II.3 Maize

F. Torney, B. Frame, and K. Wang[1]

1 Introduction

Maize (*Zea mays* L. ssp. *mays*) is one of the three most cultivated crops in the world, along with wheat and rice. Modern maize is quite different from its proposed ancestor, the teosintes (*Zea mays* L. ssp. *parviglumis* or *mexicana*), grown by the pre-Columbian and Mesoamerican civilizations. Centuries of selection and genetic improvement of domesticated maize have influenced its evolution into the hundreds of landraces now grown worldwide. Genetic improvement of maize has been sustained by an increase in the variety of uses of this crop (Johnson 2000). In addition to genetic improvement through breeding, recent developments in plant genetic transformation have introduced new possibilities for trait improvement in maize.

1.1 Old and New Uses of Maize

Maize does not exist in the wild. It was created through the domestication of teosinte by the Mesoamerican civilizations for which it was the major staple food. Today, maize is the most important food and feed crop worldwide. In the past 20 years, it has also been increasingly used in industrial processing; and in 2004, close to one-third of United States maize production was for food, feed or industrial use (Fig. 1).

New industrial uses of maize include plastic, sweeteners and ethanol. Zein, a major protein component of the maize kernel, is a good example of this diversification of use. Its thermoplastic and film-forming properties are used by industry to produce paper and paperboard adhesive, additives in oil cloth and linoleum, moisture and oxygen barriers and varnish substitutes (Johnson 2000). In the United States, maize seed is currently refined to produce six major outputs: high fructose corn syrup (HFCS), fuel alcohol, beverage alcohol, glucose and dextrose, starch and food (Fig. 2). In the past 25 years, production of sweeteners (HFCS), and in particular fuel alcohol, has increased dramatically. Production of ethanol from maize is a rapidly developing area of industrial crop utilization, due in part to the search for alternative energy sources. Although any starch-containing grain is theoretically suitable for ethanol production,

[1] Center for Plant Transformation, Plant Science Institute, and Department of Agronomy, Iowa State University, Ames, IA 50011-1010, USA, e-mail: kanwang@iastate.edu

Biotechnology in Agriculture and Forestry, Vol. 59
Transgenic Crops IV (ed. by E.C. Pua and M.R. Davey)

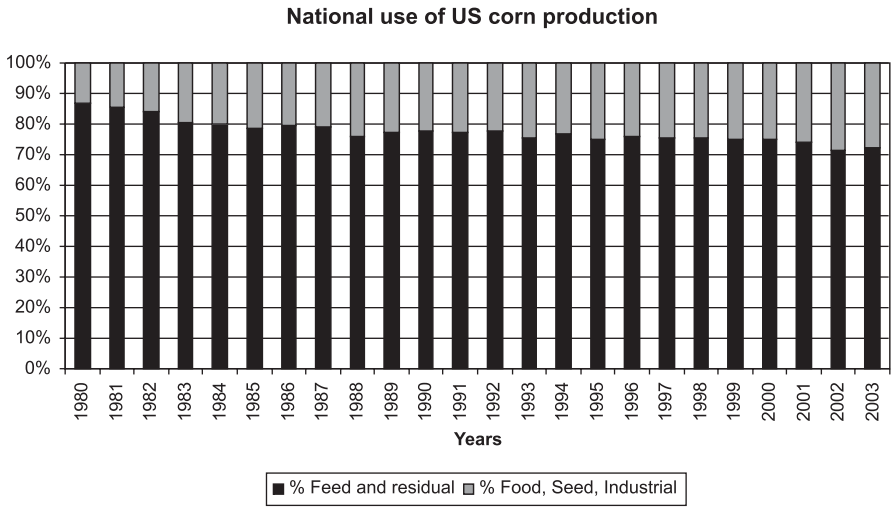

Fig. 1. United States national use of maize from 1980 to 2003. The proportion of industrial use (gray) has steadily increased in 23 years. Data compiled and published in the Corn Refiners Association Annual Report (http://www.corn.org/)

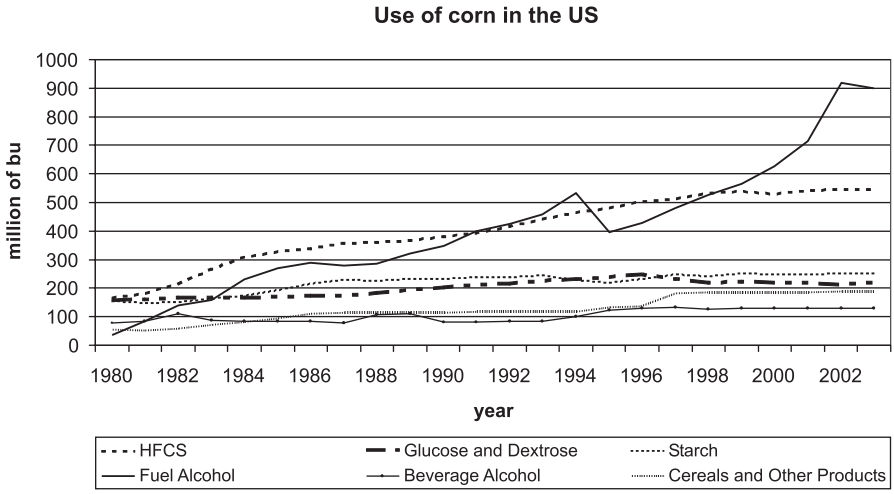

Fig. 2. Diversity and evolution of maize use in the United States from 1980 to 2003 (USDA Economic Research Service, Feed Outlook)

maize represents 95% of the current starting material for this process. For sweeteners, maize competes with sugar beet and sugar cane, the two major sweetener-producing crops in the world.

All continents (except Antarctica) produce maize, which ranks second to wheat in metric tonnes (t) of crop production world-wide. The United States produces and consumes more maize than any other country, with an annual

Table 1. Production and consumption of maize in the world ($\times 10^3$ t). Source: USDA, Foreign and Agricultural Services

Country	2002/2003		2003/2004		2004/2005	
	Production	Consumption	Production	Consumption	Production	Consumption
Brazil	44,500	37,500	42,000	38,600	35,500	38,900
Canada	8,999	12,576	9,600	11,238	8,836	10,905
China	121,300	125,900	115,830	128,400	128,000	131,500
Egypt	6,000	10,900	5,740	9,500	5,780	10,600
India	11,100	12,000	14,720	13,200	13,600	13,600
Indonesia	6,100	7,500	6,350	7,350	6,500	7,200
Mexico	19,280	24,700	21,800	26,400	22,000	27,900
Nigeria	5,200	5,200	5,500	5,500	6,500	5,900
Romania	7,300	7,200	7,020	7,200	12,000	9,400
Serbia Montenegro	5,585	4,850	3,800	4,450	6,274	5,300
South Africa	9,675	8,520	9,700	8,677	12,000	8,950
European Union	49,360	49,526	39,861	46,814	53,350	52,500
United States	227,767	200,748	256,278	211,723	299,917	224,420
World total	601,714	627,224	623,711	647,185	706,263	680,472

production nearing 257×10^6 t and an annual consumption of about 210×10^6 t (Table 1). Second is China, producing and consuming half that of the United States. Most of the United States production and, therefore, the world maize production, is localized in the "corn belt" of Illinois, Iowa, Indiana, Ohio, Minnesota, Missouri and Nebraska.

One of the major turning points in maize production occurred in the 1940s during the green revolution. Maize hybrids, a concept first outlined by Shull (1908) reached 2 t ha^{-1} during this period due to continued germplasm improvement and increased use of agronomic inputs, such as fertilizer and herbicides. Since 1962, maize yields have doubled in the United States and the world average maize yield almost tripled (Fig. 3). In recent years, novel tools such as plant genetic engineering have again revolutionized our approach to crop improvement. Tremendous resources have been directed towards using these technologies to complement traditional breeding strategies in order to improve the productivity of maize. If crop improvement in the green revolution was defined by the synergistic use of plant breeding and increased agronomic inputs, today's gene revolution (http://www.fao.org/newsroom/en/news) is catalyzed by the use of genetic engineering as a complementary tool for improving crop productivity.

1.2 Maize Improvement Through Genetic Engineering

For a ninth consecutive year, the increase in agricultural land cultivated to genetically engineered crops has reached double digits. Globally, land cultivated

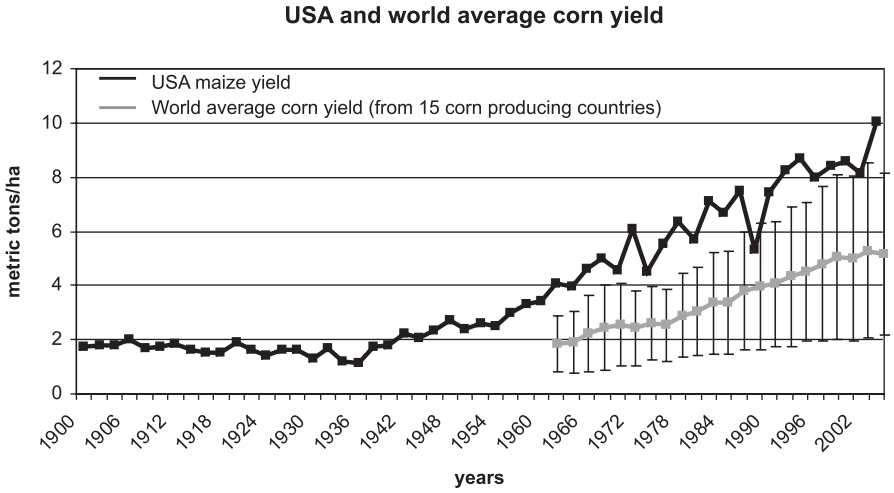

Fig. 3. Progression of maize yield in the United States from 1900 to 2004 (USDA-NASS Statistical Database) and average world maize yield increase from 1962 to 2004 (FAOSTAT 2004). The world data includes Argentina, Australia, Brazil, Canada, Chile, China, France, Germany, India, Kenya, Mexico, Nigeria, South Africa, Spain and Turkey

to biotech crops increased by 20% between 2003 and 2004; and it has increased 47-fold since 1996 (James 2004). Biotech maize (19.3×10^6 ha) is second to soybean (48.4×10^6 ha) in cultivated area devoted to biotech crops world-wide. In 2004, this represented 23% of the total land area planted to biotech crops and 14% of the total land for maize cultivation.

When these numbers are broken down into genetically engineered traits across all biotech crops, herbicide tolerance represents by far the most widely cultivated trait. For maize specifically, it is not herbicide tolerance, but Lepidoptera resistance (*Bt* technology) which represents 14% (11.2×10^6 ha) of the total biotech crop worldwide and 12.9% of the total maize cultivated area (FAOSTAT 2004). In addition, a new tendency is emerging for that of "double trait" biotech crops. The stacking of both herbicide resistance and *Bt* traits in maize, as well as in cotton, represents a major improvement in the agronomic usefulness of these genetically modified crops. More recent developments include the novel use of plant biotechnology for producing pharmaceuticals and industrial products. This remains a controversial development due to concerns about the potential for contamination of food supplies (http://www.plantpharma.org/forum/index.php).

