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DNA-Delivery Methods to Produce Transgenic Plants

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Abstract: Since the 1980s, diverse methods for plant transformation have been described including biological, chemical and physical based methods. Transformation is performed to introduce novel traits, study basic biological processes, or produce recombinant proteins of interest. We review *Agrobacterium*-mediated transformation as well as non-biological based approaches for the production of transgenic plants. This review presents the methods of gene transfer into plants, applications, advantages and disadvantages of each method.

Key words: Agrobacterium, agroinoculation, biolistics, in planta, laser, liposome, oral vaccine, pollen tube, polyfection

INTRODUCTION

The second green revolution needed for the production of enough food for the world will likely include plant biotechnology. At first glance, molecular biology is now an old technology, but its power has recently been realized and it continues to evolve. The first production of recombinant DNA molecules with the use of biochemical scissors called restriction enzymes (Smith and Wilcox, 1970; Meselson and Yuan, 1968) occurred in the early 1970s and subsequently, the first stably transformed plants were reported in the early 1980s (Herrera-Estrella, 1983). After this engineered herbicide-tolerant varieties in 1987 were allowed to be field planted in the US by the US Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS; Comai *et al*., 1985). Presently, transgenic crops are increasingly becoming a dominant feature of the agricultural landscapes of the USA and other countries. The estimated global area for 2003 was 67.7 million ha and for 2005 was 90 million ha. Since the introduction of Genetic Modified (GM) crops in 1996, the average growth in area has been more than 10% per year. This growth represents one of the highest adoption rates for any crop technology (Dunwell, 2004; James, 2005).

While transgenic plants have transformed agriculture, there are even greater potential impacts in non-agricultural uses. Transgenic crops to produce medically useful products might represent the foundations of a new pharming (pharmaceutical farming) industry. Plants have many advantages compared with traditional systems for the molecular farming of pharmaceutical proteins (Teli and Timko, 2004). These include the low cost of production, rapid scalability, the absence of human pathogens and the ability to fold and assemble complex proteins accurately (Ma *et al*., 2003). At present, transgenic plants and plant cell culture systems are becoming widely accepted as a general platform for the large-scale production of recombinant proteins like plantbodies and plantigens (Fischer *et al*., 2004; Artsaenko *et al*., 1998). Potato is one of the candidate plants as a bioreactor for recombinant protein production and is also one of the potential crops for production of the edible vaccine for oral virus immunization (Richter *et al*., 2000). Furthermore, transgenic plants are widely used in basic plant biology. All the above factors drive the continued evolution of transgenic plant technologies.

There are two requisite biotechnologies for the production of transgenic plants. First is the stable introduction of new genetic material into plant cells (transformation) which is the main focus of this review. Second is utilization of tissue culture methods to regenerate transgenic plants from single transformed cells. Gene delivery systems involve the use of several

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techniques for transfer of isolated genetic materials into a viable host cell. At present, there are two classes of delivery systems: biological and non-biological (which the latter includes physical and chemical) systems. The process of transgenic plant production is a complex multi-stage procedure involving three discrete phases as follows: 1. Exogenous DNA is delivered the host cell; 2. Exogenous DNA is stably integrated into the host cell's genome in a manner that will result in proper expression of the gene; and 3. A viable transgenic plant is recovered. In this review, we will discuss various transformation methods and their applications to develop transgenic plants. This research represents an update of other reviews on subtopics of this study (Stanton, 2003; De la Riva *et al*., 1998; Rommens *et al*., 2004; Li *et al*., 1993; Racoczy-Trijanowska, 2002; Lazzeri and Shewry, 1994).

BIOLOGICAL GENE TRANSFER

Biologically-based transformation systems are biomimetic, relying on natural processes. However, plant biotechnologists have turned often negative biological processes (at least for the host cell); e.g., infection and mutagenesis, into positive forces that are intended on increasing the fitness of the host.

Agrobacterium-mediated transformation: In the 1970s the prospect of using *Agrobacterium tumefaciens* for the rational gene transfer of exogenous DNA into crops was revolutionary. Genetic transformation of plants was viewed as a prospect. In retrospect, *Agrobacterium* was the logical and natural transformation candidate to consider since it naturally transfers DNA (T-DNA) located on the tumor inducing (Ti) plasmid into the nucleus of plant cells and stably incorporated the DNA into the plant genome (Chilton *et al*., 1977). Now, some thirty years later, this method has been the most widely-used and powerful technique for the production of transgenic plants. There still remain, however, many challenges for genotype-independent transformation of many economically important crop species, as well as forest species (Stanton, 2003; De la Riva *et al*., 1998).

The direct cloning (De Frammond *et al*., 1983; Hoekema *et al*., 1983) of cassettes into T-DNA, which are subsequently transferred into the plant genome with the vir genes residing are on a separate helper plasmid was proved to be more amenable and stable. This binary system uses smaller binary vectors (about l0-15 kb) and does not require a co-integration step. Also, this system could be used to transfer many genes at once-up to 150 kb (Hamilton, 1997; Hamilton *et al*., 1996, 1999; Rossi *et al*., 1996; Liu *et al*., 1999).

