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Microparticle Bombardment as a Tool in Plant Science and Agricultural Biotechnology

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ABSTRACT

Microparticle bombardment technology has evolved as a method for delivering exogenous nucleic acids into plant cells and is a commonly employed technique in plant science. Desired genetic material is precipitated onto micron-sized metal particles and placed within one of a variety of devices designed to accelerate these "microcarriers" to velocities required to penetrate the plant cell wall. In this manner, transgenes can be delivered into the cell's genome or plastome. Since the late 1980s microparticle bombardment has become a powerful tool for the study of gene expression and production of stably transformed tissues and whole transgenic plants for experimental purposes and agricultural applications. This paper reviews development and application of the technology, including the protocols and mechanical systems employed as delivery systems, and the types of plant cells and culture systems employed to generate effective "targets" for receiving the incoming genetic material. Current understanding of how the exogenous DNA becomes integrated into the plant's native genetic background are assessed as are methods for improving the efficiency of this process. Pros and cons of particle bombardment technologies compared to alternative direct gene transfer methods and *Agrobacterium* based transformation systems are discussed.

INTRODUCTION

THE PRODUCTION of genetically transformed plant tissues and plants is now a central aspect of experimental plant science and a basic component of agricultural biotechnology. The worldwide area committed to transgenic crops has risen from 1 to 52.6 million hectares between in 1997 and in 2001 (James, 2001). The technology of microparticle bombardment has made an important contribution to these advances, facilitating the genetic transformation of a large range of plant species for fundamental studies of gene regulation and the production of plants expressing agronomically useful traits. In a previous review of the subject, Luthra *et al.* (1997) documented 200 scientific papers published between 1987 and 1995, which reported the use of particle bombardment technologies in plant science. Searching the databases in preparation for the present review article revealed that since 1995 more than 250 additional papers have been published in this area, illustrating the continued importance of particle bombardment technology. In the following pages we describe particle bombardment, its relationship to other plant genetic transformation systems, and the impact it has had on plant science and biotechnology.

TECHNOLOGIES FOR INSERTION OF FOREIGN DNA INTO PLANT CELLS

Biotechnologists have a range of methods at their disposal for integrating foreign DNA into plant cells. These can be divided into *Agrobacterium*-mediated or direct gene transfer systems. In the former, soil bacteria of the genus *Agrobacterium* have been harnessed for their natural ability to act as genetic engineers. The bacteria contains a plasmid, known as the Ti plasmid, which codes for genetic mechanisms required to insert foreign DNA into a host plant. After the pathogen infects cells at a wound surface, a 15–30 kb portion (the T-DNA) of the Ti-plasmid is transferred and integrated into the plant genome, causing the plant cells to proliferate in a tumorous manner and produce chemical compounds required for proliferation

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of the prokaryote (Chilton *et al.*, 1977; Zupan and Zambryski, 1997). Plant scientists have capitalized on this phenomenon by removing the tumour inducing genes from the bacterium and replacing most of the T-DNA region with genetic sequences which are of interest to the investigator (Zupan and Zambryski, 1997). Strains of such "disarmed" *Agrobacterium* can therefore be used to integrate DNA into plants for experimental purposes and to transfer genes of agronomic interest into crop species.

Direct gene transfer systems have been developed to bypass the need for the *Agrobacterium* vector and deliver "naked" DNA into plant cells. Electroporation, polyethylene glycol (PEG) mediated DNA uptake, microinjection, silicon carbide fibers, and microparticle bombardment are all direct gene transfer technologies that have been successfully used to produce transgenic tissues and plants.

Gene transfer technologies

Unlike animals, plant cells process a cellulose wall which must be removed or penetrated if foreign DNA is to be delivered to the nuclear or plastomic genome. Early attempts at direct gene transfer focused on enzymic removal of the cell wall to produce protoplasts. PEG or electroporation was then used to facilitate DNA uptake through the plasma membrane. Electroporation, which causes temporary pores to be formed in membranes, has also been used to deliver DNA into intact cells and tissues (D'Halluin *et al.*, 1992; Xu and Li, 1994), but at frequencies significantly lower that that achieved for protoplasts. Although effective, genetic transformation of protoplasts is no longer a favored system for recovering transgenic plants. Producing sufficient amounts of high-quality protoplast is laborious and technically demanding, while recovery of large numbers of fertile, phenotypically normal plants from these single cells is not practical for many agronomocially important crop species. Likewise, microinjection technology has failed to deliver earlier promises, being unable to produce transgenic plants in sufficient numbers compared to required inputs in resources (Songstad *et al.*, 1995).

