz and Schell 1986), are used frequently in cereal transformation. For example, A281 derivative EHA101 (Hood et al. 1986), Ach5 derivative LBA4404 (Hoekema et al. 1983), and C58 derivatives ABI (Koncz and Schell 1986) and C58z707 (Hepburn et al. 1985) have been used successfully in maize transformation in both super-binary and standard binary vector systems (Ishida et al. 1996; Zhao et al. 2001; Frame et al. 2002; Sidorov et al. 2006; Frame et al. 2006a).

Super-binary vectors, which carry *virB* and *virG* genes of A281 (Komari 1990), have been highly effective for maize transformation. It should be noted that super-binary vectors exhibited higher transformation frequency when combined with *Agrobacterium* strain LBA4404 (Hiei et al. 1994).

High frequency *Agrobacterium*-mediated maize transformation protocols using the super-binary vectors can be found in (Ishida et al. 1996; Zhao et al. 2001; Ishida et al. 2007). Protocols using a standard binary vector can be found in (Frame et al. 2002; Frame et al. 2006b).

3 Target Tissues and Genotypes

To date, the most amenable target materials for both biolistic-mediated and *Agrobacterium*-mediated transformation methods have been IEs, IE-derived embryogenic callus and callus derived liquid suspension culture (Armstrong 1999; Hansen and Wright 1999; Torney et al. 2007). Early transformation experiments focused on target tissues such as liquid suspension culture or friable Type II callus (Fromm et al. 1990; Gordon-Kamm et al. 1990; Walters et al. 1992; Frame et al. 2000). Focus on targeting a dedifferentiated cell-state for DNA delivery was aimed at maximizing the chance of producing transgenic plants regenerated from single transformed cells (Torney et al. 2007). While a Type II callus response from targeted IEs facilitates selection and regeneration of transgenic events, this callus type is not readily produced in most inbred germplasms, with some exceptions (Lowe et al. 2006).

The most widely used laboratory genotype, Hi-II (high Type II callus production, Armstrong et al. 1991), is a hybrid line that manifests a Type II embryogenic callus induction frequency (ECIF) of 100%. Because of its inferior agronomic performance and segregating genetic background, however, it is not suitable for some functional genomic studies. As such, efforts have been made to develop IE transformation systems that target inbred lines. Although genotype-specific recalcitrance is common in maize inbred lines using the IE tissue culture system, those that do respond often produce Type I callus instead of the more friable Type II callus typical of Hi-II (Wan et al. 1995; Brettschneider et al. 1997; Frame et al. 2006b) or a mixture of the two (in the case of inbred A188). Like Type II callus, Type I callus produces somatic embryos but is a more compact and differentiated callus phenotype. Transformation of inbred line IEs exhibiting a Type I embryogenic callus response has been achieved using biolistic (Koziel et al. 1993; Brettschneider et al. 1997; Wang et al. 2003) and *Agrobacterium* methods (Ishida et al. 1996; Ishida et al. 2003; Huang and Wei 2005; Frame et al. 2006b).

Additional efforts to identify genotype-independent transformation systems have focused on using the mature seed – in particular the shoot apical meristem (SAM) of germinated seedlings – as an alternative explant to IEs. Heterogenous shoot tip cultures derived from the SAM were targeted for biolistic transformation (Zhong et al. 1992; Zhong et al. 1996; O'Connor-Sánchez et al. 2002; Zhang et al. 2002). Non-chimeric transgenic events were recovered using this approach, but at low frequency. In a recent report, the coleoptilar node region of germinated seedlings of public inbred line H99 was used to produce homogenous, embryogenic Type I callus which was subsequently *Agrobacterium*-transformed to produce stable transgenic events (Sidorov et al. 2006).

It must be emphasized that no matter what types of explants are targeted, using non-stressed healthy maize plants as starting materials is the key to the success of both biolistic and *Agrobacterium*-mediated transformation. Therefore, research efforts and funds invested in greenhouse management and equipment to improve light, temperature, soil and other growth conditions will be well rewarded in the long run.

3.1 *IEs and Type II Embryogenic Callus of the Hi-II Genotype*

Target materials (IE, Type II callus, and callus-derived suspension cultures) of the Hi-II hybrid genotype used in early transformation successes continue to play important roles in transgenic maize production, although suspension cultures have not been widely used in public laboratories. IEs have been the preferred target material for biolistic (Songstad et al. 1996) and *Agrobacterium*-mediated transformation systems

(Zhao et al. 2001). When cultured on N6 medium with proline, dichlorophenoxyacetic acid (2,4-D) and silver nitrate (Armstrong et al. 1991; Songstad et al. 1991; Songstad et al. 1996), friable, rapidly growing and highly embryogenic callus (Type II) is formed from embryo scutellum cells. The Type II callus produced is suitable for biolistic transformation (Walters et al. 1992; Frame et al. 2000), and can be used as a year-round stock material to alleviate shortages of IEs. *Agrobacterium*-mediated transformation of this callus phenotype (albeit an elite line) has also been reported (Lowe et al. 2006).