Despite the development other non-biological methods of plant transformation (Shillito *et al*., 1985; Potrykus, 1991; Uchimiya *et al*., 1986; De la Pena *et al*., 1987; Arencibia *et al*., 1992, 1995; Fromm *et al*., 1985, 1986; Sanford, 1988), *Agrobacterium*-mediated transformation remains popular and among the most effective. This is especially true among most dicotyledonous plants, where *Agrobacterium* is naturally infectious. *Agrobacterium*-mediated gene transfer into monocotyledonous plants was thought to be not possible. However, reproducible and efficient methodologies were established for rice (Hiei *et al*., 1994), banana (May *et al*., 1995), corn (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997), sugarcane (Enriquez-Obregon *et al*., 1997, 1998; Arencibia *et al*., 1998), forage grasses such as Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) (Bettany *et al*., 2003). Among the commercially important conifers, hybrid larch was the first to be stably transformed via co-cultivation of embryogenic tissue with *A. tumefaciens* (Levee *et al*., 1997). Subsequently, this method was successfully applied to several species of spruce (Klimaszewska *et al*., 2001; Arce-Johnson *et al*., 2002; Charity *et al*., 2005; Grant *et al*., 2004).

Methods relative to transformation targets can be classified into two categories: those requiring tissue culture and in-planta methods. Tissue culture based methods can lead to unwanted genetic changes such as alterations in cytosine methylation, induction of point mutations and various chromosomal aberrations (Phillips *et al*., 1994; Singh, 2003; Clough, 2004). In-planta methods obviate the need for tissue culture. Feldmann and Marks (1987) reported the successful genetically transformed *Arabidopsis thaliana* by inoculating seed with *A. tumefaciens*. Transformation rates greatly improved when Bechtold *et al*. (1993) inoculated plants that were at the flowering stage. At present, there are very few species that can be routinely transformed in the absence of a tissue culture-based regeneration system. *A. thaliana* can be transformed by several in-planta methods including vacuum infiltration (Clough and Bent, 1998), transformation of germinating seeds (Feldmann and Marks, 1987) and floral dipping (Clough and Bent, 1998). Other plants that were successfully subjected by vacuum infiltration include rapeseed, *Brassica campestris* and radish, *Raphanus sativus* (Ian and Hong, 2001; Desfeux *et al*., 2000). The labor-intensive vacuum infiltration process was eliminated in favor of simple dipping of developing floral tissues (Clough and Bent, 1998). Also, the results indicate that the floral spray method of *Agrobacterium* can achieve rates of in-planta transformation comparable to the vacuum-infiltration and floral dip methods (Chung *et al*., 2000). In-planta

procedures have some advantages to tissue culture methods. They are extremely simple, tissue culture and the resulting somaclonal variations are avoided and only a short time is required in order to obtain entire transformed individuals. However, the mean frequency of transformants in the progeny of such inoculated plants is relatively low and very variable. In many species false positive results are apparently the case leading to the conclusion that this method might be effective when in reality it is not.

However, recently, *Agrobacterium* inoculation of germinating seeds of rice has resulted in transformation efficiencies higher than 40% (Supartana *et al*., 2005) but ranged from 4.7 to 76% for the flower infiltration method and from 2.9 to 27.6% for the seedling infiltration method (Trieu *et al*., 2000). In contrast with *A. tumefaciens*, most plant cells transformed with *A. rhizogenes* develop into transgenic hairy roots that are easily recognized and cultivated in vitro. *A. rhizogenes* mediated transformation is useful for recalcitrant species (Tepfer, 1984; Mugnier, 1988; Petit *et al*., 1987) as well as for many areas include for artificial seed production (Uozumi and Kobayashi, 1997), for production of plant secondary metabolites (Hamill and Lidgett, 1997), as an experimental system to study biochemical pathways (Braun *et al*., 2002), to study of responses to chemicals and to study interactions with other organisms (Downs *et al*., 1994; Mugnier, 1997; Kifle *et al*., 1999). In addition, hairy roots have been used to express antibodies and other recombinant proteins (Wongsamuth and Doran, 1997; Medina-Bolívar and Cramer, 2004).

For inoculation of manually impossible infection of viruses a method was developed that called agroinoculation (Grimsley, 1990). Furthermore, agroinoculation almost always increases infection efficiency even when manual inoculation is possible (Hayes *et al*., 1988; Briddon *et al*., 1989; Lesser *et al*., 1992). One of the most efficient applications for agroinoculation is induction of recombination which it is able for marker gene deletion (Jia *et al*., 2006).