Two more recent developments in direct gene transfer technologies are the application of silicon carbide fibers and microparticle bombardment for the production of genetically transformed tissues and plants. Silicon whiskers are microfibers 10–80 μm in length and approximately 0.6 μm in diameter. When plant cells, fibers, and desired DNA are vortexed in a liquid medium the cell wall is wounded and/or penetrated by the needle-like whiskers, allowing the DNA to enter the cell and become integrated into the genomic material (Kaeppler *et al.*, 1992). Fertile transgenic plants have been regenerated from maize (Frame *et al.*, 1994) and at least four other monocotyledonous plants using this technology (Dalton *et al.*, 1999). However, despite being a simple and inexpensive method, silicon fiber technology has not become a widely used technique. This is probably due to the need to mate the fibers to specific cells types in order to achieve successful transformation and possible issues over property rights for the technology (Petolino, 2002). A fuller critique of the benefits and limitations of direct gene transfer systems is provided by Songstad *et al.* (1995).

In contrast to the direct gene transfer systems described above, micropartcile bombardment has proved to be a highly successful method for gene transfer to plant cells and has been widely adopted by plant biotechnologists since the early 1990s. Development and application of this technology has been a major factor in overcoming recalcitrance to genetic transformation in a range of plants and advancing basic knowledge of gene expression within plant science. It remains the genetic transformation system of choice for some crop species and is second only to *Agrobacterium* in its efficacy to produce agromomically important transgenic crop plants. The remainder of this review will examine particle bombardment in greater detail. We will examine development of the technology, the major variables affecting its successful utilization, its practical application for generating transgenic plants, perceived advantages, and disadvantages of the technology in comparison to other genetic transformation systems.

DEVELOPMENT OF PARTICLE BOMBARDMENT TECHNOLOGY

Microparticle bombardment is a technique by which micron-sized metal particles are coated with DNA and accelerated into target cells at velocities sufficient to penetrate the cell wall but below that which will cause lethal damage. In this manner, desired DNA can be transported into the cell's interior where it becomes detached from the microprojectile and integrated into the nuclear or plastronic genome. Particle bombardment was developed in the 1980s to genetically engineer plants that were recalcitrant to transformation with *Agrobacterium*. This included a large proportion of the non-Solanaceous species, most notably the cereals and legumes which contain the majority of the world's most important food crops. Initial reports were restricted to transient expression of marker genes in onion, corn, soybean, wheat, and rice (Klein *et al.*, 1992), but were soon followed by production of transgenic soybean (McCabe *et al.*, 1988) and maize plants (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). Application of particle bombardment developed rapidly through the 1990s being used successfully to produce transgenic plants in a wide range of different plant species. The technology has also been adapted to transfer exogenous DNA to bacteria (Klein *et al.*, 1992; Smith *et al.*, 1992), fungi (Armaleo *et al.*, 1990), algae (Mayfield and Kindle, 1990), insects and mammals for both gene expression studies (Klein *et al.*, 1992; Cheng *et al.*, 1993; Luo and Saltzman, 2000) and gene therapy (Yang and Sun, 1995).

The first particle delivery device was developed by Sanford and co-workers (Sanford *et al.*, 1987; Sanford, 1988) and utilized DNA coated tungsten particles (the microcarriers), which were loaded onto the leading edge of small plastic bullets (the macrocarriers). A 0.22–gun powder cartridge was used to propel the bullets at high speed into a stopping plate which contained an opening too small for the macrocarrier to pass through. Upon impacting the stopping plate the bullet was arrested and the tungsten particles launched through the opening to impact the plant tissues situated below. Although effective, this device was soon replaced by a second generation of particle bombardment technologies, which were refined to reduce unintentional damage to the targeted plant tissues and remove the need for gun powder. A degree of control was also introduced into the process allowing experimenters to adjust and optimize numerous parameters of the bombardment process for successful delivery of DNA to a variety of cell types. The most effective of the mircoparticle bombardment systems are described below.

