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Methods of Plant Transformation- A Review

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ABSTRACT

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Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species. However, the desire for higher transformation efficiency has stimulated work on not only improving various existing methods but also in inventing novel methods. The most published techniques for gene transfer into plant cells were dismissed as either disproven or impractical for use in routine production of transgenic plants. In many laboratories, virtually all the transformation work relies on particle bombardment with DNA coated microprojectiles or *Agrobacterium* mediated transformation for gene transfer to produce transgenic plants from a range of plant species.

Introduction

Plant genetic transformation permits direct introduction of agronomically useful genes into important crops and offers a significant tool in breeding programs by producing novel and genetically diverse plant materials. The directed desirable gene transfer from one organism to another and the subsequent stable integration and expression of a foreign gene in the genome is referred to as 'Genetic Transformation'. The transferred gene is known as 'transgene' and the organisms that are developed after a successful gene transfer are known as 'transgenics' (Babaoglu *et al.*, 2000).

Among the various r-DNA technologies, genetically modified plants expressing δ -endotoxin genes from *Bacillus thuringiensis* (*Bt*), protease inhibitors and plant lectins have been successfully developed, tested and demonstrated to be highly viable for pest management in different cropping systems during the last decade and a half (Gatehouse, 2008). Insect resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture. Most of the plant derived genes produce chronic rather than toxic effects and many insect pests are less or not sensitive to most of these factors. Therefore, the genes for δ -endotoxins are expected to provide better solutions.

Advances in biotechnology have provided several unique opportunities that include access to various plant transformation techniques, novel and effective molecules, ability to change the levels of gene expression, capability to change the expression pattern of genes, and develop transgenics with different insecticidal genes. With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert foreign genes that confer resistance to insects into the plant genome (Bennett, 1994). To sustain the crop yield potential and to meet the growing demand for food, crop productivity needs to be increased. However, in most crops it is believed that the genetic potential has been fully exploited for yield increase. As a result, any improvement in productivity has to revolve around the reduction of losses due to pests and diseases under optimal nutrition and abiotic factors. Recombinant DNA technology coupled with plant tissue culture has helped develop novel options for the economical management of various kinds of biotic stresses including insect pests. These technologies would be of immense value in reducing the losses caused by biotic stresses, including insect pests.

Transgenic plants display considerable potential to benefit both developed and developing countries. Transgenic plants expressing insecticidal *Bt* proteins alone or in conjunction with proteins providing tolerance to herbicide are revolutionizing agriculture (Shelton *et al.*, 2002). The use of such crops with input traits for pest management, primarily insects and herbicide resistance, has risen dramatically since their first introduction in the mid 1990s.

India, the largest cotton growing country in the world has increased productivity by up to 50% while reducing the insecticide sprays by half, with environmental and health implications, besides increased income to cultivators after introduction of *Bt* cotton in

2002. Success achieved in cotton has served as an excellent model to emulate in many other crops such as rice, wheat, pulses and oilseeds that have the potential to make agriculture a viable profession for the peasants of India.

Transformation studies

Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (DeBlock *et al.*, 1984; Horsch *et al.*, 1984; Paszkowski, 1984) has been extended to many plant species in at least 35 families.

Gene transfer successes include most major economic crops, vegetables and medicinal plants. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species. The techniques have continued to evolve to overcome a great variety of barriers experienced in the early phases of the development in the field of plant transformation.

Transformation methods

Gene delivery systems involve the use of several techniques for transfer of isolated genetic materials into a viable host cell. At present, there are two classes of delivery systems (Table 1): (a) Non-biological systems (which include chemical and physical methods) and (b) Biological systems. The desire for higher transformation efficiency has stimulated work on not only improving various existing methods but also in inventing novel methods.

Biological requirements for transformation

The essential requirements in a gene transfer system for production of transgenic plants are:

Availability of a target tissue including cells competent for plant regeneration.