The optimization of *Agrobacterium*-plant interaction is crucial for efficient transformation. Many factors (e.g., the type of explant) are also important and they must be suitable to allow the recovery of whole transgenic plants (De la Riva *et al*., 1998; Jefferson, 1987; Opabode, 2006; Hiei *et al*., 1994; Frame *et al*., 2002; Zhao *et al*., 2001; Cardoza and Stewart, 2003; Zhang *et al*., 2003; Arencibia *et al*., 1998; Enriquez-Obregon *et al*., 1999; Olhoft and Somers, 2001; Cheng *et al*., 1997, 2003; Desfeux *et al*., 2000; Jones *et al*., 2005). In addition, residual *Agrobacterium* can confound transgenic plant analysis and also be of environmental concern (Cubero and Lopez, 2004). One can test for contaminating *Agrobacterium* by culturing (Cubero, 1998; Cubero *et al*., 2006), or Polymerase Chain Reaction (PCR). Being of the viable but nonculturable (VBNC) state for the *Agrobacterium* (Manahan and Steck, 1997) and false positive results of PCR advise a combination of isolation and molecular techniques (Moore *et al*., 2001).

It is notable that the fundamental limitation of *Agrobacterium* based systems led to the reduced ability of them to transfer DNA into monocot cells. Also, this system relies completely on the functioning of the Vir proteins, which target only to the nuclear genome and not to organelle genomes. Public concerns about the permanent introduction of foreign DNA into food crops in order to produce the safe transformants has recently been caused to use plant-derived putative transfer DNAs (P-DNA) fragment which it can be used to replace the universally employed *Agrobacterium* T-DNA (Rommens *et al*., 2004; Depicker *et al*., 2001; Rommens, 2004, Darbani *et al*., 2007, 2008).

Viral based transformation: A number of biological alternative strategies have been developed over the last few years for gene delivery in higher plants, but none have led to stable plant transformation. Viral based transformation systems provide very high expression temporarily in plant cells. Potato virus X (Ruiz *et al*., 1998), barley stripe mosaic virus (Holzberg *et al*., 2002) and geminivirus vectors (Kjemtrup *et al*., 1998; Turnage *et al*., 2002) are some of plant viral vectors that are commonly used. Substrates of viral mediated transformation could be viral RNA or more conveniently, viral cDNA which can be used via direct inoculation, which has proven to be the most efficient way of introducing cDNA-derived viral RNA into plants (Ahlquist *et al*., 1984; Lu *et al*., 2003; Marillonnet *et al*., 2005; Jia *et al*., 2003). Modified viruses in which an antibiotic resistance gene replaces the viral coat protein genes, can be used to enhance integration (Elmer and Rogers, 1990). Rapidity and high level expression of transgene are the benefits of virus-based transient RNA and DNA replicons versus non-viral systems. Also, the potential of vectors based on viral genomes to spread throughout a host plant to produce systemic infection has inspired the potential combination of using viral vectors with in-planta based transformation. But they have low infectivity using average or large-length transgenes (Porta and Lomonossoff, 2002;

Marillonnet *et al*., 2005). Other disadvantageous include large number integrated transgene copies, chromosomal rearrangement, appearance of disease symptoms and low frequency for stable transformation. Viral vectors can also serve as efficient initiators of Posttranscriptional Gene Silencing (PTGS) or Virus Induced Gene Silencing (VIGS) in natural virus infections (Tenllado and Diaz-Ruiz, 2001). Also, inducible expression is applicable by viral based transformation methods, which could be useful tool for marker gene elimination (Hull *et al*., 2005; Kopertekh and Schiemann, 2004; Jia *et al*., 2006).

NON-BIOLOGICAL BASED TRANSFORMATION

Non-biological based or direct gene transfer methods use chemical or physical DNA up-take induction into protoplasts, intact cells or whole plants. These methods are potentially suitable for all species and all plant materials and do not require to specialized vector systems. Often, until *Agrobacterium*-mediated transformation systems are optimized for plant species, direct DNA techniques are used for transformation. Direct transformation systems have the following disadvantages: requirement of specialized instrumentation and consumables in many cases, fragmentation and rearrangement of introduced sequences, high copy integration and co-integration of target gene cassette with plasmid vector sequence. As regards nucleus targeting of *Agrobacterium*-mediated transformation, however, gene targeting into other intracellular compartment, e.g., chloroplast genetic transformation is feasible via direct gene transfer methods. Targeting into the chloroplast have some advantageous, ex., high level expression, gene silencing and positional effects elimination and especially from the safety point of view (Darbani *et al*., 2007). Also, chloroplasts are better able to express bacterial genes than are nuclei. Therefore, worthy is clear for these methods in which will be discussed in this section.