Detailed protocols for genetic transformation of Hi-II IEs using *Agrobacterium*-mediated (Zhao et al. 2001; Frame et al. 2002) or biolistic-mediated (Dunder et al. 1995; Songstad et al. 1996; Frame et al. 2000) methods have been published. At the Iowa State University Plant Transformation Facility (ISU PTF), the *Agrobacterium*-mediated transformation frequency (TF) ranges from 1–21% (number of bialaphos resistant callus events per 100 embryos infected) by construct and averaged 7.2% over the 36 constructs (completed during 2007). All these constructs were based on a standard binary vector, pTF101.1 (2x CaMV 35S promoter driving the *bar* gene as selectable marker, Paz et al. 2004), harbored in *Agrobacterium* strain EHA101 (Hood et al. 1986). By comparison, TF using the biolistic-mediated method ranges from 8–41%, averaging 19% over the last 28 constructs bombarded using the published protocol (Frame et al. 2000). Frequency fluctuations may be affected by various experimental factors including Hi-II seed source, ear variation, season, and construct.

In comparing two distinct sources of Hi-II germplasm, both originating from the Maize Genetics Coop (https://maizecoop.cropsci.uiuc.edu), but with one producing seed in Iowa (designated as PTFHT) and the other producing seed in Wisconsin (designated WHT, and kindly provided by H Kaeppler), we found that WHT germplasm transformed at frequencies four times higher than PTFHT germplasm using the biolistic gun. Average TF using our published protocol to co-transform 28 constructs over an 18 month period was 30% for WHT (ranging from 7–57% by construct) and 7% for PTFHT (ranging from 1–13%, L. Marcell and B. Frame, unpublished results).

Interestingly, this difference in TF between the two germplasms is not evident in our *Agrobacterium*-mediated transformation experiments, where fluctuations in TF are more strongly influenced by the construct employed. For example, average TFs for two GOI constructs, both cloned on vector pTF101.1 in EHA101, were 2% and 18% while average TF for the PTFHT and WHT germplasms in these same experiments were 9% and 8%, respectively.

The consistent, differential performance of these two Hi-II germplasms across two different transformation methods is not understood. Callus growth rate from bombarded WHT embryos is higher than that from PTFHT embryos (B. Frame, unpublished results), yet a *germplasm x media* interaction that may account for this difference in growth rate is unlikely given that switching pipeline media did not increase the relative TF of WHT using the *Agrobacterium* transformation method (B. Frame, M. Main, J. Lund, unpublished results). It is possible that there is a *germplasm x antibiotic* interaction that is limiting WHT performance using our *Agrobacterium* method.

Using PTFHT germplasm, TF also varies widely by ear for both transformation technologies. Across three separate *Agrobacterium*-mediated transformation experiments in which embryos from 18 ears were infected using the same pTF101.1-based construct, TF between ears ranged from 0–17% with an average of 5.5%. By comparison, TF between operators and embryo sizes ranged only from 4–6% and 5–6%, respectively (B. Frame, M. Main, J. Lund, unpublished results). These results suggest that if the number of embryos to be infected is fixed, it is expedient to dissect more ears, and fewer embryos per ear.

This range in TF by ear is also consistently observed using our biolistic-mediated transformation method. Embryos from 12 of the above-mentioned 18 ears were also bombarded. Average TF for these ears was 18% but ranged from 0–31% by ear (B. Frame, M. Main, J. Lund, unpublished results). Of interest was whether, for the twelve ears compared across transformation methods, the high transforming ears using the *Agrobacterium* method were also the high transforming ears using the biolistic gun. Results showed that the four lowest transforming ears in *Agrobacterium* experiments (all 2% TF) ranked from lowest (0% TF) to second highest (30% TF) in biolistic gun experiments, suggesting no obvious consistency in ear effect across transformation methods.

To investigate whether transformation rates also fluctuate throughout the year, TF was assessed four times a year for three years in an ongoing study in the ISU PTF. Using only PTFHT germplasm, greenhouse-derived IEs (1.2–2 mm) were harvested 9–12 days post pollination (depending on season) and bombarded (3 days post-dissection) at the equinox (spring and fall) and solstices (winter and summer) using the published protocol (Frame et al. 2000) with fixed parameters as follows: 0.6 μm gold coated with the construct pB184 (maize ubiquitin, *ubi*, promoter driving the *bar* gene, Frame et al. 2000), 650 psi rupture disks, 6 mm gap distance, 6 cm target distance, and 3 days pre-culture. Four to eight ears (240 embryos) were represented in each eight plate bombardment on each date. Average TF for spring and fall were 17±3% and 11±1%, respectively, and for winter and summer were 18±5% and 10±2%, respectively (L. Marcell and B. Frame, unpublished results). These results may reflect lighting constraints dictated by lack of adequate greenhouse cooling – in summer and early fall, we are often forced to turn off greenhouse lights to minimize heat stress on people and plants. In turn, this may affect the vigor, and transformation competency of embryos reaching the lab (Zhao et al. 2001).