A method to introduce DNA into those regenerable cells and

A procedure to select and regenerate transformed plants at a satisfactory frequency.

Practical requirements for transformation

Beyond the biological requirements to achieve transformation and the technical requirements for verification of reproducible transformation, desired characteristics to be considered in evaluating alternative techniques or developing new ones for cultivar improvement include:

(1) High efficiency, economy, and reproducibility, to readily produce many independent transformants for testing.

(2) Safety to operators, avoiding procedures, or substances requiring cumbersome precautions to avoid a high hazard to operators (e.g. potential carcinogenicity of Silicon carbide whiskers).

(3) Technical simplicity, involving a minimum of demanding or inherently variable manipulations, such as protoplast production and regeneration.

(4) Minimum time in tissue culture, to reduce associated costs and avoid undesirable somaclonal variation.

(5) Stable, uniform (nonchimeric) transformants for vegetatively propagated species, or fertile germline transformants for sexually propagated species.

(6) Simple integration patterns and low copy number of introduced genes, to minimize the probability of undesired gene disruption at insertion sites, or multicopy associated transgene silencing.

(7) Stable expression of introduced genes in the pattern expected from the chosen gene control sequences (DeBlock, 1993).

When tested against the above criteria, most published techniques for gene transfer into plant cells must be dismissed as either disproven or impractical for use in routine production of transgenic plants. As a result, in many laboratories, virtually all the transformation work relies on Particle bombardment with DNA coated microprojectiles or *Agrobacterium* mediated transformation for gene transfer to produce transgenic plants in a range of plant species (Birch, 1997).

Non-biological based transformation

Particle bombardment/Biolistics

Particle bombardment was first described as a method for the production of transgenic plants in 1987 (Sanford *et al.*, 1987) as an alternative to protoplast transformation and especially for transformation of more recalcitrant cereals. Unique advantages of this methodology compared to alternative propulsion technologies are discussed elsewhere in terms of 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 (Southgate *et al.*, 1995; Sanford, 2000; Taylor and Fauquet, 2002).

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 explants to use for biolistic transformation because they can be spread out as uniform targets of cells and have high recovery capacity (Kikkert *et al.*, 2004). Rice transformation has also been successfully achieved via the bombardment of 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 (DeBlock *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).

However, particle bombardment has some disadvantages. The transformation efficiency might be lower than with *Agrobacterium* mediated transformation and it is more costly, as well. Intracellular targets are random and DNA is not protected from damage. As a result, many researchers have avoided particle bombardment method because of the high frequency of 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).

Biological gene transfer

Agrobacterium mediated transformation

The natural ability of the soil bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenus*, to transform host plants has been exploited in the development of transgenic plants. In 1970s the prospect of using *A. 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 incorporates the DNA into the plant genome (Chilton *et al.*, 1977). Now forty five years later, this method has been the most widely used and powerful technique for the production of transgenic plants. However, there still remain 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).

Despite the development of other non-biological methods of plant transformation (Shillito *et al.*, 1985; Uchimiya *et al.*, 1986; Sanford, 1988; Arenchibia *et al.*, 1992, 1995), *Agrobacterium* mediated transformation remains popular and is 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 have been established for rice (Hiei *et al.*, 1994), banana (May *et al.*, 1995), corn (Ishida *et al.*, 1996), wheat (Cheng *et al.*, 1997), sugarcane (Arenchibia *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; Charity *et al.*, 2005; Grant *et al.*, 2004).

Methods relative to transformation targets can be classified into two categories: (a) those requiring tissue culture and (b) *in planta* methods.