Particle bombardment/biolistics: As an alternative to protoplast transformation, particle bombardment was first described as a method for the production of transgenic plants in 1987 (Sanford *et al*., 1987) especially for transformation of more recalcitrant cereals. John Sanford, Ted Klein and colleagues at Cornell University advanced the original bombardment concept (Sanford *et al*., 1987; Sanford, 2000) and coined 'biolistics'. A variation in the microprojectile transformation is an electric Discharge Particle Acceleration Device (DPAD). Unique advantages of this methodology compared to alternative propulsion technologies are discussed in terms of the range of species and genotypes that have been engineered and the high transformation frequencies for major agronomic crops (McCabe and Christou, 1993). In plant research, the major applications of biolistics include transient gene expression studies, production of transgenic plants and inoculation of plants with viral pathogens (Sanford, 2000; Southgate *et al*., 1995; Taylor and Fauquet, 2002). Because of the low cost, more heterogeneous tungsten particles are also widely consumed as a physical factor but transformation efficiency is highest with gold particles in the range of 0.7-1.0 μm mean diameter (Southgate *et al*., 1995; Taylor and Fauquet, 2002; Sanford *et al*., 1993; Kikkert, 1993; Sanford *et al*., 1991).

Gene constructs for biolistics can be in the form of circular or linear plasmids or a linear expression cassette. Embryogenic cell cultures are likely the best explant to use for biolistic transformation because they can be spread out as uniform targets of cells and because they have a high recovery capacity (Kikkert *et al*., 2004). Use of mannitol or sorbitol as osmotica in bombardment medium is effective and caused higher rates of stable transformants for all suspension cultured cells (Kikkert *et al*., 2004), as does brief air-drying. Rice transformation has also been successfully achieved via the microbombardment of immature embryos or embryogenic calli (Li *et al*., 1993; Sivamani *et al*., 1996; Cao *et al*., 1992; Zhang *et al*., 1996), in which transformation efficiency has been raised to 50% (Li *et al*., 1993). Particle bombardment has emerged as a reproducible method for wheat transformation (De Block *et al*., 1997; Bliffeld *et al*., 1999) and the first stable transformation in a commercially important conifer species (*Picea glauca*) was achieved via embryogenic callus tissue as explant (Ellis *et al*., 1993) which it adapted for transformation of other species of spruce (Charest *et al*., 1996; Walter *et al*., 1999), larch (Klimaszewska *et al*., 1997) as well as radiata (Walter *et al*., 1998) and loblolly pines (Connett *et al*., 2002). Hypersensitive responses to *Agrobacterium* that leads plant cell death are eliminated (Perl *et al*., 1996) via biolistics as well as the need to kill *Agrobacterium* after transformation. Operation of the biolistic device is easy. Furthermore, plasmid construction is simplified and co-transformation with multiple transgenes (Francois *et al*., 2002; Leech *et al*., 1998) is routine. The use of linear expression cassettes called clean gene technology eliminates the possible of plasmid backbone integration into the target (Fu *et al*., 2000). Biolistics is the method of choice for the study of transient gene expression 24-48 h after bombardment as well as for plastid transformation (Southgate *et al*., 1995; Bruce *et al*., 1989; Norris *et al*.,

1993). Co-integrated of this system with viral vectors can enhance expression through increased copy number by self-replication and integration (Hayes *et al*., 1988). In situ fertilization using *in vitro* biolistic based targeted microspores could be useful in order to transgenic plants production without any tissue culture phase, in-planta (Aziz and Machray, 2003).

Biolistics has some disadvantages. The transformation efficiency might be lower than with *Agrobacterium* and it is more costly as well. Intracellular targets are random and DNA is not protected from damage. Many researchers have not used biolistics because of the high frequency for complex integration patterns and multiple copy insertions that could cause gene silencing and variation of transgene expression (Dai *et al*., 2001; Darbani *et al*., 2008). But some laboratories have overcome this problem by reducing the quantity of DNA loaded onto the microcarriers and/or by use of linear cassettes (Fu *et al*., 2000). Also, biolistic mediated site-specific integration of rice is not less efficient than *Agrobacterium*-mediated transformation because single-stranded T-DNA is not a substrate for Cre/lox recombination and poly ethylene glycol-mediated (PEG-mediated) method since it requires the use of protoplasts (Srivastava and Ow, 2001). The ability of successful integration of Yeast Artificial Chromosomes (YACs) with insert up to 150 kb (Mullen *et al*., 1998) and of 106 kb BAC plasmid (Ercolano *et al*., 2004) into the plant genome by particle bombardment is an attractive proposition, especially for functional complementation analysis.

Efforts to reduce the incidence of recombination and reduction in the amount of DNA bombarded into each cell have been led to develop 'agrolistics', which was developed to increase efficiency of biolistics and yield simpler integration patterns (Taylor and Fauquet, 2002). Agrolistics is a refinement to microparticle bombardment technology which vir genes are co-bombarded. Use of agrolistics has been shown to increase the number of transgenic plants that have the clean integrate or precisely transgene as well as to reduce the frequency of degraded transgene integrations (Hansen and Chilton, 1996). This technology requires further development but has the potential as a refinement to alternative of biolistics.