Now, 2005 is the closing year of the first decade of biotech crop commercialization. The fact that the rate of increase in land area committed to these crops continues to increase, even though the purchase price of the seed is generally greater than non-biotech seed (Moschini et al. 2000), is testimony to the success of biotech crops worldwide.

1.3 Maize Genetic Transformation: The Critical Points

Genetic modification of plants through hybridization and selection is the basis of successful crop improvement in agriculture. However, genetic modification of maize using recombinant DNA technology became possible only in the final decade of the past century. The first fertile transgenic maize plants were reported in 1990 (Gordon-Kamm et al. 1990), while the first attempt at inserting a foreign gene into maize was reported as early as 1966 (Coe and Sarkar 1966). In this earliest experiment, DNA from one maize variety was injected into apical meristems of developing seedlings of another maize variety. The donor seedlings from which the DNA was extracted were homozygous for various dominant traits such as red anthers or purple sheaths. The targeted recipient seedlings were homozygous recessive for the same traits. If successful, red pigment was expected to appear in the 242 recipient plants. However, phenotypic evidence of genetic transformation was not observed. Although unsuccessful, this precedent experiment outlined two critical points for achieving maize transformation, namely: penetration and competence.

- Penetration. How effectively can DNA be delivered into plant cells? Coe and Sarkar (1966) emphasized that the cell wall may be a major barrier to DNA delivery. Since then, several DNA delivery techniques have been developed, amongst which the two most efficient techniques for maize are biolistic (Fromm et al. 1990; Gordon-Kamm et al. 1990) and *Agrobacterium*-mediated (Ishida et al. 1996) DNA delivery. The development of both methods required, of course, the correct target tissue.
- Competence. This involves a cell's capacity to first be genetically transformed and then to regenerate into a fully fertile plant. These attributes jointly address the issue of target tissue. For maize transformation, several possibilities have been tested with varying degrees of success. In vitro techniques and development of the appropriate combination of media and selection agent has often been the key for achieving genetic transformation of maize. In turn, the transformation competency of a given target tissue is tightly linked to that of the delivery method.

As techniques for maize genetic transformation improve and the number of transgenic events produced is no longer a limiting factor, considerable focus can now instead be directed towards transgene integration and the stability of transgene expression. This emphasis on the quality of transgenic events underlines the dual nature of efforts to improve maize genetic transformation technology.

This review outlines how maize genetic transformation has improved from the pioneer work of Coe and Sarkar (1966) to the present and emphasizes particular challenges overcome to transform maize. In addition, it presents the application of some new technologies to maize genetic transformation.

2 Penetration – Delivery Methods

The challenge of introducing DNA coding for a gene of interest into a cell is tightly linked to two factors, namely the delivery method and target tissue. Many combinations have been tested over the years and few were successful.

The uptake of DNA by plant cells was demonstrated beginning in the 1960s (Stroun et al. 1966; 1967a, b; Ledoux and Huart 1968; Kleinhofs and Behki 1977). However, these early studies disagreed on the fate of the DNA following uptake. Some groups reported that the DNA was integrated into the plant genome and replicated, while others suggested that the DNA was taken up by cells but then degraded. Even when needles were used to directly inject the DNA solution into maize apical meristems, the cell wall was described as a potential barrier to DNA penetration and the authors suggested that the cell wall may have to "be disrupted mechanically or chemically or otherwise circumvented" (Coe and Sarkar 1966).

2.1 Some Unsuccessful Attempts to Genetically Transform Maize

Numerous additional efforts to achieve maize transformation were based on very interesting concepts, but were unsuccessful and therefore abandoned. Attempts were made to transplant petunia nuclei into maize protoplasts (Potrykus and Hoffmann 1973), but the experiments were carried out under non-sterile conditions and no further analysis was done. Uptake of the blue-green alga *Gloeocapsa* into maize protoplasts was demonstrated, but no long-term persistence of the introduced alga was observed (Burgoon and Bottino 1976). Most of this early work in plant transformation using non-conventional methods was discussed by Kleinhofs and Behki (1977). Two less controversial attempts at introducing exogenous DNA into maize cells were reported 20 years after the work of Coe and Sarkar (De Wet et al. 1985; Ohta 1986). In these experiments, DNA was mixed with pollen and applied to the silks. Some seeds displayed predicted phenotypic changes, but molecular analysis was not provided by the authors (Ohta 1986). Later, Bennetzen and Lin (1988) attempted to rescue alcohol dehydrogenase (adh) defective maize pollen by injecting DNA carrying the cloned *adh-1* gene into spikelets. After staining the pollen for *adh-1* activity, the authors calculated delivery frequency to be 0.005% and concluded that further use of this technique was limited, since it was time-consuming, inefficient and required a large amount of glasshouse space for the donor plants.

More sophisticated strategies were undertaken. Incubating protoplasts in a polyethylene glycol (PEG) solution along with DNA proved to be a successful approach to achieving DNA uptake by maize cells (Armstrong et al. 1990; Golovkin et al. 1993; Omirulleh et al. 1993). Since protoplasts were used, the cell wall barrier was not an issue. The frequency of transformation under optimal conditions using Black Mexican Sweet (BMS) maize cell suspension cultures

was 0.3% (Armstrong et al. 1990). Although this frequency may seem low, it was calculated as 300 independent stable transformation events out of a million cells, and isolating a million protoplasts was feasible by a routine protocol. The DNA was efficiently delivered into maize protoplasts, but plants could not be regenerated from transgenic callus events. This inability to recover plants was a common problem encountered when starting with protoplasts from suspension cultures (Phillips et al. 1988) and likely one of the reasons that PEG-mediated maize transformation was not adopted extensively by the community. Only one laboratory reported successful PEG-protoplast transformation and regeneration of fertile transgenic maize (Golovkin et al. 1993; Omirulleh et al. 1993). In this study, a maize embryogenic suspension culture of genotype HE/89, a line developed by selection for its favorable tissue culture response (Mórocz et al. 1990), was used for transformation. The authors were able to obtain a large number of fertile transgenic maize plants from transformation and regeneration of protoplasts using this line. In addition to these intrinsic tissue culture difficulties, the PEG-protoplast technique itself was more difficult to perform than electroporation, another technique originally developed for protoplasts (Fromm et al. 1985).

2.2 Electroporation

Because the cell wall appeared to be a major barrier to DNA penetration, the use of protoplasts as a starting material for maize transformation was a natural choice. DNA penetration and expression of the introduced transgene was demonstrated in electroporated maize cells (Fromm et al. 1985). BMS microcalli were incubated with a mixture of cellulase, hemi-cellulase and pectinase to digest their cell walls. Purified protoplasts were electroporated in a solution containing plasmid DNA bearing the chloramphenicol acetyl transferase (CAT) gene, which was subsequently detected in the electroporated cells. Stable transformation of protoplasts derived from maize callus was subsequently achieved (Fromm et al. 1986). Eventually, transgenic maize plants were produced using embryogenic cell suspension cultures of the maize line A188 (Rhodes et al. 1988), but these plants were not fertile. Conversely, non-trangenic plants that were fertile were recovered (Prioli and Sondahl 1989; Shillito et al. 1989). Thus, although electroporation enabled penetration of DNA into maize cells, it proved to be an unsatisfactory combination of delivery method and target tissue for obtaining fertile transgenic maize plants.

Several teams developed tissue, as opposed to protoplast, electroporation techniques, including immature zygotic embryos or type I callus (D'Halluin et al. 1992), A188×B73-derived suspension cultures (Laursen et al. 1994) and type II callus (Pescitelli and Sukhapinda 1995). In all cases, successful transformation of maize cells occurred only when the target tissue was wounded either by partially digesting the cell wall (D'Halluin et al. 1992; Laursen et al. 1994), or by mechanical wounding and plasmolysis of cells (D'Halluin et al.

1992; Pescitelli and Sukhapinda 1995). Stable transgenic maize plants were recovered in all cases.

Although electroporation of either protoplasts or entire tissue has been documented, the low efficiency of this approach is unlikely to provide a workable system for the emerging area of functional genomics. Nevertheless, protoplast electroporation has proven to be a valuable tool for analysis of gene function through transient expression assays (Sheen 2001).

2.3 Silicon Carbide Whiskers

While electroporation was being developed, other research teams were following different paths for genetically transforming maize cells. Physical delivery of exogenous DNA into target cells was demonstrated using two major "brute force" methods that circumvented the cell wall barrier, namely biolistic and whiskers. Both techniques rely on the penetration of exogenous particles, and DNA with them, into plant cells.

Silicon carbide whiskers (SiC), needle-shaped crystals used by industry in the production of composite materials (Braun 2004), are one of the simplest ways to introduce DNA into maize cells. In the first report of DNA penetrating a maize cell using SiC, BMS suspension culture cells were mixed with DNA and whiskers, vortexed for one minute, and *gus* gene expression was monitored (Kaeppler et al. 1990). In all samples tested, transient β-glucuronidase activity was detected and, two years later, stable transformation of BMS cells was obtained using this method (Kaeppler et al. 1992). The first fertile transgenic maize plants generated using this method were produced from A188×B73-derived suspension cells (Frame et al. 1994).

Using whiskers to transform maize is an attractive method because it is simple and inexpensive. However, a major drawback of this delivery method is that it has been successful with only a limited number of regeneration proficient tissues, such as well dispersed maize suspension cultures (Frame et al. 1994) or callus cultures (Petolino et al. 2000), for which the latter authors concluded that the system was "not particularly efficient for large-scale transgenic production".

2.4 Biolistics

Development of a high-velocity micro-particle transformation apparatus in 1987 opened new possibilities for cereal transformation (Klein et al. 1987). At that time, *Agrobacterium tumefaciens*-mediated transformation was in its infancy and, in theory, was restricted to dicotyledonous plants. This new idea was to force the penetration of DNA into the cell by accelerating microparticles coated with DNA into the target tissue, thereby overcoming the dilemma of DNA penetration through the cell wall. Because the biolistic delivery system circumvented the need for cell wall removal, more organized tissues could be

targeted for transformation. This invention marked a turning point in the history of maize transformation. *Bt* maize, a transgenic product of this transformation methodology, was commercialized in 1996, only six years after the first reports of fertile transgenic maize production using this technique (Fromm et al. 1990; Gordon-Kamm et al. 1990). Recent research has outlined advantages to targeting maize immature embryos using *A. tumefaciens*-mediated methods instead of the biolistic gun (Zhao et al. 1998; Shou et al. 2004). Nevertheless, biolistic transformation of maize immature embryos remains a widely used technology in many laboratories (Songstad et al. 1996; Brettschneider et al. 1997; Frame et al. 2000).

2.4.1 The Biolistic Armory

"Biolistic" is a generic term for micro-particle bombardment and derives from *biological ballistic* (Armstrong 1999). The original biolistic gun was reported by Klein et al. (1987) and, a year later, its efficacy for delivering DNA to intact BMS maize suspension cells was demonstrated (Klein et al. 1988). By 1990, the production of fertile transgenic maize using this device was reported (Gordon-Kamm et al. 1990). Its original design used a gunpowder charge to propel the DNA-coated microparticles through a vacuum chamber into target cells from the surface of a plastic disk referred to as the macrocarrier (Klein et al. 1987). The macrocarrier, which is also propelled downward by the burst, is intercepted by a metal stopping screen, while the micro-particles continue through the screen to penetrate the target tissue placed towards the bottom of the chamber. Since the late 1980s, the system has been improved and helium gas has replaced gunpowder to provide the kinetic energy that propels the particles (Sanford et al. 1991).