3.2 IEs and Type I Callus Culture of Maize Inbreds

Because of inherent genetic disadvantages to using the Hi-II hybrid genotype for transformation, considerable effort has been invested in developing robust methods for transforming maize inbred genotypes. Targeting and transforming IEs that in turn respond to produce Type I callus for selection and regeneration has been used in both biolistic and *Agrobacterium*-mediated transformation systems. Using biolistic methods, stable transformation of inbred lines CG0056 (Koziel et al. 1993), H99, A188 and Pa91 (Brettschneider et al. 1997) and Oh43 (Wang et al. 2003) have been reported. Stable transformation of inbred line A188 IEs using *Agrobacterium*-mediated methods was first reported by Ishida et al. (1996) and proved to be a major breakthrough in

transgenic maize production. The relatively high TF achieved, and the high frequency of single or low copy number events obtained in this study, rendered this a useful system for functional genomic studies and for producing transgenic events that were likely to exhibit relatively stable transgene expression in progeny generations (Zhao et al. 1998; Shou et al. 2004).

Subsequently, this method was used to transform several additional proprietary or public inbred lines including H99, W117, Mo17, B104, Ky21 and B114 (Gordon-Kamm et al. 2002; Ishida et al. 2003; Huang and Wei 2005; Frame et al. 2006a). Key to success in these inbred line studies was the identification of parameters that induced targeted IEs to respond with a high ECIF after transformation, as is the case for the Hi-II, Type II maize tissue culture system. Although this is a necessary condition, it is not always a sufficient one for achieving high TF. Frame et al. (2006a) reported that Oh43 demonstrated high ECIF but was transformation incompetent using the *Agrobacterium*-mediated protocol the authors described.

Type I callus from responding embryos can also be targeted for transformation. This approach was used for electroporation (D'Halluin et al. 1992) and biolistic gun transformations (Wan et al. 1995; Wang et al. 2003). In the ISU PTF, we still rely on bombarding Type I callus for those inbred lines with low ECIF or transformation competence using our *Agrobacterium*-mediated method. Using dicamba-based media (Carvalho et al. 1997) modified to contain MS salts and vitamins (Frame et al. 2006a), we are able to biolistically transform Type I callus of inbred lines W22 (1% TF), M37W (1% TF) and W64 (6% TF, B. Frame, unpublished results).

3.3 Mature Seed-Derived Explants for Transformation

While the IE is currently the most reliable target material for maize transformation, its disadvantages (genotype dependent tissue culture response for inbred lines and greenhouse space requirements for donor embryo supply) have fueled many investigations for alternative target explants.

One attractive target tissue is the shoot apical meristem (SAM) in seedlings germinated from mature seeds. It was hypothesized that once germ-line progenitor cells in the SAM were transformed with a selectable marker gene, they would proliferate under selection pressure and subsequently regenerate into shoots without undergoing a proliferative, callus formation stage. However, the small number of predetermined germ-line cells in the meristem presented a major challenge to targeting the SAM directly (Lowe et al. 1995; Bowman and Eshed 2000; Sticklen and Oraby 2005). Only the successful delivery and integration of transgenes into germ-line cells can result in non-chimeric plant that will transmit the transgene to its progeny. To increase the population of transformation and regeneration competent target cells, protocols were established (Zhong et al. 1992, 1996) and subsequently modified (Li et al. 2002; O'Connor-Sánchez et al. 2002; Zhang et al. 2002) to induce and transform (using the biolistic gun) heterogeneous multiple-shoot meristem cultures. Non-chimeric transgenic events were recovered at a low frequency from these

experiments, including from the recalcitrant inbred line B73 (Zhang et al. 2002), and two subtropical inbred lines (O'Connor-Sánchez et al. 2002).

Although high frequency transformation of these heterogeneous cultures remains elusive, expectations that a SAM-based tissue culture system may be relatively genotype independent were upheld by Li et al. (2002) and O'Connor-Sánchez et al. (2002), respectively, in which 70% of 45 temperate maize inbred lines tested and all nine tropical and subtropical lines (inbred and hybrid lines) tested produced proliferative, regenerable cultures from SAM explants. Of interest in the literature describing the evolution of maize tissue cultures derived from SAM or explants containing the SAM is early reference to the elastic nature of these cultures (Zhong et al. 1992) and reports that, from these heterogeneous shoot tip cultures, somatic embryogenic callus could be produced through media manipulations of the cytokinin to auxin ratio (Zhong et al. 1992) or the addition of adenine (O'Connor-Sánchez et al. 2002).