Electro-transfection: Electroporation is the application of strong electric field pulses to cells and tissue is known to cause some type of structural rearrangement of the cell membrane resulting in a temporary increase in porosity and providing a local driving force for ionic and molecular transport through the pores. *In vitro* introduction of DNA into cells is now the most common application of electroporation. Several physical factors such as created transmembrane potential by the imposing pulse electric field, extent of membrane permeation, duration of the permeated state, mode and duration of molecular flow, global and local (surface) concentrations of DNA, form of DNA, tolerance of cells to membrane permeation and the heterogeneity of the cell population (Hui, 1995; Weaver, 1995) may affect the electro-transfection efficiency.

Transient expression from electroporated plant cells has been used to research at different field (Higo and Higo, 1996; Ecker and Davis, 1986; Fisk and Dandekar, 1998). Electroporation coupled with a transient expression assay is rapid, allowing for the reproducible detection of gene products within hours of the introduction of DNA in contrast with a stable transformation (Fisk and Dandekar, 2004). Electroporation has several advantages over biolistics in that it does not require the expensive particle gun apparatus, associated consumable supplies and licensing and has worked well for stable-transformation experiments (Shewry *et al*., 1994). Also, the efficiency of electroporation was relatively high: 6.25% for embryos and 54.6% for callus clusters, which is fully comparable with the best results obtained for this species after microbombardment (Deshayes *et al*., 1985). Using protoplasts as explants, regeneration of transformants limits its use for stable transformation (Lazzeri and Shewry, 1994). *Zea mays* embryos are suitable explants which enables the recovery of fertile transgenic plants recovering (D'Halluin *et al*., 1992). However, electroporation-mediated gene transfer into intact meristems was reported by Chowrira *et al*. (1995). It is mentionable that use of pollen electroporation is also able to escape regeneration phase (Saunders and Matthews, 1995). Despite apparent advantages of electroporation, it is not used very often because of sometimes irreproducible results and non-Mendelian segregation of progeny.

In order to efficiently apply electric field, especially to expel the tissue culture step, electrophoresis-mediated gene transfer was developed to force negatively charged foreign DNA into the host cells (Ahokas, 1989). It was proposed as an alternative method to biolistics, but is not nearly as efficient (Ahokas, 1989; Griesbach and Hammond, 1994). A voltage of 25 mV and amperage of 0.5 mA for 15 min are the most often used parameters (Ahokas, 1989; Griesbach and Hammond, 1994; Songstad *et al*., 1995). On average, this technique gives about 55% survival rate which up to 57% of the survivors

expressed a marker gene (Songstad *et al*., 1995). Comparison of this technique with *Agrobacterium* and particle gun delivery approaches introduced the *in vivo* electrophoresis transformation as an acceptable method by use of intact poinsettia meristems (Vik *et al*., 2001).

Silicon carbide fibers: One of the recently developed methods to delivery of DNA to plants is Silicon Carbide-Mediated Transformation (SCMT). Physical and chemical characteristics of silicon carbide fibers make them capable of puncturing the cells without killing them. The advantages of this system are: rapid, inexpensive, easy to set-up and is effective on a variety of cell types. Some of its disadvantages include, low transformation efficiency, cell damage and inhalation of the fibers can be a health hazard (Komatsu *et al*., 2006; Kaeppler *et al*., 1990, 1992; Wang *et al*., 1995; Songstad *et al*., 1995). The efficiency of SCMT depends on the fiber size, parameters of vortexing, shape of the vessels used, plant species and explant and characteristics of the plant cells and especially the thickness of the cell wall (Racoczy-Trijanowska, 2002). There are several known examples SCMT success including maize (Kaeppler *et al*., 1992; Wang *et al*., 1995; Petolino *et al*., 2000), rice (Komatsu *et al*., 2006), wheat (Brisibe *et al*., 2000), tobacco (Kaeppler *et al*., 1990), *Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea* and *Agrostis stolonifera* (Dalton *et al*., 1998). Recently, the efficiency of rice callus transformation based on whisker fibers was 30 to 50% (Komatsu *et al*., 2006). SCMT has been shown to be comparable in efficiency to biolistics in some instances and therefore, represents an alternative option for soybean embryogenic tissue transformation (Terakawa *et al*., 2005). Also, silicon carbide fibers can be used for wounding to improve frequency of *Agrobacterium*-based transformation (Singh and Chawla, 1999).

Polymer based transfection (polyfection): A barrier to more widespread use of genetic manipulation techniques is the difficulty of transforming some of the major crop plant species. Some cereal and grass species are recalcitrant to genetic manipulation. Nevertheless, because of the agronomic importance of monocots as staple food plants, there is great interest in transforming these species. Cereal transformation has been achieved by several different techniques of direct gene transfer. The first successful method was protoplast transformation (Rhodes *et al*., 1988) and subsequently particle bombardment (Gordon-Kamm *et al*., 1990), cell electroporation (D'Halluin *et al*., 1992) and vortexing tissues with silicon carbide whiskers (Wang *et al*., 1995) have all resulted in transgenic cereal plants.