Other particle acceleration methods based on similar concepts have also been developed. Like the biolistic gun, the air gun (Oard et al. 1990) uses a macro-carrier and tungsten micro-projectiles, but it has the advantage of being less costly (Songstad et al. 1995). Transient *gus* gene expression has been observed in bombarded maize suspension cells. In the ACCELL system, DNA-coated gold particles are loaded onto a metal sheet, also called a macro-carrier. An electrical impulse accelerates the particles at a precise velocity controlled by the voltage. The greater control of particle penetration by this device makes it a versatile tool for delivering DNA to a variety of crops, genotypes and tissues (McCabe and Christou 1993).

The Particle Inflow Gun (PIG) was first described by Takeuchi et al. (1992) and later improved by Finer et al. (1992). It directly accelerates DNA-coated particles into plant cells with a gentle burst of gas without the use of a macro-carrier. Minimal damage to target tissue transformed using this gun is attributed to the low-pressure helium stream required to accelerate only micro- and not macro-projectiles. Stable transformation of embryogenic maize suspensions has been achieved (Vain et al. 1993a).

2.4.2 Optimization of Biolistic Parameters

The biolistic gun is a relatively simple, robust and reproducible method for targeting a variety of maize explants for transformation. Stable maize transformation has been achieved using BMS cell suspensions (Klein et al. 1989), embryogenic maize suspension cultures (Fromm et al. 1990; Gordon-Kamm et al. 1990; Register et al. 1994), type II callus (Fromm et al. 1990; Walters et al. 1992; Armstrong et al. 1995; Pareddy and Petolino 1997; Frame et al. 2000), type I callus (Wan et al. 1995) and immature zygotic embryos (Koziel et al. 1993; Songstad et al. 1996; Brettschneider et al. 1997; Frame et al. 2000). The biolistic gun has also been used to reproducibly (although inefficiently) recover stable transgenic maize from shoot apical meristem-derived cultures (Lowe et al. 1995; Zhong et al. 1996; O'Connor-Sanchez et al. 2002; Zhang et al. 2002).

Following original optimization experiments using non-regenerable BMS maize cells (Klein et al. 1988), several reports undertook to optimize biolistic transformation parameters for regenerable maize tissues. A four-fold reduction in the amount of gold used at bombardment increased stable transformation efficiency of pre-cultured H99 immature embryos (Brettschneider et al. 1997). Similarly, a reduction in gold particle size from 1.0 μm to 0.6 μm (Frame et al. 2000) increased stable transformation efficiency in Hi II (Armstrong et al. 1991) type II callus and transient expression of an anthocyanin marker gene in type I callus (Randolph-Anderson et al. 1997). These authors attributed improved transformation rates to a reduction in damage to targeted cells at bombardment, as proposed by Kausch et al. (1995).

Increasing particle velocity by increasing rupture disk pressure from 900 psi to 1,300 psi (1,000 psi = ca. 6.89 MPa) increased stable transformation efficiency for H99 immature zygotic embryos, which produced a type I callus response in culture (Brettschneider et al. 1997). Interestingly, neither transient *gus* gene expression nor the frequency of post-bombardment somatic embryogenesis differed at these two helium pressures; only the rate of stable clone recovery was affected. The authors concluded that the greater rupture disk pressure facilitated DNA delivery to cell layers in which cells competent for transformation and regeneration in these target embryos were located. Similar observations by Dunder et al. (1995) led the authors to recommend greater rupture disk pressures when targeting type I maize cultures and reduced pressures for type II callus cultures. In our laboratory, rupture disk pressures as low as 650 psi are routinely used for targeting both immature embryos and type II callus (Frame et al. 2000).

The type of particle used in biolistic delivery also plays an important role in stable transformation efficiency. Gold particles cost more per unit weight than tungsten particles but have a more homogeneous size and smoother surfaces. These attributes are believed to minimize cell damage at bombardment (Russell et al. 1992). Moreover, tungsten is reported to have a toxic effect on some cells and may impair regeneration (Russell et al. 1992). Comparisons of transient

expression following bombardment of BMS maize cells with gold or tungsten particles coated with the CaMV35S-driven *gus* gene or the rice actin-driven *gus* gene showed more expressing cell clusters when gold was used (Hunold et al. 1994).

In addition to physical parameters, improvements in culture methods have contributed to increased transformation efficiencies using biolistic delivery methods. Four hours prior to bombardment, embryogenic cell suspensions were placed on a medium containing 0.2 M sorbitol and 0.2 M mannitol (Vain et al. 1993b). Osmotic treatment prior to bombardment was hypothesized to minimize cell damage at particle impact by reducing turgor pressure in the cell. The same treatment was applied after bombardment to help cell recovery. The transformation efficiency was significantly greater when both pre- and post-osmotic treatments were applied to the target tissue than when one or the other or neither was done (Vain et al. 1993b). Similar results were achieved using 12% sucrose to increase the osmotic potential of the medium (Dunder et al. 1995). In addition, pre-culturing explanted immature embryos for a period of days prior to bombardment was shown to increase biolistic transformation efficiency (Songstad et al. 1996).

2.5 *Agrobacterium tumefaciens*

While using "brute force" to facilitate penetration of exogenous DNA into plant cells was proven to be very effective, an alternative approach was also being developed.

The role of the soil-borne bacterium *A. tumefaciens* in tumor development in plants was described as early as 1907 (Smith and Townsend 1907). It was confirmed subsequently that this tumor-inducing ability could be attributed to the transfer of a bacterial DNA sequence into the plant host genome (Chilton et al. 1977). Finally, evidence that a non-oncogenic (disarmed) strain of *A. tumefaciens* could be used to introduce a T-DNA sequence modified with exogenous DNA into a plant cell, and that this foreign DNA sequence could be expressed, was reported independently in 1983 by three laboratories (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983). Since *A. tumefaciens* was known to be a pathogen of dicotyledonous and not monocotyledonous plants, its use as a vector for stable plant transformation was initially limited to dicotyledons. Maize infection by *A. tumefaciens* was demonstrated as early as 1986 (Grimsley et al. 1986) and transgene expression in maize cells after *A. tumefaciens* infection was reported by several teams (Schlappi and Hohn 1992; Ritchie et al. 1993; Shen et al. 1993). Recovery of maize plants containing the *gus* and *npt*II genes after inoculation of cut shoot apices with an *Agrobacterium* suspension was reported as early as 1991 (Gould et al. 1991), but not until 1996 was a robust protocol reported for production of stable transgenic maize using an *Agrobacterium*-mediated gene-delivery method (Ishida et al. 1996).

2.5.1 Penetration of the T-DNA into Plant Cells

Penetration of T-DNA from bacterial cells into plant cells is efficient enough to produce transgenic plants, although the mechanism is not completely understood. Two 25-bp border sequences bracket and define the T-DNA region, which is the only part of *Agrobacterium* Ti (tumor-inducing) plasmid DNA transferred into the plant cell. Another critical component on the Ti plasmid is the virulence (*vir*) region located outside of the T-DNA. The *vir* genes produce a number of enzymes and proteins that facilitate the T-DNA transfer. The T-DNA is believed to be cleaved from the Ti plasmid as a single-strand molecule. This single-stranded T-DNA, the T-strand, is coated by *vir*E1 and E2 proteins and capped by *vir*D2 protein in the bacteria. In a process similar to conjugation, *A. tumefaciens* uses a type IV secretion system to transfer this nucleoprotein complex into the plant cell (Gelvin 2000).

Until recently, most efforts to enhance transformation efficiency using *A. tumefaciens* have focused on modifying bacterial components. Using strain LBA4404 to harbor a binary vector containing extra copies of certain *vir* genes (called the super binary vector) resulted in high transformation efficiencies in rice (Hiei et al. 1994) and maize (Ishida et al. 1996; Zhao et al. 1998).

Using forward and reverse genetics, plant mutants have been identified that react differently to the same *A. tumefaciens* strain (Gelvin 2000, 2003; Hwang and Gelvin 2004). This indicates that plant factors are also involved in mediating the transformation process. Modifying the target plant so that it becomes the "ideal" recipient for *A. tumefaciens* may also be one way to enhance transformation efficiency. Gelvin's group identified *Arabidopsis thaliana* "rat" mutants (resistant to *Agrobacterium* transformation; Zhu et al. 2003). One of them was deficient for the *HTA-1* gene coding for H2A-1 histone. Transient expression was observed in this mutant, but stable expression was not obtained (Mysore et al. 2000). Penetration of the T-DNA was observed in the rat-5 mutant by transient *gus* expression, although the plant cells were not competent for stable transformation. Interestingly, *Agrobacterium* transformation by the flower dip procedure (Clough and Bent 1998) was still possible for this mutant. Expression of the wild-type *HTA-1* gene in the rat-5 mutant of *Arabidopsis* restored the competency for stable transformation via tissue culture and enhanced efficiency for transformation using the floral dip method (Mysore et al. 2000).

2.5.2 The Super Binary Vector

The super binary transformation system for maize was the second major turning point in maize genetic transformation after the 1990 reports using the biolistic gun. Ishida et al. (1996) used immature zygotic embryos from the maize line A188 as a target tissue for their seminal work in stably transforming maize using *Agrobacterium tumefaciens*. One of the key points in their success was the use of a Ti plasmid "super binary" system, in which a DNA segment containing *vir*B, *vir*C and *vir*G genes were cloned into the binary vector that

normally carries only genes of interest (Komari and Kubo 1999). The presence of extra copies of these *vir* genes, in addition to the endogenous *vir* genes on the disarmed Ti plasmid, allowed the authors to reach transformation efficiencies as high as 30%. The transformation system used the *bar* gene as a selectable marker and the intron-containing *gus* marker gene, both driven by the CaMV35S promoter. Molecular analysis by Southern blot showed that *A. tumefaciens*-mediated transformation produced large numbers of single- and low copy number transformation events. This high transformation efficiency and high proportion of single-copy events using the super binary vector system was later confirmed using the maize Hi II genotype (Zhao et al. 1998).

2.5.3 The Standard Binary Vector

A. tumefaciens-mediated transformation of maize proved to be a low-cost technique generating transgenic events with simple transgene integration patterns (Ishida et al. 1996) and improved transgene expression stability (Shou et al. 2004) and reports of using standard binary vector system for DNA delivery have also been forthcoming (Frame et at. 2002; Zhang et al. 2003). One advantage of the standard binary vector is that no homologous recombination step is required for introducing the gene of interest into the *Agrobacterium* strain. In our laboratory, *A. tumefaciens* strain EHA101 harboring the standard binary vector pTF102 (Frame et al. 2002) is used to infect 10- to 13-day-old Hi II immature zygotic embryos. Co-cultivation is carried out for three days on medium supplemented with the anti-oxidant L-cysteine, following earlier work in soybean (Olhoft and Somers 2001). Bialaphos-resistant type II callus events are recovered eight weeks after infection at 5% frequency and regenerated to produce stably transgenic R_0 plants and stably transformed R_1 progeny.