By altering growth regular regimes and duration of explant exposure, Sidorov et al. (2006) capitalized on the elastic nature of apical and axillary bud meristems in the coleoptilar node of germinated seedlings to produce homogeneous Type I callus from several proprietary inbred lines and the public inbred genotype, H99. Briefly, coleoptilar node explants were first cultured on a cytokinin/auxin media before being moved to media containing only the auxins 2,4-D and picloram. On this latter media, a vigorous, fast growing Type I embryogenic callus was produced, primarily from axillary bud meristems, and moved into the dark for proliferation. The Type I callus was then stably transformed using *Agrobacterium*-mediated methods at frequencies ranging from 1.5–11%. Furthermore, 60% of the transgenic events recovered were low copy number events (1–2 copies of the transgene). Although genotype independence has yet to be demonstrated using this alternative explant, the work by Sidorov et al. (2006) marks a new direction in maize transformation. In combining the benefits of using *Agrobacterium*-mediated methods over the biolistic-mediated methods already described (Ishida et al. 1996; Zhao et al. 1998; Shou et al. 2004), with those of transforming maize inbred lines using a mature seed explant, this new method overcomes some of the major limitations imposed by dependency on an IE-based transformation system (continuous plant growing that often requires a large greenhouse space) and on the Hi-II hybrid genotype (not fully suitable for genomic and genetic studies).

Recently, a leaf-based regeneration and transformation system was reported in which a relatively high callus induction frequency was achieved from young leaf bases of the maize hybrid Pa91 x H99 (Ahmadabadi et al. 2007). Addition of polyamines such as spermidine to the medium increased callus induction rates and transgenic plants were produced from bombarded, leaf-derived, Type I callus. Significant in this recent report was the author's claim that production of transformation-competent callus is independent of the presence of the SAM in explanted leaves. Furthermore, the authors describe the production and maintenance of embryogenic callus from leaf explants cultured in the light using a recently described growth regulator, phytosulfokine-alpha (Matsubayashi et al. 1997). Light-maintained maize cultures would be particularly useful in chloroplast transformation because effective antibiotic selection to obtain tissue carrying transformed chloroplasts needs to be carried out in the light (Maliga 2004).

4 Selectable Markers for Maize Transformation

Only a small fraction of the cells that are subjected to transformation actually integrate the DNA into the genome and become progenitors of transgenic plants. Therefore, an efficient selection system, which consists of a selective pressure and a selectable marker gene, is required so that transformed cells may preferentially proliferate. Genes that confer resistance to antibiotics or herbicides, such as kanamycin, hygromycin, phosphinothricin (PPT) and glyphosate, are widely used in higher plant research. If the development of herbicide resistant plants is the goal, the trait gene itself could also serve as a selectable marker gene. A selectable marker gene is an intrinsic component of a plant transformation method and the choice of the system is a key factor in successful transformation. In general, a system is deemed effective if transformed and untransformed cells can clearly be differentiated and transformed cells are not irreversibly damaged by the selective pressure. There is no single selection system that works universally well for all plants species. For example, kanamycin resistance has been most frequently employed in the transformation of dicotyledonous plants whereas hygromycin resistance is most effective in rice transformation (Hiei et al. 1994). Several selective agents that have worked reasonably well in maize are listed in Table 2 and discussed below. It is possible that selections better than any of those listed may yet be discovered because more than 15 selectable marker genes that have already been tested in various other plants remain to be examined in maize (Komari et al. 2006). For a comprehensive review of selectable marker genes in transgenic plants, see Miki and McHugh (2004).

4.1 *Major Markers in Maize Transformation*

The combination of the phosphinothricin acetyltransferase (*bar* or *pat*) genes and the selective agent phosphinothricin (PPT) or its derivatives (bialaphos, PPT+two alanine, or glufosinate, an ammonium salt of PPT) provides a very efficient system for selection in maize and has been effectively used throughout the maize transformation literature since it was first introduced (Fromm et al. 1990; Gordon-Kamm et al. 1990; Ishida et al. 1996; Zhao et al. 2001; Frame et al. 2002, 2006a). Growth of transformed cells is uninhibited whereas untransformed cells are severely inhibited on selective media. Selection of transformed cells is straightforward and deteriorative effects on growth and regeneration of maize cells under the selection pressure is negligible. It is important to note that among the three selective agents used in this system (PPT, bialaphos and glufosinate), bialaphos appears to be the most effective agent for transformation of Hi-II and a number of inbred lines (Dennehey et al. 1994; Wang et al. 2003). Secondly, for selection to work effectively in maize, the *bar* gene should be controlled by a strong promoter.