PDS-1000/He, Biolistic® particle delivery system

The Sanford device was extensively modified to produce the PDS-1000/He machine, which was trademarked as the Biolistic system and licensed to duPont. The PDS-1000/He is commercially available from BioRad, (Hercules, CA) and at the time of writing costs between $18,000 and $20,000 to purchase and bring to operation. Details of the system are available at the BioRad website (BioRad, 2002). As the name suggests, helium gas is used as the propellant. This is continuously fed into a chamber at the top of the device, and held back by a plastic "rupture" disc. A variety of rupture discs are available, and designed to break at pressures ranging from 450 to 2200 psi. DNA is precipitated onto gold or tungsten particles (the microcarriers) approximately 1 μm in diameter and spread evenly on a circular plastic film (the macrocarrier) placed below the rupture disc in the main vacuum chamber of the Biolistic device. When the rupture disc breaks a shock wave is released, which impacts the macrocarrier causing it to fly a short distance before striking a wire-mesh stopping screen. The macrocarrier is arrested and the mirocarriers launched through the mesh into the plant target tissues situated on a shelf in the chamber below (Kikkert, 1993). The gold or tungsten particles impact the tissue in a circular pattern with a diameter of one to several centimeters depending on the distance at which the tissue is positioned below the stopping screen.

A vacuum chamber was introduced to prevent the microcarriers from being decelerated by air friction prior impacting the target tissues. Ability to vary the vacuum is one of several parameters of the Biolistic system that can be manipulated to optimize transformation frequencies for a given plant cell type. These are used to vary the momentum with which the microcarriers strike the target tissue and number of "hits" achieved per unit area. After optimization for a given target cell type, transient transformation efficiencies of greater than 10,000 cells per shot (Kikkert, 1993) or more than 1000 per cm^2 of bombarded tissues (Schopke *et al.*, 1997) are common with the PDS-1000/He system.

The Biolistic device became commercially available in the early 1990s and has changed little since that time. It quickly became and remains the most widely used of the particle bombardment systems and is a common fixture in plant biotechnology laboratories, where it is utilized for both transient gene expression studies and the production of transgenic tissues and plants (Finer *et al.*, 1999). It is robust in nature, efficient, relatively easy to operate, and capable of producing reproducible results across experiments and between laboratories in different locations around the world.

Particle inflow gun

The Particle Inflow Gun (PIG) was developed as a low-cost alternative to the Biolistic and other particle bombardment systems and can be assembled from commonly available parts for under $1000. The PIG is also cheaper to operate as there is no requirement for rupture discs or macrocarriers. The target tissues are placed within a vacuum chamber. DNA-coated microparticles are precipitated onto the screen of a syringe filter, which is then screwed into a Leur-lok needle adapter attached to a solenoid. The solenoid is used to generate a burst of low-pressure helium, which accelerates the mirocarriers and projects them into the plant cells situated below (Finer *et al.*, 1992; Vain *et al.*, 1993).

PIG technology has been successfully used to generate stably transformed cells of maize (Finer *et al.*, 1992) and soybean (Vain *et al.*, 1993a) and more recently to recover transgenic plants of corn (O'Kennedy *et al.*, 2001), banana (Becker *et al.*, 2000), and cassava (Zhang and Pounti-Kaerlas, 2000). A modified particle inflow gun, in which the vacuum chamber was eliminated, was developed and used by Sudhakarm *et al.* (1998) to generate transgenic rice plants. Despite the low costs of acquiring and operating PIG technology, which make it an attractive proposition in developing countries, reports of its successful application are less common in the literature than those for Biolistics.

Electrical discharge particle acceleration: ACCELL™ technology

ACCELL technology involves the use of high voltage electrical discharge to vaporize a droplet of water. DNA-coated microprojectiles are spread on a mylar sheet and positioned within a vacuum chamber. Shock waves generated by electrical discharge into the water droplet are channeled to accelerate the macro- and microcarrier complex into a stopping screen and project the microprojectiles into the target cells (McCabe and Christou, 1993). The major benefit of the ACCELL system is that the strength of the shock waves can be finely controlled, allowing the gold or tungsten particles to be accurately targeted to different cells layers within the plant tissue (Christou and McCabe, 1995). Lack of a gaseous propellant also reduces damage to the cells.

Although more elegant in design and operation, electrical discharge particle acceleration has not had as great an impact on plant biotechnology as the Biolistic system. It is certainly an effective technology and has been successfully utilized to generate transgenic plants from a variety of species (Brar *et al.*, 1994; Christou and Ford, 1995; Cooley *et al.*, 1995; Keller *et al.*, 1997). However, it is not widely available and consequently its application has remained somewhat limited.

Microtargeting bombardment device

Researchers at the Swiss Federal Institute of Technology developed a particle bombardment system capable of delivering 80% of the DNA to within an area as small as 150 microns in diameter (Sautter, 1993). The microtargetting device was designed primarily to utilize plant shoot meristems as the target tissue for transgene insertion. Ability to effectively target exogenous DNA to totipotent cells within shoot meri-stems removes the need for extensive tissue culture systems in plant genetic engineering and would potentially facilitate production of transgenic plants in a species and genotype independent manner.