3 Competence and Regenerability – Target Tissue

Discussion of maize genetic transformation in this chapter focuses on systems that lead to stable transformation. In order to achieve this end, a transformation-competent targeted cell must first be able to receive DNA fragments and stably integrate them into the nuclear genome. The transformed cell must then, in most cases, proliferate under a suitable selection scheme to distinguish it from non-transformed cells. Finally, a fertile maize plant must be recovered that is germ-line transformed and thus transmits the introduced trait to its progeny.

3.1 Selectable Markers

As aptly emphasized by Vain et al. (1993a), the effectiveness of any target tissue and gene-delivery method is highly dependent on a selectable marker system

and the promoters for recovering transformed cells, although promoters will not be a subject of this chapter. Recent successes in efficient maize transformation have, in part, been attributed to the move away from the aminoglycoside selection agents that had proven so effective for the rapid advancement of dicotyledon transformation in the1980s to the use of the *bar* (bialaphos-resistant) selectable marker gene which confers resistance to phosphinothrycin, the active ingredient in several commercial herbicides (Gordon-Kamm et al. 1991). The use of this selectable marker gene for recovering stable transformants of bombarded maize cells was described by Spencer et al. (1990) using non-regenerable BMS cultures and, shortly thereafter, by Gordon-Kamm et al. (1990) using embryogenic suspension cells in the first report describing the recovery of fertile transgenic maize. More recently, transgenic maize events produced using *Agrobacterium*-mediated methods have been recovered at high frequency, also using this selection system (Ishida et al. 1996, 2003; Zhao et al. 2001). Other herbicide-resistant genes that have been used effectively as selectable marker genes to recover fertile transgenic maize in combination with their complementary herbicides are ALS (acetolactate synthase and chlorsulfuron; Fromm et al. 1990) and EPSPS (5-enolpyruvylshikimate-3-phosphate synthase and glyphosate; Howe et al. 1992).

An effective selectable marker gene for maize that is neither antibiotic nor herbicide-based is the *pmi* (phosphomannose isomerase) gene. A cell expressing this gene can metabolize mannose as a carbon source, thereby conferring a positive advantage to transformed cells under selection on mannose-containing medium (Evans et al. 1996). This selectable marker system has also been reported to work very effectively in *Agrobacterium*-mediated maize transformation systems (Negrotto et al. 2000). The search for so-called "safe" selectable marker systems (Reed et al. 2001), such as *pmi*/mannose, is likely to maintain its momentum and interest as one approach to producing transgenic events that do not carry antibiotic- or herbicide-resistant genes. Recently, the use of D-amino acid oxidase (DAAO) in *Arabidopsis* has shown that, depending on the D-amino acid used, negative selection or a positive selection for transformed cells can be achieved (Erikson et al. 2004). D-Alanine, for example, was an effective negative selectable marker agent for *Arabidopsis* and these authors also described its toxicity in maize. This preliminary evidence suggests the possibility of using D-alanine and the DAAO gene as a new selection system for maize.

3.2 Stable Integration of Transgenes

The first requirement for competency of a targeted cell is that it be capable of stably integrating exogenous DNA. Depending on the transfer method, exogenous DNA penetrates the cell in different forms. Physical delivery methods such as electroporation, whiskers and the biolistic gun use naked DNA, whereas *Agrobacterium* transformation transfers a nucleoprotein complex.

These differences lead to downstream consequences regarding exogenous DNA integration. Both processes remain largely unknown, but recent progress has shed light on various aspects of transgene integration into the plant genome. Most of the information available on the integration mechanism comes from sequencing and analysis of transgene integration sites.

3.2.1 Transgene Integration via Physical Delivery Methods

Current understanding of the process of exogenous DNA integration is quite poor. The transgene is understood to integrate into the genome by illegitimate recombination (IR) or non-homologous end-joining (NHEJ; Kohli et al. 2003). One of the hypotheses is that DNA integration is a two-phase process (Kohli et al. 1998).

The first phase is a pre-integration one in which the exogenous DNA is processed by the cell before being integrated into the genome. After bombardment, the cells enter a stress-response phase as demonstrated by callose formation (Hunold et al. 1994). During this phase, multiple enzymes are activated, including those for DNA repair and DNA degradation. It is believed that a combined action of those two types of enzymes on the freshly introduced DNA may result in multimerization of complete and incomplete exogenous DNA molecules (Kohli et al. 1998), which may explain the complex integration patterns found when sequencing transgene integration sites (Makarevitch et al. 2003).

The second phase occurs at integration. Double-stranded break-points in the host genome may serve as landing sites for the intact or rearranged exogenous DNA by stimulating the cell's recombination machinery (Kohli et al. 1998). Such temporary "hot spots" may become the site for IR or NHEJ, since separately introduced transgenes frequently integrate at the same site in the genome (Register et al. 1994; Kohli et al. 2003). These sites vary in targeted cells, resulting in the multitude of integration patterns observed when a number of independent transgenic events are compared. Interestingly, transgene copy numbers assessed by detailed Southern blot analysis could not reveal the real complexity of an insertion site as effectively as could the use of sequencing (Kohli et al. 2003).

3.2.2 T-DNA Insertion

How the T-DNA is inserted into the host genome remains somewhat of a mystery. Some pieces of the puzzle have, however, been elucidated. T-DNA enters the cell as a nucleoprotein complex. The VirD2 protein that caps the T-DNA and the VirE2 protein both contain nuclear localizing signals (NLS). The VirD2 NLS signal interacts with a host importin-α (Ballas and Citovsky 1997). Modification of the VirD2 NLS alters the nuclear localization of the T-DNA nucleoprotein complex and affects transformation efficiency (Shurvinton et al. 1992; Mysore

et al. 2000). Interestingly, the VirE2 NLS does not interact with the same host protein, but rather with the VirE2 interacting protein 1 (VIP-1) to allow nuclear import (Tzfira et al. 2001). *A. tumefaciens* seems to have developed two parallel systems to insure the nuclear import of its transferred T-DNA. The molecular process of the insertion itself is not yet fully understood, although some groups suggest that micro-homologies between the T-DNA and the insertion site aid in integration (Mayerhofer et al. 1991; Kohli et al. 2003).

3.3 From Targeted Cell to Fertile Plant

Several plants, like tobacco, can be regenerated from leaf explants (Vasil et al. 1964; Vasil and Hildebrandt 1965). However, early work with maize tissue cultures demonstrated totipotency only from shoot meristem or some embryo-derived cells (Green and Phillips 1975). Although immature embryos from six inbred lines were cultured in this study, embryogenic scutellar callus capable of regenerating plants was produced from only inbred line A188, or its reciprocal crosses with inbred line R-navajo. These results demonstrated the totipotency of immature embryo-derived callus but also emphasized that its production was genotype dependent, a characteristic that still presents problems for maize genetic transformation 30 years later.

The ideal maize explant to target for transformation would be one for which germ-line transformation is ensured with as short a period of in vitro culture as possible. Potential maize transformation target explants, ranked from high to low for morphological complexity, are presented in Fig. 4. In this simplistic depiction, the less complex the targeted explant, the greater the probability of germ-line transformation. In turn, if prolonged culture is re-

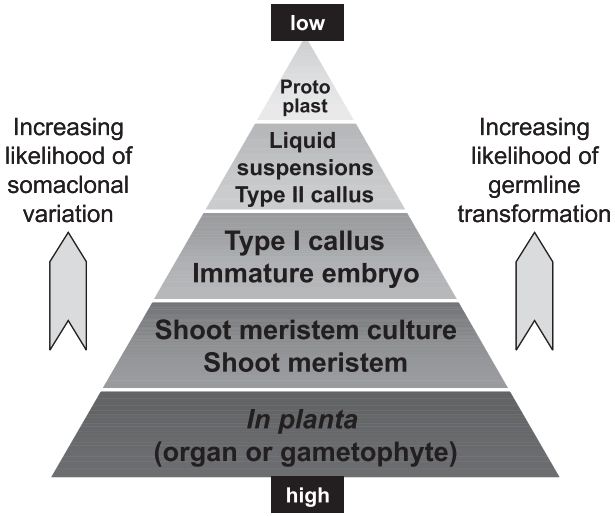

Fig. 4. Morphological complexity of potential maize target explants

quired to achieve this undifferentiated or dissociated state, the probability of somaclonal variation increases (Lee and Phillips 1987). Unicellular protoplasts were considered an ideal target to ensure germ-line transformation and recovery of non-chimeric maize plants, but failure to recover fertile transgenic plants from transformed protoplasts was attributed to loss of totipotency after prolonged culture (Rhodes et al. 1988). Tissue culture-induced variation would be all but eliminated were *in planta* transformation achieved, but targeting a plant organ would also reduce the likelihood of germ-line transformation unless it was a gametophyte. Although the latter scenario has been achieved in *Arabidopsis thaliana* using the floral dip method (Bechtold et al. 1993), no conclusive evidence for maize gametophyte or *in planta* transformation has yet been reported. Interestingly, this *in planta* approach to transformation was that taken in some of the earliest attempts to transform maize and remains an intriguing prospect today.

Since the introduction of the biolistic gun as a method for transforming maize, three maize target tissues have been commonly used, namely the immature zygotic embryo, immature embryo-derived scutellar callus and callus-derived liquid suspension cultures. Although the immature embryo is the starting ex-plant for all these target tissues, it was the last amongst them to be targeted for stable transformation, in part because of the trade-offs illustrated in Fig. 4. Instead, early biolistic experiments were carried out using liquid suspension cultures or friable type II callus, based on the premise that targeted explants had to be reduced to their most dissociated state (least morphologically complex) prior to DNA delivery to ensure that non-chimeric transgenic plants would be regenerated from single transformed cells. The first report demonstrating stable maize transformation (using electroporation) from more organized explants such as immature embryos and type I callus (D'Halluin et al. 1992) stated that "embryogenic, friable, type II cell cultures were no longer a prerequisite for genetic transformation of maize". Furthermore, the authors argued that Mendelian inheritance of their transgene demonstrated the non-chimeric nature of their primary transformants and, therefore, the single cell origin of their transgenic events. By challenging the premise that morphologically complex explants were less competent transformation targets, these results helped expand the range of potential target tissues in maize.

3.3.1 Callus or Suspension Cultures

Embryogenic callus can be produced from scutellum cells of maize immature zygotic embryo explants dissected 10–14 days after pollination and placed with their adaxial side in contact with auxin containing medium. Beginning 2–3 days later, embryogenic callus forms from rapidly dividing cells in the abaxial, basal region of the scutellum (Fransz and Schel 1990).

Maize callus phenotypes are characterized as type I or type II. Using maize genotype A188, high concentrations of proline and N6 salts (Chu et al. 1975),

friable, rapidly growing, highly embryogenic callus was developed (Armstrong and Green 1985) and called type II to differentiate it from that which had been observed previously (designated type I). In contrast to type II callus, in which embryogenic structures are independently suspended in the friable callus matrix, the latter is compact and embryogenic lobes show a high degree of association.