The most common method of delivering foreign DNA into plant protoplasts involves treatment with PEG as a hydrophilic long-chain polymer (Paszkowski *et al*., 1984). Protoplast transformation has several advantages. Many cells can be handled with ease, many independent transformants can be produced, the selection of transformants is relatively simple and no specialized equipment is needed. As mentioned earlier, regenerating whole plants from transgenic protoplasts has proven difficult (Lazzeri and Shewry, 1994; Golovkin *et al*., 1993; Shimamoto *et al*., 1989). The system is only rarely employed due to the low frequency of transformants and the inability of many species to be regenerated into whole plants from protoplasts (Paszkowski *et al*., 1984). However, it has been reported that the PEG based method is efficient: 10-20% for *Physcomitrella* transformation (Schaefer, 2002) and 65 to 28% for rice protoplasts (Bart *et al*., 2006) according to plasmid size (4.5 to 12 kb). Recently, a fast PEG-mediated transient expression system was reported that is suitable for production of biopharmaceuticals (Buar *et al*., 2005). In addition, long-chain cations such as poly-L-ornithine, poly-L-lysine, dextran sulphate and high concentrations of divalent ions such as Zn^{2+} and Cu^{2+} have been applied to achieve high levels of DNA uptake (Potrykus, 1991). Among the polycations combined polybrene-spermidine based callus treatment was introduced as an efficient method for cotton transformation which it is less toxic than the other polycations and spermidine protects DNA from shearing because of its condensation effect (Sawahel, 2001).

Liposome-mediated transfection (lipofection): A derivation of PEG-mediated transformation is the liposome-mediated transformation technique. Cationic liposomes are positively charged lipids and are increasingly used for DNA uptake due to their favorable interactions with negatively charged DNA and cell membranes. In this approach foreign DNA must be encapsulated in a spherical lipid bilayer termed a liposome to prepare lipoplexes (Gad *et al*., 1990). After endocytosis (Fukunaga *et al*., 1983), the DNA is free to recombine and integrate into the host genome. As with other transformation systems, a variety of vectors including viral vectors can be applied with this system. Successful transformation based on this system was reported for tobacco (Dekeyser *et al*., 1990), wheat (Zhu *et al*., 1993) and potato (Sawahel, 2002). In the recent case frequency of stable transformation was 7% of calli regenerating from protoplasts. This method is relatively non-toxic (Antonelli

and Stadler, 1989), is simple to perform with readily available chemical reagents, is highly reproducible and efficient and requires no sophisticated equipment (Antonelli and Stadler, 1990; Felgner *et al*., 1987). Transformation of intact YACs into plant cell was achieved via lipofection-like particle bombardment. A lipofection-PEG combination method was more efficient than each one of them separately (Wordragen *et al*., 1997; Freeman *et al*., 1984). It is determined lipoplex size is a major factor determining lipofection efficiency which large lipoplex particles showed, in general, higher lipofection efficiency than small particles (Almofti *et al*., 2003).

Injection based methods: Microinjection is one of the most effective techniques of transforming animal cells and has been used for the transformation of plant cells as well (Banks and Evans, 1976). An important feature of the method is the use of Low Melting Point (LMP) agarose, both for holding protoplasts during microinjection and for their subsequent culture (Harwood and Davies, 1990). This direct and precise delivery of DNA into the plant cell or its nucleus is achievable by means of a glass microcapillary-injection pipette (Crossway *et al*., 1986; Morikawa and Yamada, 1985). The procedure is very slow and requires an expensive micromanipulator. However, it allows the introduction not only of plasmids but also whole chromosomes into plant cells (Griesbach, 1987; Jones-Villeneuve *et al*., 1995). Several plant species such as tobacco (Schnorf *et al*., 1991), petunia (Griesbach, 1987), oilseed rape (Neuhaus *et al*., 1987) and barley (Holm *et al*., 2000) have been transformed using microinjection. Also, the technique has been used to study intercellular communication and macromolecular trafficking in plants (Storm *et al*., 1998). This technique is very precise from the delivery point of view, is capable for transformation of any protoplast or single-cell system and has extremely high transformation efficiencies, but requires expensive equipment and tedious procedures.