In the microtargetting system, DNA is not precipitated onto metal microcarriers, but mixed with them and delivered as a small droplet to the end of a capillary tube within a chamber. A blast of high-pressure gas, most usually nitrogen, is used to rupture the droplet, forming an aerosol of the DNA/microcarrier, and forcing it through a small aperture. The particles are thereby accelerated and fly through a partial vacuum to impact the target tissue (Sautter, 1993).

Microtargeting meristems for the production of transgenic plants has not delivered on its early promises and although the microtargetting device has produced transgenic plants, only a few such devices have been built, and it is not a widely used technology (Finer *et al.*, 1999).

Helios gene gun

The Helios Gene Gun differs from the devices described above in being a hand-held, semiportable particle bombardment system. Manufactured by BioRad, it has been on the market since the mid-1990s (BioRad, 2002). It does not utilize a vacuum chamber and thus can be used to deliver DNA to tissues, organs, and whole organisms that will either not survive a vacuum or are too large to fit into such limited spaces.

DNA is precipitated onto gold particles and spread evenly onto the inside of a narrow plastic tube. The prepared tubing is cut into sections about 1 cm in length and inserted into a cartridge holder, in a similar manner as bullets in a revolver. The holder is then placed into the gun mechanism to sit between the source of helium and the barrel of the gun. The device is attached to a helium tank via a flexible cable, the nozzle placed within 1–2 cm of the target and the trigger pulled. A pulse of helium sweeps the microcarriers off the inside of the tubing and accelerates them down the barrel to strike the target tissue. Up to 12 shots can be held in each chamber allowing many bombardments to be carried out in a short time (BioRad, 2002). The Helios system is designed to be robust and flexible enough to be taken out of the laboratory and used in locations such as the greenhouse and animal facilities. Major applications of the Helios Gene Gun are proving to be delivery of nucleic acids to animals, such as mice and rabbits, for transient gene expression in plants and animals and inoculation of plants with viral pathogens (Briddon *et al.*, 1998; Finer *et al.*, 1999).

APPLICATION OF PARTICLE BOMBARDMENT TECHNOLOGIES

Microparticle bombardment has found three major applications within plant research.

Transient gene expression studies

Particle bombardment offers a simple and convenient method for inserting nucleic acids into plant cells. As a result, it has been widely used as an experimental tool for investigating transient expression of genes and gene constructs in plants cells. In such studies, tissues are bombarded and cultured for a limited time, usually 24–48 h, before being assessed for transgene expression. Visual reporter genes including the *uidA* (GUS) gene (Jefferson *et al.*, 1987), luciferase (Millar *et al.*, 1992) and more recently green fluorescent protein (Davis and Vierstra, 1998) are powerful components of such studies, allowing qualitative and quantitative assessment of transgene expression levels. Researchers are therefore able to study the effect of mutations, introns, codon use and efficacy of promoters, determining how these affect gene expression in various plant tissues. Deletions and mutations can be made in regulatory sequences and bombarded into plant cells to dissect and identify their control elements and ascertain under what physiological conditions a given promoter is up or down-regulated (Vain *et al.*, 1996; Komari *et al.*, 1998; Finer *et al.*, 1999). Transient expression studies are often carried out in this manner to test the efficacy of transgenes and promoters, prior to initiating the production of genetically transformed plants.

Production of genetically transformed plants and tissues

Transgenic tissues and whole plants are an important laboratory tool for investigating gene regulation, development, biotic and abiotic defense mechanisms and the complexities of metabolic pathways under different conditions. In addition, crop plants genetically modified to express traits with agronomic value are an important component of agricultural systems in North America, China, and Argentina and are becoming so in additional countries (James, 2001). Particle bombardment has been widely exploited to produce tissues and plants for the above applications and has had a major impact on basic plant science research and plant biotechnology.

Particle bombardment technology facilitated recovery of the first transgenic maize, wheat and soybean plants and advanced many other transformation programs, most notably rice, to the stage where large numbers of genetically transformed plants could be recovered. More than 40 rice varieties have now been engineered in this manner (Christou, 1996). Table 1 provides a list of the major crop species for which particle bombardment has been applied to produce transgenic plants. A significant range of traits have been transferred and imparted to crop plants via particle bombardment including resistance to viral, bacterial and fungal pathogens, nematodes and arthropod pests, herbicide resistance, drought tolerance, extended shelf life, improved processing traits, and enhanced nutritional content among others. It is not the intention of this paper to review this large topic and for more information the reader is directed to the available literature (Repellin *et al.*, 2001 and references therein). Field trials of many transgenic crop species developed by particle bombardment have taken place or are underway (Christou, 1996; Horvath *et al.*, 2001).