Type II embryogenic callus, like non-regenerable BMS callus (Sheridan 1982), could be used to produce finely aggregated liquid suspension cultures. While BMS cell cultures were used for preliminary optimization experiments with the biolistic gun (Klein et al. 1988) and whiskers (Kaeppler et al. 1990, 1992), it was suspension cultures produced from highly embryogenic type II (A188×B73) callus that facilitated production of the first fertile transgenic plants using the gene gun (Gordon-Kamm et al. 1990) and whiskers (Frame et al. 1994). Recovery of some somaclonal variant transgenic lines using these target cells (Gordon-Kamm et al. 1990) was attributed to prolonged time in culture and cryo-preservation was used by these and other authors (Register et al. 1994) to extend the "shelf-life" of particularly desirable cell lines. As early as 1990, stable biolistic transformation of type II callus was also reported (Fromm et al. 1990) and was rapidly followed by several reports of targeting type II callus using the gun (Walters et al. 1992; Pareddy and Petolino 1997; Frame et al. 2000) and whiskers (Petolino et al. 2000).

Stable transgenic maize plants have been regenerated from type I callus transformed using electroporation (D'Halluin et al. 1992) and the biolistic gun (Wan et al. 1995; Wang et al. 2003). Because many inbred or elite maize genotypes often produce type I callus instead of type II callus from cultured immature embryo scutella (Duncan et al. 1985), establishing the transformation competence of this more structurally complex tissue was a significant development for expanding the inventory of maize genotypes accessible to transformation. However, when compared with friable type II callus, the compact, differentiated and slow growing characteristics of type I callus require that increased selection pressure and labor be used for effective recovery of transgenic events after transformation (Pareddy and Petolino 1997).

Finally, targeting callus cultures for transformation provides access to maize genotypes that display notoriously low embryogenic callus induction frequency from immature zygotic embryos. Callus cultures (type I or type II) can be bulked up from the few responding embryos of a recalcitrant genotype, providing access via biolistic transformation (Wan et al. 1995; Wang et al. 2003) to a callus phenotype and genotype of choice.

3.3.2 Immature Zygotic Embryos

Seventeen years ago, Klein et al. (1988) observed transient *gus* gene expression in scutellar cells of maize immature zygotic embryos following particle bombardment. Armed with the additional knowledge that totipotent callus could

be produced from this explant (Green and Phillips 1975; Armstrong and Green 1985), the authors stated that the immature embryo would be a prime target for transformation. Today, where dedicated glasshouse or growth chamber space is available for embryo donor plant production, this has become the explant of choice for biolistic or *Agrobacterium*-mediated transformation.

While the target explant is the immature embryo scutellum, it acts as a repository for abaxial somatic cells located from one (Fransz and Schel 1990) to five cell layers deep (Pareddy and Petolino 1997) which, when precultured in the presence of auxin, undergo rapid division and differentiation into embryogenic cell clusters to form embryogenic callus. It is from these proliferating, totipotent cells that transgenic events are recovered after selection. Importantly, prolonged tissue culture steps are not required to produce this population of transformation competent cells, thereby minimizing loss of totipotency due to somaclonal variation (Songstad et al. 1996). Immature embryos have been directly targeted for routine stable transformation using both the biolistic gun (Koziel et al. 1993; Songstad et al. 1996; Frame et al. 2000) and *Agrobacterium*-mediated methods (Ishida et al. 1996, 2003; Zhao et al. 1998, 2001; Negrotto et al. 2000; Frame et al. 2002).

Development of the hybrid Hi II line (Armstrong et al. 1991), in conjunction with a medium regime on which 100% of F_1 or F_2 immature embryos produced type II embryogenic callus (Armstrong and Green 1985; Songstad et al. 1996), has enabled widespread use of the immature embryo explant for transgenic research and production using either biolistic or *Agrobacterium*-mediated transformation methods. Our laboratory, for example, routinely transforms Hi II immature zygotic embryos using the PSD-1000/He delivery system with an average transformation frequency of 15 bialaphos resistant events per 100 bombarded embryos.

Recently, this "transformability" of Hi II germplasm was transferred to an elite stiff stalk line using marker assisted breeding; and an efficient *Agrobacterium*-mediated protocol for transforming this type II elite line callus was developed (Lowe et al. 2004).

3.3.3 Maize Inbred Line Transformation

In spite of the outlined advantages to targeting immature embryos, transformation of maize inbred or elite lines for crop improvement using this explant is limited by the quality and frequency of embryogenic callus induction from immature embryos of a given genotype on a given medium. Except for some inbred lines such as A188 (Armstrong and Green 1985), the response frequency is generally low, despite attempts to identify a genotype-independent culture system for this explant (Duncan et al. 1985; Carvalho et al. 1997). Use of breeding to improve the tissue culture response is one approach to targeting elite lines (Armstrong et al. 1992; Lowe et al. 2004). Some success has also been reported for directly targeting immature embryos of inbred or elite lines (other

than A188) using the biolistic gun (Koziel et al. 1993; Brettschneider et al. 1997; Wang et al. 2003) or *A. tumefaciens* (Ishida et al. 2003; Frame et al. 2005; Huang and Wei 2005).

3.3.4 Shoot Meristems

Poor induction of embryogenic callus from the immature embryo explants of many maize inbred lines led researchers to investigate alternative explants from which to develop transformation competent cells. If, in addition, mature seeds could be used as starting material for producing target cells, one would not have to rely on greenhouse space to grow maize plants for weekly production of immature embryos. Targeting shoot apical meristems of germinated seeds was the subject of the first attempt at maize genetic transformation 40 years ago (Coe and Sarkar 1966) and recently has been one of renewed interest.

Because the fate of cells in the apical meristem is predetermined by their location in that meristem (Bowman and Eshed 2000), the challenge to using these cells as transformation targets is either to deliver DNA into a germ-line progenitor cell, or to reprogram the cell by manipulating culture conditions before or after transformation, to ensure stable transformation. If not, meristem transformation and regeneration produces a chimeric plant which does not transmit the transgene to its progeny (Cao et al. 1990; Gould et al. 1991).

Two approaches have been used to produce stable transgenic progeny from maize shoot apical meristem explants. In the first system, the apical domes of coleoptile-stage immature embryos were bombarded with an antibiotic selectable marker and a *gus* reporter gene, and plants germinated. Sectors in the apical meristems of these regenerated plants that were chimeric for the introduced transgene were proliferated on medium containing a cytokinin and the antibiotic selection agent that inhibited chloroplast development. In this way, chimeric plantlets could be regenerated and, through sequential vegetative propagation of de novo meristems, transgenic plants and progeny were eventually obtained (Lowe et al. 1995).

In the second system, the authors used medium supplemented with both cytokinin and auxin to induce heterogenous multiple shoot meristem cultures from the shoot apical meristem (SAM) of germinated mature seeds (Zhong et al. 1996). In spite of concerns that biolistic targeting of morphologically complex shoot tip cultures would lead to chimeric plant production, stable transformation and transmission of the transgene to progeny was demonstrated, although at low frequency, and chimeric plants were not reported. This was attributed to the morphologically elastic phenotype of the bombarded SAM cultures used (Zhong et al. 1992). For example, by varying relative concentrations of cytokinin and auxin, these cultures could also be induced to produce somatic embryos directly from the SAM, or from callus derived from the SAM. Inter-conversion between embryogenic and organogenic callus in SAM-derived cultures of subtropical and tropical maize was observed

using a similar medium supplemented with adenine (O'Connor-Sanchez et al. 2002). Using the biolistic gun, stably tranformed plants and progeny were also produced in this latter study.

One important advantage to targeting SAM-derived cultures for transformation is that their production is reported to be somewhat genotype-independent. SAM cultures were produced in 70% of 45 temperate zone inbred and hybrid lines tested (Li et al. 2002a) and all nine tropical and subtropical lines reported (O'Connor-Sanchez et al. 2002). In addition, SAM cultures of the well known but notoriously recalcitrant inbred line B73 have been transformed using the biolistic gun, although at low frequency (Zhang et al. 2002).

Progress towards stably transforming these heterogeneous, shoot meristem-derived tissues using *Agrobacterium* has been reported (Li et al. 2002b). Alternatively, shoot apical meristems were first incubated with *Agrobacterium* cells and then induced to form either somatic embryogenic callus or organogenic shoot meristem cultures from which R_0 transgenic plants, but no progeny, were recovered (Sairam et al. 2003). When embryogenic callus was first induced (directly or indirectly) from mature seed derived nodal sections and then co-cultivated with *A. tumefaciens* (Sidorov et al. 2006), stable transformation was achieved and transgenic progeny plants were recovered.

In a recent study, potentially novel, embryogenic tissues derived from the embryo explant of mature seeds of seven maize inbred lines, including Mo17, were described (Huang and Wei 2004) but not targeted for transformation. While implementation of this methodology has yet to be reported by other laboratories, it is indicative of the widespread interest in developing readily available, genotype-independent, competent cells for maize crop improvement using genetic transformation technology.

4 Future Prospects

As already discussed, targeting a wider range of maize genotypes, and some inbred lines in particular, for research or trait improvement purposes will continue not least of all in an effort to marry transformation technology with current genomic characterization of specific inbred lines. Maize transformation (both biolistic and *Agrobacterium*-mediated methods) is now a routine but not a trivial task. The production and maintenance of large numbers of transgenic maize plants requires committed resources and efficient organization. As functional genomics demands more transgenic plants for gene analysis, the importance of improving transgenic plant quality becomes imminent.

Currently, the quality of transgenic events is addressed by using the "numbers game" strategy. Hundreds of independent events are produced routinely to select for a few "successful" events (Z.Y. Zhao, personal communication). For most academic laboratories, this production is an unattainable task. In the next stage of improving maize transformation, the quality issue will likely be addressed using different strategies.

For research programs, it may be sufficient to simply choose transgenic events that strongly and stably express the transgene under study. However, for a commercial program, the quality of a transgenic event also pertains to how "clean" it is; i. e., does it (1) only contain one copy of the gene of interest, (2) only contain the desired and essential sequences and (3) not contain the selectable marker. Furthermore, control of pollen dissemination to avoid cross-pollination with non-transgenic maize may be of particular interest especially if maize producing pharmaceutical and industrial products is commercialized.

4.1 Clean Insertion Strategies

4.1.1 Isolated Cassette Bombardment

Biolistic transformation is known to generate complex integration patterns, which is believed to cause transgene silencing (Fagard and Vaucheret 2000) and may also be a legal concern for biotech crop commercialization. In order to overcome these drawbacks, several strategies have been tested.

In a standard biolistic transformation experiment, the entire vector sequence is delivered together with the transgene into a targeted cell, such that they cannot be separated as Mendelian traits. One solution to avoid their insertion is to use transgene expression cassettes only instead of complete plasmids carrying the vector backbone and the antibiotic resistance gene for bacterial selection. In rice, using a supercoiled plasmid, or a double-stranded isolated gene expression cassette for transformation, resulted in no loss of transformation frequency using the latter (Breitler et al. 2002). Furthermore, the occurrence of silencing decreased drastically in T_0 plants. However, this approach did not seem to decrease the complexity of the integration site based on the Southern blots shown in the work (Breitler et al. 2002).