The injection of inheritable materials using a hypodermic syringe is called macroinjection. Macroinjection is an approach to the delivery of exogenous DNA that could circumvent the expense, time and genome stress associated with the transformation of cells in culture and subsequent plant regeneration. Also, plant regeneration from transformed protoplasts, still remains a problem. Therefore, cultured tissues, which facilitate the development of immature structures, provides an alternate cellular target for transformation. These immature structures may include immature embryos, meristems, immature pollen, germinating pollen, etc. (Soyfer, 1980; Zhou *et al*., 1983). The main disadvantage of this technique is the likelihood for the production of chimeric plants with only a part of the plant transformed. However, from this chimeric plant, transformed plants of single cell origin can be subsequently obtained. To escape these problems Pollen-Tube Pathway (PTP) utilizing the normal fertilization cycle was developed to eliminate the regeneration phase. PTP based transformation is an injection/delivery of naked DNA into ovaries to produce transformed progeny (Touraev *et al*., 1997). In this approach the stigma is cut off and a drop of DNA solution is applied to the cut end of the style of recently pollinated plant florets. This procedure was used for the first time in rice (Luo and Wa, 1988). Afterward, other species such as wheat (Mu *et al*., 1999), soybean (Hu and Wang, 1999; Shou *et al*., 2002), *Petunia hybrida* (Tjokrokusumo *et al*., 2000) and watermelon (Chen *et al*., 1998) were subjected. In rice, the transformation efficiency was higher after excising the palea than after cutting top floret (Xie *et al*., 1990). A variation is the injection of a bacterial inoculum or plasmid DNA into inflorescences with pollen mother cells in the premeiotic stage without removing the stigma. Such an approach has been employed for rye (De la Pena *et al*., 1987). Although the results of the described experiments could be encouraging, the final transformation efficiency was about 10-fold lower than with biolistics. However, this approach has recently been introduced as a potential method for stable plant transformation (Peffley *et al*., 2003).

Wave and beam mediated transformation: Ultrasound treatment increases membrane permeability (Bommannan *et al*., 1992), thereby facilitating the entrance of macromolecules into cells for transformation (Wyber *et al*., 1997). Ultrasound for transfection has been widely applied in animal cells or tissues. However, there has been little research using ultrasound in plant cells or tissues. Ultrasound has been reported to mediate gene uptake in plant protoplast, suspension cells and intact pieces of tissues. In this technique explants are suspended in a few milliliters of sonication medium in a microcentifuge tube. Plasmid DNA (and possibly carrier DNA) is then added and after rapid mixing the samples are ready for sonication. The cells are finally transferred to fresh growth medium. In this term, sound frequency and exposure time determined the uptake efficiency (Liu *et al*., 2005). Early papers report transient expression of chloramphenicol acetyltransferase (cat) gene was reported in sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana tabacum* L.) via a brief exposure of the protoplasts to 20 kHz ultrasound in the presence of

plasmid DNA (Joersbo and Brunstedt, 1990a). Also, ultrasound treatment can be effective to transfection of virus particles (Joersbo and Brunstedt, 1990b). Stable transformation of tobacco by leaf pieces sonication was achieved via 1500 to 2000 fold longer time ultrasound treatment compared with protoplast sonication (Zhang *et al*., 1991). Another example for intact tissue sonication based transformation is potato tuber discs ultrasonication (Sawahel, 1996). Much of the ultrasound technique is aimed at Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT) in plant cells or tissues (Trick and Finer, 1997; Horsch *et al*., 1985; Weber *et al*., 2003) as introduced earlier. SAAT is a new technology and method involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. Experiments have been demonstrated that SAAT tremendously improved the efficiency of *Agrobacterium* infection by introducing large numbers of micro-wounds into the target plant cells or tissues (Santarem *et al*., 1998; Tang, 2003). Trick and Finer (1997) reported that, in all tissues tested, the SAAT treatment greatly enhanced the levels of transient expression. In addition, 2.2-fold, 2.5-fold and 4.1-fold increase, consequently resulted by sonication, sonication with $CaCl_2$ treatment and sonication with acetosyringone treatment in transformation frequency in contrast to manual wounding based method (Kumar *et al*., 2006). Also, SAAT could be efficiently useful for transformation of woody trees, particularly *Eucalyptus* sp. (Monica *et al*., 2004). In order to in-planta based transformation via SAAT, germinating seeds and seedlings were subjected that enhanced the efficiency of transformation (González *et al*., 2002). Recently, laser beam was applied to introduce genetic materials. Laser-mediated transformation works by a focused laser microbeam to puncture momentarily-made self-healing holes (~ 0.5 μm) in the cell wall and membrane. Therefore, exogenous DNA could simply be taken up by cells (Badr *et al*., 2005). The method was newly developed and requires further assessing for both of the different experimental conditions and plant species.

Desiccation based transformation: Since dried embryos have the unique ability to take up DNA during rehydration (Topfer *et al*., 1989), it was hypothesized that they could be mixed with a nutrient solution containing the foreign DNA for direct transformation. DNA would be taken up as the embryo rehydrates and seedlings would germinate in the presence of a selection medium to assess the incorporation of the foreign DNA (Topfer *et al*., 1989). The old and immature approach is potent at least theoretically to expel many problems associated with biological or chemical and/or physical methods but it needs further surveys.