Transgenic maize can also be effectively recovered using the 5–enolpyruvylshikimate–3–phosphate synthase (EPSPS) selectable marker gene which

Table 2 Selectable markers for maize transformation

Selective pressure	Nature of pressure	Selectable marker gene	Enzyme for protection	Success in maize*	Reference
Resistance to herbicides					
Phosphinothricin (PPT)	Inhibition of glutamine synthase	• *bar* from *Streptomyces hygroscopicus*	PPT-N-acetyltransferase (PAT)	+++	Gordon-Kamm et al. 1990
Bialaphos (alanyl PPT)		• *pat* from *Streptomyces viridochromogenes*			Brettschneider et al. 1997
Glufosinate (PPT ammonium) in BASTA®					
Glyphosate in Roundup®	Inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)	• *epsps* from maize or *Agrobacterium*	EPSPS insensitive to glyphosate	++	Huang et al. 2004
		• Synthetic *gox*	Glyphosate oxidoreductase	+	Howe et al. 2002
Butafenacil	Inhibition of protoporphyrinogen oxidase (PPO)	*ppo* from *Arabidopsis thaliana*	PPO insensitive to butafenacil	+	Li et al. 2003
Sulfonylurea (SU) Imidazolinone (IZ)	Inhibition of acetolactate synthase (ALS)	*als* from maize or *Arabidopsis thaliana*	ALS insensitive to SU and IZ	+	Fromm et al. 1990
Resistance to other drugs					
Kanamycin G418 Paromomycin	Inhibition of protein synthesis	*nptII* from *Escherichia coli*	Neomycin phosphotransferase	+	D'Halluin et al. 1992
Hygromycin	Inhibition of protein synthesis	*hpt* from *Escherichia coli*	Hygromycin phosphotransferase	++	Walters et al.1992
Methotrexate (MTX)	Inhibition of dihydrofolate reductase (DHFR)	*dhfr* from mouse	DHFR insensitive to MTX	+	Golovkin et al. 1993
Positive selection marker					
Mannose as a sole carbon source	Non-transformed cells cannot assimilate mannose	*pmi* from *Escherichia coli*	Phosphomannose isomerase	++	Negrotto et al. 2000

* +++, extremely effective and broadly applicable; ++, effective; +, useful but low in transformation frequency or not extensively studied

confers resistance to glyphosate (Howe et al. 2002). Glyphosate resistance is a deregulated trait used widely in commercialized Roundup Ready™ GE maize varieties in the USA. As such, it can serve as a selectable marker for producing transgenic maize lines destined for commercialization. It should be noted that larger clusters of maize cells may be less sensitive to glyphosate and some non-transgenic calli may be expected to grow using this selection (Y. Ishida, unpublished results). However, selection at the stage of regeneration is more effective and, as also observed in wheat (Zhou et al. 1995; Hu et al. 2003), escapes are rarely regenerated.

The neomycin phosphotransferase II gene (*nptII*) and the antibiotic kanamycin (or its analogs G418 or paromomycin) were employed in early maize transformation attempts (Rhodes et al. 1988; Gould et al. 1991; D'Halluin et al. 1992; Lowe et al. 1995). Although growth of single cells or small clusters of cells is inhibited by these antibiotics, larger clumps of maize cells are much less sensitive. The concentration of antibiotics required in later stages of selection may therefore be high enough to damage selected cells or plants (Y. Ishida, unpublished results). Because of these limitations, the *nptII*/kanamycin selection system is not widely used for maize transformation. In our experience, effective selection pressure for maize was easier to apply using paromomycin than kanamycin (Y. Ishida, unpublished results). For example, we infected IEs of inbred A188 with an *Agrobacterium* strain that carried an *nptII* gene under the control of the *nos* promoter. We modified a standard protocol (Ishida et al. 2007) to include an initial selection pressure of 25 mg/l paromomycin followed by later selection pressure of 50 mg/l paromomycin. Transgenic maize plants were obtained from 7.5% of the infected IEs (Y. Ishida, unpublished results).

The hygromycin phosphotransferase (*hpt*) gene in conjunction with the selection agent, hygromycin, has been used for particle bombardment and *Agrobacterium*-mediated transformation in maize (Walters et al. 1992; Ishida et al. 1996). Growth of untransformed maize cells is suppressed (although not to the degree seen using bialaphos) while transformed cells can be clearly identified on hygromycin containing media. TF using the *hpt* system is typically less than half or one third of that with the *bar* system (Ishida et al. 2007), depending on the genotype used.