4.1.2 Site-Directed Recombination

Site-specific DNA integration, as well as specific excision of a DNA fragment, are attractive ways of solving many issues inherent to genetic engineering (randomness of the transgene insertion or presence of undesired sequences in the transgene integration site, for example). Both phenomena can be achieved through site-specific recombination: the recombination of two precisely oriented sequences (target sites), either in two separate DNA molecules or in the same DNA molecule, catalyzed by a specific enzyme, the recombinase (Ow 2002). For instance, if the target sites bracket the selectable marker gene (e. g., *bar* or *npt*II) in the same orientation, it becomes possible to remove the undesirable gene by expressing a recombinase (Lyznik et al. 1996; Ow 2001; Zhang et al. 2003). Other applications can be envisaged for this sequence removal strategy. Simplification of complex integration sites by removing extra sequences is possible. This was demonstrated using wheat as a model and a modified

Cre/lox system (Srivastava et al. 1999). In all four independent transgenic events produced with lox target site-containing plasmids, and crossed with a Cre recombinase expression line, simplification of transgene integration pattern was observed as well as selectable marker sequence removal (*bar* gene). In similar experiments with maize, simple insertion events were also obtained (Srivastava and Ow 2001).

Another application for site-directed recombination is site-directed insertion, the most desired feature for transgenic transformation. However, to achieve this illusive goal, an integration site (a sequence to be targeted) must first be established, either through genomic characterization or genetic transformation to allow insertion of the gene of interest by the second round of transformation (Albert et al. 1995; Srivastava and Ow 2002). The concepts and possible applications are reviewed by Ow (2006).

Site-directed recombination appears to be operable, but more data is required to substantiate its effectiveness. Thanks to the numerous site-specific recombination systems in existence, several variations to this approach are available (Lyznik et al. 1996; Sugita et al. 2000; Ow 2001). However, some issues may be associated with future adoption of the method. For example, the lox sites are undesirable for validation of commercial products because of their bacterial origin. The presence of the recombinase gene in the final product is also not desirable and requires an extra breeding step for removing it through segregation.

4.1.3 A. tumefaciens Vector/Strain Improvement

One of the important features in *Agrobacterium*-mediated transformation is that the bacterium transfers only its T-DNA region to the recipient plant genome, such that transgenic events generated by this method should contain only the desired T-DNA segment. In fact, it appears that the *Agrobacterium* method is not as "clean" as had been hoped. Using this method, a high percentage of vector backbone elements were carried into transgenic plants (Kononov et al. 1997; Meza et al. 2002; Shou et al. 2004). Even if these elements do not influence transgene expression (Meza et al. 2002), undesirable backbone sequences can hinder legal validation of a transgenic event. This problem is far from negligible, since the presence of the vector backbone has been detected in 50–75% of independent events investigated in two separate studies (Kononov et al. 1997; Shou et al. 2004). Strategies, such as using double T-DNA border sequences (Kuraya et al. 2001) and negative selection markers inserted into the backbone, are currently being tested to reduce the occurrence of vector backbone integration.

Another feature of the *Agrobacterium*-mediated method has been to allow removal, by segregation, of the selectable marker gene. Two separate T-DNAs are used instead of one. One carries the gene of interest and the other carries the selection marker. This approach has been tested in rice and tobacco (Depicker

et al. 1985; Komari et al. 1996; McCormac et al. 2001; Breitler et al. 2004). Depending on the strategy used to deliver both T-DNAs, the co-transformation efficiency varies from study to study. However, in all of them, some transgenic events, both in rice and tobacco, show segregation of the two T-DNA in the progeny. A two T-DNA binary system was used in maize to achieve high-efficiency transgene segregation in co-transformed maize plants (Miller et al. 2002).

Recently, the ability has been demonstrated of other bacteria (*Sinorhizobium meliloti*, *Rhizobium* sp. NG234, *Mesorhizobium loti*) to transfer DNA into plant cells (Broothaerts et al. 2005). Although these transformation efficiencies are low compared to *Agrobacterium*-mediated transformation, using other bacteria to mediate exogenous DNA delivery to plant cells may open new possibilities for crop biotechnology (Gelvin 2005).

4.2 Control Pollen

Pollen dissemination, leading to unwanted out-crossing of transgenic pollen with non-transgenic plants, is a major issue for maize transgenic crop production. Several approaches can be envisaged to address this issue. One strategy is simply to physically and temporally isolate a transgenic maize crop from other non-transgenic maize fields. In 2003, a one mile (1.6 km) distance was defined as the minimal isolation radius for open pollination tests for pharmaceutical-producing maize plants in addition to delayed planting for 28 days (USDA-APHIS 2003). An extensive study on transgenic maize pollen flow showed that very little cross-pollination could be found at 300 m away from the transgenic pollen source (Stevens et al. 2004).

Although physical and temporal isolation minimizes any possible pollen contamination, biological containment measures are being developed. Two other possibilities can be investigated for pollen control, namely male-sterile lines (natural or induced) and organelle transformation.

4.2.1 Cytoplasmic Male Sterility

One way to implement transgenic pollen containment is to prevent the production of pollen. Cytoplasmic male sterility (CMS) has been studied in maize in the context of hybrid seed production because it prevents labor costs associated with manual de-tasseling (Levings 1993). CMS is a maternally inherited trait preventing formation of viable pollen. It is generally associated with mitochondrial defects (Mackenzie et al. 1994). The first CMS to be described in maize was the Texas cytoplasm (cms-T; Rogers and Edwardson 1952). It was used for hybrid seed in the United States until the Southern corn leaf blight epidemic in the 1970s (Ullstrup 1972) demonstrated a high susceptibility of this male sterile germplasm to the disease (Levings 1993). Despite this drawback, it is

possible to envisage the use of CMS as one approach to pollen containment when transgenic maize is produced on a small scale.

Another possibility is to use genic male sterility (GMS), which is triggered by a defect in a nuclear gene and therefore segregates as a normal Mendelian trait. Recent work described a system in which transcription of an inverted repeat of the Ms45 gene promoter led to transcriptional gene silencing of the Ms45 gene. This induced a high frequency of male-sterile plants lacking the Ms45 transcript (Cigan et al. 2005). Such a system, if added to a traditional gene of interest expression system, would bring about the desired biological containment of the transgene by producing male sterile plants.

4.2.2 Plastid Transformation

Another biological containment approach for transgenic pollen drift is to place the transgene in the plastid genome. Because inheritance of chloroplast traits is maternal in maize (Daniell and Varma 1998), Mendelian transmission of a transgenic locus associated with the nuclear genome would be circumvented and pollen grains would not carry the transgene of interest. The first successful plastid transformation was achieved using the biolistic gun to transform *Chlamydomonas reindhartii* (Boynton et al. 1988) and this delivery method is still the tool of choice for plastid transformation. Plastid transformation offers many advantages over nuclear transformation, but is lagging behind nuclear transformation because of its current low efficiency and the issue of heteroplasty.

Two major plastid transformation strategies have been used so far, namely (1) transformation of the chloroplast genome by homologous recombination (Maliga 2004) and (2) use of an autonomous replicating vector in the plastid (Staub and Maliga 1994). Using the first approach, the gene of interest is bracketed by two regions highly homologous to a precise part of the chloroplast genome and the construct is introduced by biolistic transformation allowing a targeted insertion within the chloroplast genome (Lutz et al. 2001; Maliga 2004). A derivative of a homologous recombination strategy using a phiC-31 phage site-specific recombination system has been described (Lutz et al. 2004), in which a target site was first inserted using homologous recombination. In a second round of transformation, a simpler vector was used in combination with either a nuclear integrated or a transiently expressed recombinase gene. Targeted insertion was observed as frequently as 17 independent transformation events per bombardment.

The use of plastids as targets for transformation offers the added advantage that recombination protein production yields are higher than in nuclear transformants. Such an approach was recently described by Tregoning et al. (2004) for the production of vaccine in plants.

5 Concluding Remarks

Maize is an economically important crop with rich genetic information that, like wheat, has benefited from human intervention for agronomic trait improvement. Because of its importance, the need for improving production levels and broadening the uses of maize has warranted development of genetic engineering technologies to meet this goal. Although maize genetic transformation is now a routine application in some laboratories, it is far from being a trivial technique. Since the first attempts of genetic transformation, many approaches have been explored by the research community to achieve stable and quality maize transformation events. Through the aim of achieving reproducible inbred line transformation, it is feasible that completely new approaches may evolve from those presently described. The issue of what approach is taken towards crop genetic engineering remains a society debate that scientists, politicians and the public will undertake for maize and all other biotech crops.

Acknowledgements. This work is partly supported by the Plant Genome Research Program of the National Science Foundation (DBI 0110023).

References

Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. Plant J 7:649–659

Armstrong CL (1999) The first decade of maize transformation: a review and future perspective. Maydica 44:101–109

Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214

Armstrong CL, Petersen WL, Buchholz WG, Bowen BA, Sulc SL (1990) Factors affecting PEG-mediated stable transformation of maize protoplasts. Plant Cell Rep 9:335–339

Armstrong CL, Green CE, Phillips RL (1991) Development and availability of germplasm with high Type II culture formation response. Maize Genet Coop News Lett 65:92–93

Armstrong CL, Romero-Severson J, Hodges TK (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, Parker GB, Pershing JC, Brown SM, Sanders PR, Duncan DR, Stone T, Dean DA, Deboer DL, Hart J, Howe AR, Morrish FM, Pajeau ME, Petersen WL, Reich BJ, Rodriguez R, Santino CG, Sato SJ, Schuler W, Sims SR, Stehling S, Tarochione LJ, Fromm ME (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

Ballas N, Citovsky V (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. Proc Natl Acad Sci USA 94:10723–10728

Bechtold N, Ellis J, Pelletier G (1993) In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci Paris Life Sci 316:1194–1199

Bennetzen JL, Lin C (1988) Transformation of Adh null pollen to Adh+ by microinjection. Maize Genet Coop News Lett 62:113–114

Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187

Bowman JL, Eshed Y (2000). Formation and maintenance of the shoot apical meristem. Trends Plant Sci 5:110–115

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534–3158

Braun HJ (2004) Ceramic material. In: Hempstead CA, Worthington WE Jr (eds) Encyclopedia of 20th century technology. Taylor & Francis/Routledge, Kentucky, pp 134–136

Breitler JC, Labeyrie A, Meynard D, Legavre T, Guiderdoni E (2002) Efficient microprojectile bombardment-mediated transformation of rice using gene cassettes. Theor Appl Genet 104:709–719

Breitler JC, Meynard D, Van Boxtel J, Royer M, Bonnot F, Cambillau L,Guiderdoni E (2004). A novel two T-DNA binary vector allows efficient generation of marker-free transgenic plants in three elite cultivars of rice (*Oryza sativa* L.). Transgenic Res 13:271–287

Brettschneider R, Becker D, Loerz H (1997) Efficient transformation of scutellar tissue of immature maize embryos. Theor Appl Genet 94:737–748

Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LM, Yang W, Mayer JE, Roa-Rodriguez C, Jefferson RA (2005) Gene transfer to plants by diverse species of bacteria. Nature 433:629–633

Burgoon AC, Bottino PJ (1976) Uptake of the nitrogen fixing blue-green algae *Gloecapsa* into protoplasts of tobacco and maize. J Hered 67:223–226