CONCLUSION

Plant genetic transformation is of particular benefit to molecular genetic studies, crop improvement and production of pharmaceutical materials. These applications and the desire for higher transformation efficiency have stimulated work on inventing and improving various methods (Table 1). Aside from the elimination of tissue culture for Arabidopsis and a few other species, no great breakthroughs on basic transformation techniques have taken place since *Agrobacterium* and biolistics. *Agrobacterium*-based methods are usually superior for many species including dicots and monocots. For some angiosperms and non-angiosperms biolistics is usually used. The others are typically not done on a routine basis (Table 1). Biolistics is by far the most widely used direct transformation procedure both experimentally in research and commercially. In addition, it has proven to be the most efficient. So why have all these other methods emerged in the past 20 years if we already have efficient transformation techniques in *Agrobacterium* and biolistics? Then are there all these other methods? There are two reasons. First of all, there is hope that a more efficient and less expensive method will be developed. The commercial biolistics device costs thousands of US dollars and while less expensive home-made devices can be built fairly easily, they usually do not work as well. The second and most important reason is that biolistics and *Agrobacterium* are patented and obtaining a commercial license is not trivial, thus, commercialization pathways might be blocked without new technology.

Chloroplast targeting is one of the advantageous for direct transformation methods in sharp contrast with *Agrobacterium* based methods. High level recombinant protein expression is one of advantageous for chloroplast targeting but it have some drawbacks, e.g., low transformation frequency, prolonged selection procedures under high selection pressure and green part dependency for transgene products. However, maternal inheritance transgenes is one of the other advantageous for transplastomes, which has benefits relative to biosafety. In-planta transformation methods are immensely attractive; however, this has proven to be effective and efficient in Arabidopsis. Among these methods

Table 1: Juxtaposition of gene delivery methods

Delivery method			Factors: Transformation efficiency	Rang of transformable plant species	Tissue culture phase	Type of explant	Equipments	Others
Agrobacterium-based method			High and stable	Many species, specially dicotyledonous	With and without tissue culture phase	Different intact cells, tissues or whole plant	-	Possibility of agroinfection, combination with sonication and biolistic methods and transgene size up to 150 Kb
Viral-based method			High and transient	Virus-host specific limitation	Without tissue culture phase	In-planta inoculation	-	Rapid, efficient PTGS, inducible expression and with mosaic status
Bombardment based methods	Biolistics		High	Unrestricted	With and without tissue culture phase	Intact tissues or microspores	Very expensive apparatus	Efficient for viral infection, complex integration patterns, without specialized vectors and backbone-free integration and transformation of YAC with insert up to 150 kb
	Agrolistic		High	Unrestricted	With and without tissue culture phase	Different intact explants or whole plant	Very expensive apparatus	Clean and precisely integration with reduced degraded transgene events
Elctro-transfection	Electro-poration		Low to high	Unrestricted	With and without tissue culture phase	Protoplasts, meristems or pollen grains	+	Fast, simple and inexpensive in contrast with biolistics
	Electro-phoresis		Moderate	Unrestricted	Without tissue culture phase	Intact tissue on whole plant	-	Rapid and simple but yields shimeric
Polyfection			Low	Recoverable species from protoplast	With tissue culture phase	Protoplasts	-	Difficulty of regeneration, toxicity and DNA sharing
Lipofection			Low	Recoverable species from protoplast	With tissue culture phase	Protoplasts	-	High efficiency with combination of PEG based method, simple, non-toxic and positive correlation between efficiency and lipoplex size and highly reproducible
Silicon carbide mediated			Low to high	Unrestricted	With tissue culture phase	Variety of cell types	-	Rapid, inexpensive and easy to set-up
Injection based methods	Micro-injection		High	Recoverable species from protoplast	With tissue culture phase	Protoplasts	Expensive micro-manipulator	Very slow, precise, single cell targeting possibility, requires high skill, the chimeric nature of transgenic plants and ability of whole chromosome transformation
	Macro-injection	PTP*	Low	Plants with sexual reproduction	Without tissue culture phase	Gametes	-	Rapid and simple
		Others	Low	Limited (with few species)	Without tissue culture phase	Immature embryos or meristems	-	Production of chimeric plants and damaging of cells
Wave or beam mediated transformation	Sonoporation		Low	Unrestricted	With and without tissue culture phase	Protoplast, cells, tissues and seedlings	+	Effective to transfect by virus particles and able to increase the *Agrobacterium* based transformation efficiency
	Laser mediated		Low	Unrestricted	With tissue culture phase	Variety of cell types	+	Rapid and simple
Desiccation based method			Low	Unrestricted	With tissue culture phase	Rehydrated embryos	-	Simple

*Pollen-tube pathway, +: Necessary, -: Unnecessary

Agrobacterium based in-planta transformation is efficient than others especially using sonoporation, ex., seedling sonication, in order to eliminate the chimeric results. The reader should be cautious about investing many resources in techniques that are touted as effective but unproven. Even though plant transformation can no longer be described as a new technology, it continues to evolve. Moreover, tools go on to expand, leading to more facile and efficient transformation.

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