Inoculation with viral pathogens

The ability of microparticle bombardment to deliver nucleic acids into intact plant cells and organs has been exploited to inoculate whole plants and cell cultures with viral pathogens. Both DNA and RNA viruses have been manipulated in this manner, allowing controlled experimentation in the laboratory, circumventing the need to utilize insect or other vectors for transmission of the pathogen (Gal-On *et al.*, 1997). Fusion of the viral genome with visual markers, such as GFP, produces powerful techniques for investigating virus replication and movement within and between plant cells. Bombardment of whole plants allows resistance mechanisms to be investigated and the pathogenic components of the virus to be dissected and identified (Briddon *et al.*, 1998; Brown *et al.*, 1999).

In our laboratory, particle bombardment is a central component of the research activities. Transgenes imparting putative

TABLE 1. MAJOR CROP SPECIES FOR WHICH PARTICLE BOMBARDMENT HAS BEEN SUCCESSFULLY UTILIZED TO PRODUCE TRANSGENIC PLANTS

Crop species	*Target tissue*	*Reference*
Wheat (*Triticum* spp)	ET	(Juahar *et al.*, 1999; Rasco-G. *et al.*, 2001)
Rice (*Oryza sativa*)	ET	(Christou, 1997; Chen *et al.*, 1998a)
Maize (*Zea mays*)	ET, M	(Gordon-K. *et al.*, 1990; Lowe *et al.*, 1995)
Barley (*Hordeum vulgaris*)	ET	(Wan *et al.*, 1994; Horvath *et al.*, 2001)
Oat (*Avena sativa*)	ET	(Cho *et al.*, 1999; Kaeppler *et al.*, 2000)
Sorghum (*Sorghum bicolor*)	ET, Inf.	(Casas *et al.*, 1993, 1997)
Cassava (*Manihot esculenta*)	ET	(Schopke *et al.*, 1996; Zhang *et al.*, 2000)
Banana/plantain (*Musa* spp.)	ET	(Sagi *et al.*, 1995; Beker *et al.*, 2000)
Potato (*Solanum tuberosum*)	OT	(Romano *et al.*, 2001)
Sugarcane (*Saccharum officinarum*)	ET	(Falco *et al.*, 2000)
Sugarbeet (*Beta vulgaris*)	ET	(Snyder *et al.*, 1999)
Cotton (*Gossypium hirsutum*)	M	(Keller *et al.*, 1997)
Soybean (*Glycine max*)	ET, M	(Christou, 1995b; McCabe *et al.*, 1988)
Peanut (*Arachis hypogea*)	ET	(Ozias-Akins *et al.*, 1993; Yang *et al.*, 1998)
Bean (*Phaseolus vulgaris*)	EA	(Aragao *et al.*, 1996; Kim *et al.*, 1996)
Oilseed rape (*Brassica napus*)	pollen	(Fukuoka *et al.*, 1998)
Spruce (*Picea* spp.)	ET	(Brukhin *et al.*, 2000; Clapham *et al.*, 2000)
Citrus (*Citrus* spp.)	ET	(Yao *et al.*, 1996)
Cacao (*Theobroma cacao*)	ET	(Perry *et al.*, 2000)

ET—embryogenic tissue; EA—embryonic axes; Inf.—immature inflorescences; and OT—organogenic tissues.

resistance to geminiviruses are inserted into cassava tissues by the Biolistic system (Schopke *et al.*, 1996; Taylor *et al.*, 2001). Regenerated transgenic plants are then inoculated with cloned viral DNA using Biolistics or the Helios Gun to challenge the transgenic plants with the pathogen and determine the degree of resistance imparted by the transgenes.

PROCEDURES AND VARIABLES DETERMINING SUCCESSFUL GENE TRANSFER BY PARTICLE BOMBARDMENT

Successful gene transfer using particle bombardment technology requires the development and co-optimization of numerous variables. These include; (1) the physical parameters involved in the bombardment process itself; and (2) the biological requirements of the plant tissues prior to and following transgene insertion. A large body of literature was generated during the 1990s across a range of plant species, describing experimental evidence for the optimization and relative importance of the variables within (1) and (2) above. Nevertheless, any new transgenic program still requires a certain degree of empirical research in order adjust and mate the physical and biological parameters described below to ensure efficient transgene insertion into a given target tissue.