Cao J, Wang YC, Klein TM, Sanford J, Wu R (1990) Transformation of rice and maize using the biolistic process. In: Lamb CJ, Beachy RN (eds) Plant gene transfer. (UCLA Symp Mol Cell Biol, vol 129) Wiley–Liss, New York, pp 21–33

Carvalho CHS, Bohorova N, Bordallo PN, Abreu LL, Valicente FH, Bressan W, Paiva E (1997) Type II callus production and plant regeneration in tropical maize genotypes. Plant Cell Rep 17:73–76

Chilton MD, Drummond MH, Merio DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11:263–271

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu YC, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Cigan M, Unger-Wallace E, Haug-Collet K (2005) RNAi-mediated transcriptional silencing of anther-expressed genes results in male sterile maize. Annu Maize Genet Conf Proc Abstr 47:93, Abstr 93

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Coe E, Sarkar K (1966) Preparation of nucleic acids and a genetic transformation attempt in maize. Crop Sci 6:432–435

Daniell H, Varma S (1998) Chloroplast-transgenic plants: panacea – no! Gene containment – yes! Nat Biotechnol 16:602

De Wet JMJ, Bergquist RR, Harlan JF, Brink DE, Cohen CE, Newell CA,De Wet AE (1985) Exogenous gene transfer in maize (*Zea mays*) using DNA-treated pollen. In: Chapman GP, Mantell SH, Daniels RW (eds) Experimental manipulation of ovule tissues. Longman, London, pp 197–209

Depicker A, Herman L, Jacobs A, Schell J, Montagu MV (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol Gen Genet 201:477–484

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

Duncan DR, Williams ME, Zehr BE, Widholm JM (1985) The production of callus capable of plant regeneration from immature embryos of numerous *Zea-mays* genotypes. Planta 165:322–332

Dunder E, Dawson J, Suttie J, Page G (1995) Maize transformation by microprojectile bombardment of immature embryos. In: Potrykus I, Spangenberg G (eds) Gene transfer to plants. Springer, Berlin Heidelberg New York, pp 127–138

Erikson O, Hertzberg M, Nasholm T (2004) A conditional marker gene allowing both positive and negative selection in plants. Nat Biotechnol 22:455–458

Evans R, Wang A, Hanten J, Alterdorf R, Metter I (1996) A positive selection system for maize transformation. In Vitro Cell Dev Biol Plant 32:72A

Fagard M, Vaucheret H (2000) (Trans)gene silencing in plants: how many mechanisms? Annu Rev Plant Physiol Plant Mol Biol 51:167–194

FAOSTAT (2004) FAOSTAT statistical database. Food and Agriculture Organization of the United Nations, Rome. Available at: apps.fao.org/faostat/form?collection=Production.Crops.Primary&Domain=Production&servlet=1&hasbulk=0&version=ext&language=EN

Finer JJ, Vain P, Jones MW, McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep 11:323–328

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP,Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803–4807

Frame BR, Drayton PR, Bagnall SV, Lewnau CJ, Bullock WP, Wilson HM,Dunwell JM, Thompson JA, Wang K (1994) Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. Plant J 6:941–948

Frame BR, Zhang H, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE,Zhen S, Schnable P, 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

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

Frame B, McMurray J, Fonger TM, Main M, Torney F, Margie P, Taylor K, Wang K (2005) *Agrobacterium*-mediated stable transformation of multiple maize inbred lines using a standard binary vector system. Annu Maize Genet Conf Proc Abstr 47:68, Abstr 60

Fransz PF, Schel JHN (1990) Cytodifferentiation during the development of friable embryogenic callus of maize (*Zea mays*). Can J Bot 69:26–33

Fromm M, Taylor LP, Walbot V (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82:5824–5828

Fromm ME, Taylor LP, Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. Nature 319:791–793

Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833–839

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256

Gelvin SB (2003) Improving plant genetic engineering by manipulating the host. Trends Biotechnol 21:95–98

Gelvin SB (2005) Agricultural biotechnology: gene exchange by design. Nature 433:583–584

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

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WGO, Brien JV, Chambers SA, Whitney J, Adams R, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Gordon-Kamm WJ, Spencer TM, O'Brien JV, Start WG, Daines RJ, Adams TR, Mangano ML, Chambers SA, Zachwieja SJ, Willetts NG, Adams WR Jr, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1991) Transformation of maize using microprojectile bombardment: update and perspective. In Vitro Cell Dev Biol Plant 27:21–27

Gould J, Devey M, Hasegawa O, Ulian EC, Peterson G, Smith RH (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol 95:426–434

Green CE, Phillips RL (1975) Plant regeneration from tissue cultures of maize. Crop Sci 15:417–427

Grimsley N, Hohn B, Hohn T, Walden R (1986) "Agroinfection" an alternative route for viral infection of plants by using the Ti plasmid. Proc Natl Acad Sci USA 83:3282–3286

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
Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282
Howe AR, Tamayo F, Brown Armstrong C, Fromm M, Hart J, Padgette S, Parker G, Horsch R (1992) Development of glyphosate as a selectable marker for the production of fertile transgenic corn plants. In Vitro Cell Dev Biol 28:124A
Huang XQ, Wei ZM (2004) High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays* L.). Plant Cell Rep 22:793–800
Huang X, Wei Z (2005) Successful *Agrobacterium*-mediated genetic transformation of maize elite inbred lines. Plant Cell Tissue Organ Cult 83:187–200
Hunold R, Bronner R, Hahne G (1994) Early events in microprojectile bombardment: cell viability and particle location. Plant J 5:593–604
Hwang HH, Gelvin SB (2004) Plant proteins that interact with VirB2, the *Agrobacterium tumefaciens* pilin protein, mediate plant transformation. Plant Cell 16:3148–3167
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
Ishida Y, Saito H, Hiei Y, Komari T (2003) Improved protocol for transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Plant Biotechnol 20:57–66
James C (2004) Preview: global status of commercialized biotech/GM crops: 2004. International Service for the Acquisition of Agri-Biotech Applications Briefs 32
Johnson LA (2000) Corn, the major cereal of the americas. In: Kulp K, Ponte J (eds) Handbook of cereal science and technology. Dekker, New York, pp 31–80
Kaeppler HF, Gu W, Somers DA, Rines HW, Cockburn AF (1990) Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Rep 9:415–418
Kaeppler HF, Somers DA, Rines HW, Cockburn AF (1992) Silicon carbide fiber-mediated stable transformation of plant cells. Theor Appl Genet 84:560–566
Kausch AP, Adams TR, Mangano M, Zachwieja SJ, Gordon-Kamm W, Daines R, Willets NG, Chambers SA, Adams W Jr, Anderson A (1995) Effects of microprojectile bombardment on embryogenic suspension cell cultures of maize (*Zea mays* L.) used for genetic transformation. Planta 196:501–509
Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73
Klein TM, Fromm M, Weissinger A, Tomes D, Schaaf S, Sletten M, Sanford JC (1988) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc Natl Acad Sci USA 85:4305–4309
Klein TM, Kornstein L, Sanford JC, Fromm ME (1989) Genetic transformation of maize cells by particle bombardment. Plant Physiol 91:440–444
Kleinhofs A, Behki R (1977) Prospects for plant genome modification by nonconventional methods. Annu Rev Genet 11:79–101
Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci USA 95:7203–7208
Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247–258
Komari T, Kubo T (1999) Methods of genetic transformation: *Agrobacterium tumefaciens*. In: Vasil IK (ed) Molecular improvement of cereal crops. Kluwer Academic, Dordrecht, pp 43–82
Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174
Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J 11:945–957

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merline E, Rhodes R, Warren GW, Wright M, Evola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11:194–200

Kuraya Y, Komari T, Hiei Y (2001) Vectors for transforming plants. Patent WO 2001/025459 A1

Laursen CM, Krzyzek RA, Flick CE, Anderson PC, Spencer TM (1994) Production of fertile transgenic maize by electroporation of suspension culture cells. Plant Mol Biol 24:51–61

Ledoux L, Huart R (1968) Integration and replication of DNA of *M. lysodeikticus* in DNA of germinating barley. Nature 218:1256–1259

Lee M, Phillips R (1987) Genomic rearrangements in maize induced by tissue culture. Genome 29:122–128

Levings CS III (1993) Thoughts on cytoplasmic male sterility in cms-T maize. Plant Cell 5:1285–1290

Li W, Masilamany P, Kasha KJ, Peter Pauls K (2002a) Developmental, tissue culture, and genotypic factors affecting plant regeneration from shoot apical merstems of germinated (*Zea mays* L.) seedlings. In Vitro Cell Dev Biol Plant 38:285–292

Li W, Masilmany P, Kasha KJ, Peter Pauls K (2002b) *Agrobacterium tumefaciens*-mediated transformation of corn multi-shoot cultures. In: IAPTC&B (ed) Tenth IAPTC&B congress proceedings. IAPTC&B, Orlando, Abstr P-1416

Lowe BA, Way MM, Kumpf JM, Rout JR, Johnson R, Warner D, Armstrong C, Spencer TM, Chomet PS (2004) Development of a transformation competent elite maize line by marker assisted breeding. World Congr In Vitro Biol 40:P-2030

Lowe K, Bowen B, Hoerster G, Ross M, Bond D, Pierce DA, Gordon Kamm W (1995) Germline transformation of maize following manipulation of chimeric shoot meristems. Bio/Technology 13:677–682

Lutz KA, Knapp JE, Maliga P (2001) Expression of *bar* in the plastid genome confers herbicide resistance. Plant Physiol 125:1585–1590

Lutz KA, Corneille S, Azhagiri AK, Svab Z, Maliga P (2004) A novel approach to plastid transformation utilizes the phiC31 phage integrase. Plant J 37:906–913

Lyznik LA, Rao KV, Hodges TK (1996) FLP-mediated recombination of FRT sites in the maize genome. Nucleic Acids Res 24:3784–3789

Mackenzie S, He S, Lyznik A (1994) The elusive plant mitochondrion as a genetic system. Plant Physiol 105:775–780

Makarevitch I, Svitashev SK, Somers DA (2003) Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. Plant Mol Biol 52:421–432

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55:289–313

Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K,Redei GP, Schell J, Hohn B, Koncz C (1991) T-DNA integration: a mode of illegitimate recombination in plants. EMBO J 10:697–704

McCabe D, Christou P (1993) Direct DNA transfer using electric discharge particle acceleration (ACCELL^{TM} technology). Plant Cell Tissue Organ Cult 33:227–236

McCormac AC, Fowler MR, Chen DF, Elliott MC (2001) Efficient co-transformation of *Nicotiana tabacum* by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. Transgenic Res 10:143–155

Meza TJ, Stangeland B, Mercy IS, Skarn M, Nymoen DA, Berg A,Butenko MA, Hakelien AM, Haslekas C, Maza-Zepeda LA, Aalen RB (2002) Analyses of single-copy *Arabidopsis* T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. Nucleic Acids Res 30:4556–4566

Miller M, Tagliani L, Wang N, Berka B, Bidney D, Zhao ZY (2002) High efficiency transgene segregation in co-transformed maize plants using an *Agrobacterium tumefaciens* 2 T-DNA binary system. Transgenic Res 11:381–396