Maize may also be transformed using other resistance markers, as listed in Table 2. Although no system has yet been reported that outperforms the *bar*/bialaphos selection system, data related to alternative marker systems are not extensive enough to draw firm conclusions as to their relative efficacy.

Non-antibiotic and non-herbicide selectable marker genes have been developed to overcome concerns about the presence of drug or herbicide resistance genes in commercial transgenic lines. For example, plant cells expressing the phosphomannose isomerase gene (*pmi*) can grow on media with mannose as the sole carbon source. Such markers are referred to as positive selection markers (Joersbo et al. 1998). In maize, the *pmi* gene has been successfully employed with the *Agrobacterium*-mediated transformation method, and direct gene transfer methods such as PEG-mediated and particle bombardment (Evans et al. 1996; Negrotto et al. 2000; Wang et al. 2000; Wright et al. 2001; Ahmadabadi et al. 2007). Generally, transformation frequency using the *pmi* selection system is as high as with the *bar* selection system. However, distinguishing transformants from this selection system may not be obvious for less experienced researchers due to considerable background growth of untransformed cells on mannose-containing selection media (Y. Ishida, unpublished results).

4.2 *Removal of Selectable Markers*

A selectable marker gene is indispensable for identification of transformed cells. However, once the transgenic plants are obtained, the selectable marker genes are not only unnecessary but can also be problematic. Concerns have been expressed over the safety of marker genes and unnecessary DNA segments in plants (Miki and McHugh 2004), therefore the demand for marker-free transgenic plants is high. Technically, the presence of a selectable marker gene in transgenic lines is undesired if further transformations are necessary to introduce additional genes. In this case, a different selectable marker gene must be used to facilitate the secondary transformation. A number of studies focused on this issue in many plants, including maize (Depicker et al. 1985; Schocher et al. 1986; Odell et al. 1990; De Block and Debrouwer 1991; Komari et al. 1996; Ebinuma et al. 1997; Daley et al. 1998; McCormac et al. 2001). Marker genes may be removed from transgenic plants or progeny either by genetic segregation following co-transformation or by specific recombination systems.

4.2.1 Co-Transformation

If a gene of interest and a selectable marker gene are placed on separate DNA segments and a plant cell is co-transformed with these segments, there is a high probability that the segments are integrated into different chromosomes and will therefore segregate in later generations. Co-transformation is a classical approach, in which a GOI and a marker gene is purposely placed on separate plasmids and introduced into plant cells using direct transformation methods such as particle bombardment. Because the number of transgene copies tends to be high in direct transformation, and co-transformed segments tend to link together in one locus in these transformants (Zhong et al. 1999), many transformants may need to be screened for low copy transgenics, from which marker-free progeny may be obtained.

For *Agrobacterium*-mediated co-transformation, Komari et al. (1996) designed super-binary vectors that carried two separate T-DNAs. The first T-DNA contained a selectable marker gene, and the second T-DNA carried the GOI. This system was first tested in tobacco and rice and later confirmed to be effective in maize (Miller et al. 2002; Ishida et al. 2004). About 50–90% of initial transformants were found to be co-transformants (carrying both T-DNAs). In these studies, marker-free progeny were obtained from about half of the co-transformants. In another approach, Huang et al.

(2004) took advantage of the fact that the backbone of a standard binary vector could be considered as a second T-DNA delimited by the left border and the right border in the other orientation. Instead of placing both selectable marker gene and GOI in the T-DNA region, they put the selectable marker gene in the backbone and the GOI in the T-DNA (Huang et al. 2004). Transgenic maize lines were successfully generated by this configuration, and marker-free transgenics were subsequently obtained.

4.2.2 Site-Specific Recombination

Recombinases from phages and yeasts, such as Cre, FLP and R, which recombine specific target sites, *loxP*, *FRT* and *RS*, respectively, are powerful tools to remove selection marker genes and other unneeded segments from plants (Ow 2005, 2007). A selectable marker gene may be placed between two directly orientated target sites (e.g., *lox*) in a transformation vector. Once a transformant is obtained, the recombinase (e.g., *cre* gene in this *lox* example) can be introduced into the cell by cross-pollination or additional rounds of transformation. The *lox*-flanked marker gene will be removed by the Cre recombinase and the *cre* gene can be removed by subsequent genetic segregation. In maize, Zhang et al. (2003) confirmed that the *nptII* marker gene could be efficiently removed by Cre/*lox* recombination via either crossing or auto-excision. Most recently, this practice has led to regulatory approval in the USA of a commercial corn line, LY308, a high lysine corn targeted for use in the poultry industry (http://www.aphis.usda.gov/brs/aphisdocs2/04_22901p_com.pdf).