Physical parameters of the particle bombardment process

As the Biolistic process remains the most widely used particle bombardment system, an outline of the major variables determining its successful operation are described. A comprehensive review is provided by Southgate *et al.* (1995).

Preparation of the microcarriers. Genetic material for direct delivery by particle bombardment must be cloned into a suitable vector, amplified and purified to produce sufficient quality and quantity of the desired DNA for replicated experiments. pUC plasmids are commonly employed as the vector of choice as they are relatively small in size and replicate efficiently in *E coli.* If stably transformed tissues and plants are to be recovered, a selectable marker gene, most often expression cassettes coding for antibiotic or herbicide resistance, will be transferred to the plant genome in addition to the gene of interest.

Purified DNA is coated onto washed, disaggregated tungsten or gold particles, mixed with spermidine and precipitated onto the microcarriers by the addition of $CaCl_2$. The supernatant is removed, replaced with 100% ethanol and aliquots spread onto the macrocarrier membranes, where they dry to form a finely dispersed layer. The whole process must be performed under sterile conditions if prolonged tissue culture of the target tissues is intended after transgene insertion. Several versions of the above procedures have been developed, often to include polyethylene glycol in microcarrier preparation and utilizing sonication to prevent aggregation of the microprojectiles (Christou, 1995; Southgate *et al.*, 1995). In our laboratory 5 μg of plasmid DNA is precipitated onto 3 μg of gold particles, which provides enough coated mircocarriers for six bombardments.

Choice of microparticle type and size is important, as this will determine the mass, and thus depth of penetration, of the accelerated microcarrier. Although tungsten was widely utilized in early experiments and is significantly cheaper than gold, the latter has become more commonly used. Some groups have reported toxic effects of tungsten (Russell *et al.*, 1992), while the denser nature and more even surface of the gold particles allows better control of penetration into the plant tissue (Hunold *et al.*, 1994; Southgate *et al.*, 1995). Commercially available gold particles are available at sizes varying from at 0.6 to 3.0 μm in diameter.

Acceleration and delivery of the microparticles. For successful transgene integration the microcarrier/DNA complex must be delivered into the target tissue without causing excessive injury or stress to the plant cells. The degree of penetration required will depend on the thickness of the cell wall, the type of tissue being transformed, and the depth of the cell layers being targeted. Variations in the helium pressures, level of vacuum generated, size of the gold particles (including any unintentional aggregation) and position of the target tissues below the stopping screen within the particle gun's chamber, will determine the momentum and thus penetrating power with which the microprojectiles strike the tissue. All these parameters are under the experimenter's control, and must be optimized for a given target tissue (Christou, 1996; Rasco-Gaunt *et al.*, 1999). GUS, luciferase and GFP visual marker genes are commonly used to aid the worker in this process, allowing him/her to assess the number of "hits" achieved under various combinations of the bombardment variables.

Each tissue sample can also be bombarded more than once to increase the number of transformed cells. However, while this is often successful for maximizing the frequency of transient transformation events, it increases the level of damage and stress imparted to the plant cells and is rarely effective for improving the frequency of recovered transgenic tissue lines or plants (Schopke *et al.*, 1997; Klein *et al.*, 1988).

Importance and treatment of the target tissue

An attractive feature of particle bombardment is its ability to transfer foreign DNA into any cell or tissue type in which the cell wall and plasma membrane can be penetrated. This circumvents the strong host and tissue specificity of *Agrobacterium*-mediated transformation and the cell-specific requirements of other direct gene transfer systems. For example, efficacy of silicon fibers is largely restricted to cell suspension cultures (Songstad *et al.*, 1995). As a result, particle bombardment has been successfully applied to insert transgenes into a wide range of plant tissues including; intact mature organs of leaves, roots and stems, immature embryos and seedling parts, pollen, styles, meristems, undifferentiated callus and suspension cultures (see references in Christou, 1995a; Southgate *et al.*, 1995; Finer *et al.*, 1999).

If the purpose of the experiment is to achieve transient transgene expression, the tissues are usually assessed 24 to 48 h after bombardment and continued viability and division of the transgenic cells is not required. In this case the status of the target tissue is not critical and minimal tissue preparation is necessary prior to gene insertion. In contrast, if the aim is to recover useful numbers of stably transformed cell lines and/or transgenic plants, strict axenic culture conditions must be maintained and the choice of the target tissues and their treatment before and after particle bombardment becomes paramount. Failure to appreciate the importance of the type and status of the targeted biological material is considered to have been a weakness in many early plant genetic transformation programs (Christou, 1996).