In tissue culture systems for plant transformation, the most important requirement is a large number of regenerable cells that are accessible to the gene transfer treatment and that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation and selection treatments. A high multiplication ratio from a micropropagation system does not necessarily indicate a large number of regenerable cells accessible to gene transfer (Livingstone and Birch, 1995). Some time gene transfer into potentially regenerable cells may not allow recovery of transgenic plants if the capacity for efficient regeneration is short lived (Ross *et al.*, 1995). Further, tissue culture based methods can lead to unwanted somaclonal variations such as alterations in cytosine methylation, induction of point mutations and various chromosomal aberrations (Phillips *et al.*, 1994; Singh, 2003; Clough, 2004). On the other hand, realization of whole plant transformants has been a problem in a large number of crop species as these plants have proven to be highly recalcitrant *in vitro*. As a result, other strategies are being evolved wherein the tissue culture component is obviated in the procedure and these are known as *in planta* methods.

Plant genetic transformation is of particular benefit to molecular genetic studies, crop improvement and production of pharmaceutical materials. *Agrobacterium*-based methods are usually superior for many species including dicots and monocots. The others are typically not done on a routine basis (Table 2). Biolistics is by far the most widely used direct transformation procedure both experimentally in research and commercially.

So why have all these other methods emerged in the past 20-30 years, if we already have efficient transformation techniques in *Agrobacterium* and biolistics? There are two reasons. First of all, there is hope that a more efficient and less expensive method would be developed. The second and most important reason is the biolistics and *Agrobacterium* are patented.

In planta transformation

Although successful plant regeneration methods have been developed, the technology has not provided regeneration in several other crops for use in transformation protocols which is a serious limitation to the exploitation of gene transfer technology to its full potential. In the light of this major constraint, it becomes necessary to evolve transformation strategies that do not depend on tissue culture regeneration or those that substantially eliminate the intervening tissue culture steps. *In planta* transformation methods provide such an opportunity. Methods that involve delivery of transgenes in the form of naked DNA directly into the intact plants are called as *in planta* transformation methods. These methods exclude tissue culture steps, rely on simple protocols and required short time in order to obtain entire transformed individuals.

In many cases *in planta* methods have targeted meristems or other tissues with the assumption that at fertilization, the egg cell accepts the donation of an entire genome from the sperm cell that will ultimately give rise to zygotes (Chee and Slighton, 1995; Birch, 1997) and therefore is the right stage to integrate transgenes. For non-tissue culture based approaches of *in planta* transformation, *Agrobacterium* co-cultivation or microprojectile bombardment have been directed to transform cells in or around the apical meristems (Chee and Slighton, 1995;

Birch, 1997). Injection of naked DNA into ovaries has also been reported to produce transformed progeny (Zhou *et al.*, 1983).

Arabidopsis thaliana was the first plant that saw successful *in planta* transformation. Early stages of success in *Arabidopsis* transformation came from the work of Feldmann and Marks (1987). 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. *Arabidopsis* 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 high rates of *in planta* transformation comparable to the vacuum infiltration and floral dip methods (Chung *et al.*, 2000).

Table.1 DNA delivery methods available to produce plant transformants

Plant transformation	
Non-biological based transformation (Direct method)	Biological gene transfer (Indirect method)
A) DNA transfer in protoplasts 1) Chemically stimulated DNA uptake by protoplast 2) Electroporation 3) Lipofection 4) Microinjection 5) Sonication	1) Agrobacterium mediated transformation Primarily two methods a) Co-cultivation with the explants tissue b) <i>In planta</i> transformation 2) Transformation mediated by viral vector
B) DNA transfer in plant tissues 1) Particle bombardment / Biolistics 2) Silicon carbide fiber mediated gene transfer 3) Laser microbeam (UV) induced genetransfer	

(Birch *et al.*, 1997)