5 Improvement of Maize Transformation

The past 20 years have seen tremendous technological advances in maize genetics and genomics research and rapid adoption of GE maize in agriculture. The pace of research has been accelerated by the enhanced efficiency of genetic transformation, a powerful technology that allows us to complement mutants, analyze gene functions, and dissect signaling or metabolic pathways. While we are no longer concerned about whether we can produce transgenic maize, we are now challenged by how we can produce transgenic maize of better quality and greater quantity to meet the increasing demands of genomic research.

5.1 Expression and Copy Number of Transgenes

Transgene silencing or unpredictable transgene expression is one of the major concerns in transgenic plant research. Once considered experimental outliers, transgenic events that show gene silencing are now accepted as an important aspect of gene regulation systems in all living organisms. While increased knowledge of the mechanisms for gene silencing is mounting, we are still struggling to take control of this phenomenon and design strategies to minimize its undesired effects in transgenic plants.

It has been widely recognized and also discussed in Subheading 2.3 that if a large number of transgene copies are integrated into the plant genome, there is an increased chance of transgene silencing or suppression of the endogenous genes. Because the *Agrobacterium*-mediated method offers low copy number and simple transgene integration, it is currently the favored method over biolistic gun transformation. In our experience, transformation mediated by *Agrobacterium* in maize has a big advantage over that in rice in terms of copy number of the transgenes. A number of rice and maize (A188) transformation experiments have been carried out using similar super-binary vectors by *bar*, *hpt* and *pmi* selectable markers for years in our laboratory. No matter which marker was employed, the ratio of single copy events was usually 25% to 45% in rice and 50% to 70% in maize (Y. Ishida and Y. Hiei, unpublished results). In these experiments, TF fluctuated to some extent in both rice and maize, but the ratio of single copy events remained quite stable. Although this suggests that overall efficiency of transformation is probably not a reason for this difference, no other explanation has yet been forthcoming

It has also been observed that the type of promoter may affect transgene copy number and the level of silencing of a non-selected transgene (Y. Ishida, unpublished results). Maize inbred line A188 was *Agrobacterium*-transformed with two similar strains that carried identical selectable *bar* gene cassettes but different *gus* gene cassettes (one with CaMV 35S and the other with the *ubi* promoter). Strong GUS expression was detected from almost all of the *ubi* transformants but only 60% of the 35S transformants. Interestingly, Southern analysis revealed that, among these strong expressers, 46% of *ubi* transformants and 74% of 35S transformants, respectively, were single copy events. Among the 40% of weak expressers of 35S transformants, 73% were multiple copy events. One interpretation could be that the 35S promoter might be prone to silencing in multi-copy transformants. Thus, strong 35S-expressers tended to be low-copy transformants.

5.2 Transfer of Vector Backbones

One major drawback of the *Agrobacterium*-mediated method is that high frequencies of vector backbone DNA can be detected in transgenic lines. This has been observed in both monocot and dicot plants (Wenck et al. 1997; De Buck et al. 2000; Shou et al. 2004; Wu et al. 2006). This not-so-precise transgene integration caused by the *Agrobacterium*-mediated transformation process can be a serious issue, especially in development of transgenic events for commercial purposes. Failure of the termination in the process of generation of T-DNA transfer intermediates at the left border of T-DNA appeared to be a major cause of this undesired phenomenon in rice (Kuraya et al. 2004). A method recently examined in rice was to place one, two or three additional copies of the left border sequences in transformation vectors, thereby suppressing the transfer of the segment outside T-DNA to plant cells in a

nearly perfect fashion (Kuraya et al. 2004). This approach was recently tested in maize (Y. Ishida, unpublished results). Immature embryos of A188 were infected with *Agrobacterium* strains, in which one, two, three or four repeats of the left border were designed. A *gus* gene was placed outside the T-DNA in these vectors to monitor the carry over of backbone DNA in transformants. Transient expression of GUS in the embryos infected with multiple left border constructs was markedly lower when compared to the embryos infected with the standard one left border construct, suggesting that the strategy used in rice can also be effective in maize.

5.3 Location of Transgene Integration

Another problem, in addition to the copy number issue of transgene integration, is the unpredictability of transgene location in the targeted genome. It is believed that this transgene "position effect" can significantly affect its expression and stability (Matzke and Matzke, 1998; Day et al. 2000; Ow 2005; Kumar et al. 2006). To overcome this limitation, large numbers of independent transformation events are frequently screened to obtain a few that display simple integration structures and satisfactory expression levels. Numerous molecular strategies were also explored. It was found earlier that matrix attachment regions (MAR) could be used to shield the transgene from the genomic environment. For example, if the *gus* gene was flanked by the tobacco RB7 MAR elements, the number of low GUS expression transformants was significantly reduced (Mankin et al. 2003; Halweg et al. 2005).