Target tissues for recovery of transgenic plants. Recovery of transgenic plants requires transgenes to be targeted at tissues capable of: (1) receiving and integrating the introduced DNA, (2) undergoing selection for the successful transgenic events and (3) regenerating to produce fertile, phenotypically normal plants. In the case of transgenic crop improvement programs, they must also facilitate the recovery of large numbers (hundreds) of genetically modified plants. Because stable transformation frequencies are as low as 0.01% of the targeted cells, regeneration efficiencies of the target tissues must be high if transgenic plants are to be reliably recovered from bombarded tissues.

Embryogenic and meristematic tissues are the most commonly employed target tissues for the production of genetically transformed plants.

Embryogenic tissues. Embryogenic cultures fulfill the criteria described above and have become the most commonly used target tissue for the recovery of nonchimeric, genetically transformed plants (Vain *et al.*, 1995; Christou, 1996; Finer *et al.*, 1999; Repellin *et al.*, 2001). Embryogenic tissues result from the inherent ability of plant cells to return to the totipotent state when appropriately manipulated *in vitro.* Such cultures consist of rapidly proliferating tissues in which totipotent cells are located at the surface of small embryogenic units. Large numbers of progenitor cells are therefore accessible to incoming microcarriers when such tissues are subjected to particle bombardment. The size of the embryogenic units, which range from 0.1 to several millimeters in diameter, and their disorganized nature, facilitates the penetration of chemical selection agents and recovery of independent transgenic callus lines, from what are initially single cell transformation events. Cells which have successfully integrated and are expressing a selectable marker gene can be identified, isolated and encouraged to proliferate under the selection pressure. Regeneration of whole plants from transgenic callus lines generated in this manner is achieved by subsequent manipulation of growth regulators within the culture medium. An average of more than 22 genetically transformed rice plant lines were regenerated by Chen *et al.* (1998a) from each bombarded tissue sample following such procedures.

Particle bombardment of embryogenic tissues has been successfully exploited to produce transgenic plants in a wide range of agronomically important plants, including legumes, tuber crops, starchy staples, trees, commodity crops, and all the major cereals (see Table 1) and has had a major impact on plant and agricultural biotechnology. However, despite their beneficial characteristics, embryogenic tissues do not provide a universal answer for the production of genetically transformed plants.

Three drawbacks are associated with these culture systems. Firstly, initiation and maintenance of high-quality embryogenic tissues is a labor-intensive process, requiring significant input from skilled personnel. Ensuring a continuous supply of tissue of the quality required for transgene insertion is challenging and can often be the rate-limiting factor within a transgenic program. A more serious limitation is the genotype-specific nature of embryogenic culture systems. This phenomenon is consistent across all the major crop species and represents a significant hurdle for maximizing the full benefits of plant biotechnology. Thus, while a few cultivars within a given species may respond well in tissue culture and produce high-quality embryogenic target tissues for transgene insertion, the majority remain difficult to manipulate in this manner (Christou, 1996; Finer *et al.*, 1999). If transgenic plants of a given species are required as laboratory tools for basic studies of gene expression, researchers can work with easily transformable "model" genotypes. The problem can also be addressed by genetically transforming amenable varieties and introgressing the transgenic trait into agriculturally important cultivars through conventional breeding (Jauhar and Chibbar, 1999; Horvath *et al.*, 2001). However, for many agriculturally important plants, for example trees and vegetatively propagated crops such as potato, cassava and banana/plantain, breeding is often not an attractive option, and the laborious process of generating target tissues for transgene insertion must be developed *de novo* for each cultivar that is to be genetically engineered (Taylor *et al.*, 2002).

A third concern regarding the use of embryogenic tissues as the target for transgene insertion, is reduction in fertility and increasing incidence of phenotypically abnormal plants recovered from long-term callus cultures, and most especially from cell suspension systems. This has forced plant biotechnologists to develop tissue culture protocols which minimize the time, both before and after transgene insertion, that the target tissues spend in a disorganized state (Southgate *et al.*, 1995, Chen *et al.*, 1998a; Taylor *et al.*, 2001). For example, in the cereals the preferred systems for microparticle bombardment now utilize zygotic embryos which undergo only a few days, or at most a few weeks, culture prior to gene insertion (Vain *et al.*, 1995; Bommineni and Jauhar, 1997; Finer *et al.*, 1999).