Table.2 Summary of gene delivery methods and their features

Gene delivery method	Transformation efficiency	Range of transformable plant species	Tissue culture phase	Type of explant	Remarks
Electroporation	Low to high	Unrestricted	With and without tissue culture phase	Protoplasts, meristems or pollen grains	Fast, simple and inexpensive in contrast with biolistics
Lipofection	Low	Recoverable species from protoplast	With tissue culture phase	Protoplast	High efficiency with combination of PEG based method, simple and non-toxic
Microinjection	High	Recoverable species from protoplast	With tissue culture phase	Protoplast	Very slow, precise, single cell targeting possibility, requires high skill, the chimeric nature of transgenic plants and ability of whole chromosome transformation
Sonication	Low	Unrestricted	With and without tissue culture	Protoplast cells, tissues and seedlings	Effective to transfect by virus particles and able to increase the <i>Agrobacterium</i> based transformation efficiency
Particle bombardment	High	Unrestricted	With and without tissue culture phase	Intact tissue or microspores	Efficient for viral infection, complex integration patterns, without specialized vectors and backbone free integration

(Darbani *et al.*, 2008)

Gene delivery method	Transformation efficiency	Range of transformable plant species	Tissue culture phase	Type of explant	Remarks
Silicon carbide mediate transformation	Low to high	Unrestricted	With tissue culture	Variety of cell types	Rapid, inexpensive and easy to set up
Laser beam mediated transformation	Low	Unrestricted	With tissue culture phase	Variety of cell types	Rapid and simple
<i>Agrobacterium</i> mediated method	High and stable	Many species, specially dicotyledonous plants	With and without tissue culture method	Different intact cells, tissues or whole plant	Possibility of <i>Agroinfection</i> , combination with sonication and biolistic methods and transgene size up to 150 kb
Virus based method	High and transient	Virus host specific limitation	With tissue culture	In planta inoculation	Rapid, inducible expression and with mosaic status

Utilizing naked DNA, cotton transformants were recovered following injection of DNA into the axil placenta about a day after self-pollination (Zhou *et al.*, 1983). Similarly, a mixture of DNA and pollen was either applied to receptive stigmatic surfaces or DNA was injected directly into rice floral tillers, or soybean seeds were imbibed with DNA (Trick and Finer, 1997; Langridge, 1992). These procedures, intriguing as they are, are impractical at present because of their low reproducibility.

Recent studies with *Agrobacterium* inoculation of germinating seeds of rice has provided transformation efficiencies higher than 40% (Supartana *et al.*, 2005), while providing 4.7 to 76% efficiency for the flower infiltration method and from 2.9 to 27.6% efficiency for the seedling infiltration method (Trieu *et al.*, 2000).

Crop species that were successfully transformed by injuring the apical meristem of the differentiated embryo of the germinating seeds and then infecting with *Agrobacterium* include peanut, *Arachis hypogaea* L. (Rohini and Rao, 2000b & 2001), sunflower, *Helianthus annuus* L. (Rao and Rohini, 1999), safflower, *Carthamus tinctorius* L. (Rohini and Rao, 2000a), field bean, *Dolichos lablab* L. (Pavani, 2006), and cotton, *Gossypium* sp. (Keshamma *et al.*, 2008). Maize, *Zea mays* L., was transformed by treating the silks with *Agrobacterium* and afterwards pollinated with the pollen of the same cultivar (Chumakov *et al.*, 2006).

The above successes have in fact provided a great leverage for easy development of transgenic plants, as the methodology is simple, cost effective, does not call for high infrastructural requirement even to handle recalcitrant crops such as groundnut. Thus the technology of gene transfer for the development of recalcitrant crops has become

a practical possibility for experimenting and producing viable transformants. However, the optimization of *Agrobacterium*-plant interaction is crucial for efficient transformation. Many factors including type of explant are important and they must be suitable to allow the recovery of whole transgenic plants (De la Riva *et al.*, 1998; Opabode 2006; Cheng, *et al.*, 1997; Jones *et al.*, 2005; Darbani *et al.*, 2008).

Although, biotechnological advances, have provided many technologies for gene transfer into plant cells, virtually all the transformation work rely only on particle bombardment with DNA coated microprojectiles or *Agrobacterium* mediated transformation for gene transfer to produce transgenic plants. The review thus overwhelmingly emphasizes the importance of this method.

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