Mórocz S, Donn G, Németh 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
Moschini G, Lapan H, Sobolevsky A (2000) Roundup Ready® soybeans and welfare effects in the soybean complex. Agribusiness 16:33–55
Mysore KS, Nam J, Gelvin SB (2000) An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. Proc Natl Acad Sci USA 97:948–953
Negrotto D, Jolley M, Beer S, Wenck AR, 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
Oard JH, Paige DF, Simmonds JA, Gradziel TM (1990) Transient gene expression in maize, rice, and wheat cells using an airgun apparatus. Plant Physiol 92:334–339
O'Connor-Sanchez A, Cabrera-Ponce JL, Valdez-Melara M, Tellez-Rodriguez P, Pons-Hernandez JL, 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
Ohta Y (1986) High-efficiency genetic transformation of maize by a mixture of pollen and exogenous DNA. Proc Natl Acad Sci USA 83:715–719
Olhoft PM, Somers DA (2001) L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep 20:706–711
Omirulleh S, Abraham M, Golovkin M, Stefanov I, Karabaev MK, Mustardy L,Mórocz S, Dudits D (1993) Activity of a chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. Plant Mol Biol 21:415–428
Ow DW (2001) The right chemistry for marker gene removal? Nat Biotechnol 19:115–116
Ow DW (2002) Recombinase-directed plant transformation for the post-genomic era. Plant Mol Biol 48:183–200
Ow DW (2006) Transgene management via multiple site-specific recombination systems (2004 SIVB congress symposium proceedings). In Vitro Cell Dev Biol Plant (in press)
Pareddy D, Petolino J (1997) Maize transformation via helium blasting. Maydica 42:143–154
Pescitelli SM, Sukhapinda K (1995) Stable transformation via electroporation into maize type II callus and regeneration of fertile transgenic plants. Plant Cell Rep 14:712–716
Petolino JF, Hopkins NL, Kosegi BD, Skokut M (2000) Whisker-mediated transformation of embryogenic callus of maize. Plant Cell Rep 19:781–786
Phillips RL, Somers DA, Hibberd KA (1988) Cell/Tissue culture and in vitro manipulation. In: Sprague GF Jr, Dudley JW (eds) Corn and corn improvement. American Society of Agronomy, Madison, pp 345–387
Potrykus I, Hoffmann F (1973) Transplantation of nuclei into protoplasts of higher plants. Z Pflanzenphysiol 69:287–289
Prioli LM, Sondahl MR (1989) Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L). Bio/Technology 7:589–594
Randolph-Anderson B, Boynton JE, Dawson J, Dunder E, Eskes R, Gillham NW, Johnson A, Perlman PS, Suttie J, Heiser WC (1997) Sub-micron gold particles are superior to larger particles for efficient Biolistic® transformation of organelles and some cell types. Bio-Rad Tech Bull 2015
Reed J, Privalle L, Luann Powell M, Meghji M, Dawson J, Dunder E, Suttie J, Wenck A, Launis K, Kramer C, Chang YF, Hansen G, Wright M (2001) Phosphomannose isomerase: an efficient selectable marker for plant transformation. In Vitro Cell Dev Biol Plant 37:127–132
Register JC III, Peterson DJ, Bell PJ, Bullock WP, Evans IJ, Frame B, Greenland AJ, Higgs NS, Jepson I, Jiao S, Lewnau CJ, Silick JM, Wilson HM (1994) Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol Biol 25:951–961
Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer JJ (1988) Genetically transformed maize plants from protoplasts. Science 240:204–207

Ritchie SW, Liu CN, Sellmer JC, Kononowicz H, Hodges TK, Gelvin SB (1993) *Agrobacterium tumefaciens*-mediated expression of *gusA* in maize tissues. Transgenic Res 2:252–265
Rogers JS, Edwardson JR (1952) The utilization of cytoplasmic male-sterile inbreds in the production of corn hybrids. Agron J 44:8–13
Russell JA, Roy MK, Sanford JC (1992) Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol 98:1050–1056
Sairam RV, Parani M, Franklin G, Lifeng Z, Smith B, MacDougall J, Wilber C, Sheikhi H, Kashikar N, Meeker K, Al-Abed D, Berry K, Vierling R, Goldman SL (2003) Shoot meristem: an ideal explant for *Zea mays* L. transformation. Genome 46:323–329
Sanford JC, Devit MJ, Russell JA, Smith FD, Harpending PR, Roy MK, Johnston SA (1991) An improved, helium-driven biolistic device. Technique 3:3–16
Schlappi M, Hohn B (1992) Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. Plant Cell 4:7–16
Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127:1466–1475
Shen WH, Escudero J, Schläppi M, Ramos C, Hohn B, Koukoliković-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of β-glucuronidase activity in maize tissues. Proc Natl Acad Sci USA 90:1488–1492
Sheridan WF (1982) Black Mexican sweet corn: Its use for tissue cultures. In: Sheridan WF (ed) Maize for biological research. Plant Molecular Biology Association, Charlottesville, pp 385–388
Shillito RD, Carswell GK, Johnson CM, DiMaio JJ, Harms CT (1989) Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technology 7:581–587
Shou H, Frame B, Whitham S, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Mol Breed 13:201–208
Shull GH (1908) The composition of a field of maize. Am Breed Assoc Rep 4:296–301
Shurvinton CE, Hodges L, Ream W (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. Proc Natl Acad Sci USA 89:11837–11841
Sidorov V, Gilbertson L, Addae P, Duncan D (2006) *Agrobacterium*-mediated transformation of seedling-derived maize callus. Plant Cell Rep 25:320–328
Smith EF, Townsend CO (1907) A plant-tumor of bacterial origin. Science 25:671–673
Songstad DD, Somers DA, Griesbach RJ (1995) Advances in alternative DNA delivery techniques. Plant Cell Tissue Organ Cult 40:1–15
Songstad DD, Armstrong CL, Petersen WL, Hairston B, Hinchee MA (1996) Production of transgenic maize plants and progeny by bombardment of Hi-II immature embryos. In Vitro Cell Dev Biol 32:179–183
Spencer TM, Gordon-Kamm WJ, Daines RJ, Start WG, Lemaux PG (1990) Bialaphos selection of stable transformants from maize cell culture. Theor Appl Genet 79:625
Srivastava V, Ow DW (2001) Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct. Plant Mol Biol 46:561–566
Srivastava V, Ow DW (2002) Biolistic mediated site-specific integration in rice. Mol Breed 8:345–350
Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA 96:11117–11121
Staub J M, Maliga P (1994) Extrachromosomal elements in tobacco plastids. Proc Natl Acad Sci USA 91:7468–7472
Stevens WE, Berberich SA, Sheckell PA, Wiltse CC, Halsey ME, Horak MJ,Dunn DJ (2004) Optimizing pollen confinement in maize grown for regulated products. Crop Sci 44:2146–2153
Stroun M, Anker P, Charles P, Ledoux L (1966) A biochemical and cytological study of the penetration of deoxyribonucleic acid in plants. Arch Int Physiol Biochim 74:320–321
Stroun M, Anker P, Ledoux L (1967a) DNA replication in *Solanum lycopersicum* esc. after absorption of bacterial DNA. Curr Mod Biol 1:231–234
Stroun M, Anker P, Charles P, Ledoux L (1967b) Translocation of DNA of bacterial origin in *Lycopersicum esculentum* by ultracentrifugation in caesium chloride gradient. Nature 215:975–976

Sugita K, Kasahara T, Matsunaga E, Ebinuma H (2000) A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. Plant J 22:461–469

Takeuchi Y, Dotson M, Keen NT (1992) Plant transformation: a simple particle bombardment device based on flowing helium. Plant Mol Biol 18:835–839

Tregoning J, Maliga P, Dougan G, Nixon PJ (2004) New advances in the production of edible plant vaccines: chloroplast expression of a tetanus vaccine antigen, TetC. Phytochemistry 65:989–994

Tzfira T, Vaidya M, Citovsky V (2001) *VIP1*, an *Arabidopsis* protein that interacts with *Agrobacterium VirE2*, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. EMBO J 20:3596–3607

Ullstrup AJ (1972) The impact of the southern corn leaf blight epidemics of 1970–1971. Annu Rev Phytopathol 10:37–50

USDA-APHIS (2003) Field testing and plants engineered to produce pharmaceutical and industrial compounds. Fed Regul 11:337

Vain P, Keen N, Murillo J, Rathus C, Memes C, Finer JJ (1993a) Development of the particle inflow gun. Plant Cell Tissue Organ Cult 33:237–246

Vain P, McMullen MD, Finer JJ (1993b) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep 12:84–88

Vasil IK, Hildebrandt AC, Riker AJ (1964) Endive plantlets from freely suspended cells and cell groups grown in vitro. Science 146:76–77

Vasil V, Hildebrandt AC (1965) Differentiation of tobacco plants from single, isolated cells in microcultures. Science 150:889–892

Walters DA, Vetsch CS, Potts DE, Lundquist RC (1992) Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. Plant Mol Biol 18:189–200

Wan Y, Widholm JM, Lemaux PG (1995) Type I callus as a bombardment target for generating fertile transgenic maize (*Zea mays* L.). Planta 196:7–14

Wang K, Frame B, Marcell L (2003) Genetic transformation of maize. In: Jaiwal PK, Singh R (eds) Plant genetic engineering. Sci Tech, Houston, pp 175–217

Zhang S, Williams-Carrier R, Lemaux PG (2002) Transformation of recalcitrant maize elite inbreds using in vitro shoot meristematic cultures induced from germinated seedlings. Plant Cell Rep 21:263–270

Zhang W, Subbarao S, Addae P, Shen A, Armstrong C, Peschke V, Gibertson L (2003) Cre/lox-mediated marker gene excision in transgenic maize (*Zea mays* L.) plants. Theor Appl Genet 107:1157–1168

Zhao ZY, Gu W, Cai T, Tagliani LA, Hondred D, Bond D,Krell S, Rudert ML, Bruce WB, Pierce DA (1998) Molecular analysis of T0 plants transformed by *Agrobacterium* and comparison of *Agrobacterium*-mediated transformation with bombardment transformation in maize. Maize Genet Coop News Lett 72:34–37

Zhao ZY, Gu W, Cai T, Tagliani LA, Hondred D, Bond D,Schroeder S, Rudert M, Pierce D (2001) High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Mol Breed 8:323–333

Zhong H, Srinivasan C, Sticklen MB (1992) In-vitro morphogenesis of corn (*Zea mays* L.). Planta 187:483–489

Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T,Sticklen MB (1996) The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiol 110:1097–1107

Zhu Y, Nam J, Humara JM, Mysore KS, Lee LY, Cao H, Valentine L, Li J, Kaiser AD, Kopecky AL, Hwang HH, Bhattacharjee S, Rao PK, Tzfira T, Rajagopal J, Yi H, Veena Yadav BS, Crane YM, Lin K, Larcher Y, Gelvin MJK, Knue M, Ramos-Oliva C, Zhao X, Davis SJ, Kim SI, Ranjith-Kumar CT, Choi YJ, Hallan VK, Chattopadhyay S, Sui X, Ziemienowicz A, Matthysse AG, Citovsky V, Hohn B, Gelvin SB (2003) Identification of *Arabidopsis* rat mutants. Plant Physiol 132:494–505