Using recombinase-based site specific integration to circumvent position effect has been explored extensively (Ow 2005). The strategy is to first establish a founder line in which the construct carrying the recombination sites (e.g., *lox* or *FRT*) is integrated into a suitable chromosome location in the plant that allows adequate gene expression. The existing integrated construct in the founder line can then be used for sequential insertion of transgenes into the same target locus. This approach has been tested in a number of plants (Ow 2002). In tobacco, Cre-directed site-specific integration places a precise single-copy DNA fragment into the target site in about a third of the selected events. In rice, nearly half of the selected events consist of a single precise copy at the target site (Srivastava et al. 2004). These rates are significantly higher than those reported for homology-dependent insertions (Terada et al. 2002). Moreover, half of the precise single copy insertions in tobacco, and nearly all of those in rice, express the transgene within a range that is predictable and reproducible (Day et al. 2000; Srivastava et al. 2004). This indicates that once a suitable target site is found, the plant line can be used for predictable insertion and expression of genes.

The use of zinc finger nucleases (ZFNs) as molecular scissors for gene targeting is gaining increasing attention in both animal and plant systems (Durai et al. 2005; Porteus and Carroll 2005). ZFNs can be used to induce double-stranded breaks (DSBs) in specific DNA sequences, thereby stimulating site-specific homologous recombination in the targeted genomic loci. Successes in plants include Arabidopsis (Lloyd et al. 2005), tobacco (Wright et al. 2005) and maize (Arnold et al. 2007). While still at its early stage, these studies showed that the use of ZFNs may be promising in targeted plant genome modification.

Recently, two groups reported the development of maize minichromosomes in attempting to overcome the transgene position effect problem. The group of James Birchler (Yu et al. 2006; 2007) used telomere-mediated chromosomal truncation approach to introduce transgenes to an existing chromosome lacking essential genes (such as B chromosomes in maize). They designed a vector carrying Arabidopsis telomere repeats, a selectable marker and a *lox* site-specific recombination site to transform maize IEs using the *Agrobacterium*-mediated method. The addition of transgenic telomeric sequences caused chromosome fragmentation at the site and left the transgenes at the tip of the fractured chromosome. Because the engineered minichromosome provided the *lox* site for site-specific recombination, additional transgenes could then be targeted to the site via the Cre/*lox* recombination system.

The other approach, led by Daphne Preuss, focused on the development of an in vitro-assembled autonomous maize minichromosome (MMC, Carlson et al. 2007). The authors constructed plasmids that carried selectable marker gene *nptII*, fluorescent reporter gene *DsRed*, as well as various segments (7 – 190 kb) of maize genomic DNA containing centromeric repeats and delivered the purified plasmids into maize IEs using the biolistic gun method. They found that 90% of the constructs carrying various centromeric sequences were able to form an autonomous MMC in plant cells. One transgenic line carrying a 19 kb centromeric DNA showed efficient mitotic and meiotic inheritance of MMC through 4 generations.

While these technologies are still at an early stage, they demonstrate the possibility of using chromosome engineering for basic and applied research. These approaches will enable the introduction of several trait genes or genes comprising a complete metabolic pathway on a single DNA fragment with a defined sequence order. More consistent transgene expression may also be expected from these engineered chromosome environments.

5.4 Genome Information for Improvement of Transformation

As maize genetic transformation technology becomes increasingly critical to maize genomic studies, genomic information, conversely, is also becoming important to the improving transformation methods. For example, the tissue culture ability and transformation competency of different maize genotypes may be addressed at the molecular level using various genomic tools. In rice, it was found that ferredoxinnitrite reductase (NiR) was correlated with callus culture regenerability. Overexpressing NiR in a poorly regenerable rice variety increased its regeneration rate significantly (Nishimura et al. 2005). In maize, stimulation of the cell cycle by disruption of the plant retinoblastoma pathway can lead to enhanced transformation frequency (Gordon-Kamm et al. 2002). Efforts in investigating and improving tissue culturability and transformability of maize using marker-assisted breeding and genomic techniques have been reported (Che et al. 2006; Krakowsky et al. 2006;

Lowe et al. 2006). Also, a number of plant genes, including chromatin proteins, have been shown to participate in the T-DNA integration process (Gelvin and Kim 2007).

5.5 Future Goals

By manipulating *Agrobacterium* strains and improving tissue culture conditions, we are now able to transform maize, once considered a plant recalcitrant to transformation using *Agrobacterium* (Hernalsteens et al. 1984). Future improvement of the technology will focus on transformation efficiency (less input for greater output), genotype spectrum (broaden inbred line transformation), integration precision (targeted gene insertion), and delivery size (large DNA fragment transformation for gene stacking or pathway engineering). Increased overall transformation efficiency for a few widely used public inbred genotypes would provide the maize community with immediate, expanded opportunities for genomic research and crop improvement.

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