Meristems. The inherent limitations of embryogenic cultures described above are not specific to particle bombardment *per se*, however, they do presently limit the application and impact of the technology. Shoot meristems were identified as an alternative target tissue for the production of transgenic plants, which are free from these drawbacks and provide the potential to genetically engineer all genotypes from any species. Indeed the microtargeting bombardment device previously described was designed specifically with this application in mind. Shoot meristems consist of undifferentiated cells that divide and develop to produce all the plant's aerial tissues, including the germline cells. Due to the organized nature of meristems it is inevitable that chimeric structures will be produced after particle bombardment. However, under the correct tissue culture and selection procedures, fully transformed plants can be generated from the initial transformation events. Alternatively, successfully transformed meristem cells may divide and develop to produce transgenic haploid cells, thereby passing the transgene onto subsequent progeny (Lowe *et al.*, 1995; Christou, 1995a).

Meristems have been successfully used to produce genetically transformed plants in a range of crop species (McCabe *et al.*, 1988; Lowe *et al.*, 1995; Keller *et al.*, 1997; Finer *et al.*, 1999). This has most often been achieved in conjunction with ACCELL technology, which, with its finer control, is better suited than Biolistics for targeting mircocarriers into the required cell layers within these explants. The use of meristems as the target for particle bombardment, however, carries its own problems. Transformation efficiencies are low, necessitating the production of large numbers of meristems prior to particle bombardment. In addition, subsequent, labor-intensive processes are often required to select and screen for transgenic lines among many hundreds of nontransgenic and chimeric plants. This problem is compounded if large numbers of independently transformed plants are required, as in the case of crop improvement programs. Consequently, the use of meristems, although circumventing many problems associated with embryogenic tissues, presents its own challenges and requires levels of resources that make it unattractive for many research organizations.

Treatment of Target Tissues. Treatment of the target tissues prior to and after particle bombardment can have a significant effect on the frequency of successful transformation events and most especially the number of recoverable transgenic cell lines and plants. The exact tissue culture conditions required to generate high quality embryogenic tissues vary between species and even between genotypes within a species. Efforts to develop optimum totipotent tissues through classical plant tissue culture techniques therefore remain a central component of most plant genetic transformations programs. Such factors as the age of the target tissues, or time since last subculture, must be considered, as actively dividing cells are often the most effective targets for transgene insertion (Iida *et al.*, 1991; Vasil *et al.*, 1991; Chen *et al.*, 1998a). In the case of apical meristems, the treatment and physiolologcal status and age of the mother plants prior to excision of the explants must be taken into consideration.

Use of an osmotic pretreatment or partial drying of the target cells prior to bombardment is a commonly used technique to increase the frequency of successful transformation (Vain *et al.*, 1993b, Schopke *et al.*, 1997; Chen *et al.*, 1998). Tissues are exposed to an osmotic agent such as mannitol or sorbitol for several hours prior to transgene insertion to cause partial plasmolysis of the cell. This is thought to prevent, or reduce, cell death due to extrusion of the protoplasm through the cell wall at wound sites created by incoming microparticles (Finer *et al.*, 1999). In our laboratory, in addition to being given an osmotic pretreatment, cassava embryogenic tissues are briefly pressed onto sterile filter paper in order to draw off any surface fluids before being bombarded with the Biolistic device (Taylor *et al.*, 2001). This step was introduced when it became apparent that even a thin film of liquid covering the target tissues was capable of impeding penetration of the microcarriers into the embryogenic cells.

DEVELOPMENTS IN PARTICLE BOMBARDMENT CAPABILITIES

Co-transformation with multiple transgenes

Most agronomic characteristics are polygenic in nature. Expression of one transgene in a plant can impart beneficial characteristics but the ability to integrate and co-express multiple transgenes is highly desirable. In this way, complex biochemical pathways can be manipulated, defense mechanisms more effectively optimized, novel compounds synthesized and multiple beneficial traits built into crop plants.

Recovery of transformed tissues and plants usually requires integration of a selectable marker to allow recovery of successful integration events, in addition to the gene of interest. (In the case of herbicide resistance, the one gene performs both functions, but this is an exception.) Both genes may be fused within the same plasmid and bombarded into the target tissues. Alternatively, co-transformation is carried out in which the two genes are cloned into separate plasmids and then mixed together, prior to coating onto the microcarriers. Varying the ratio in which the two plasmids are mixed influences the number of transgenic plants which can be recovered, with frequencies as high as 80% co-integration achievable for a selectable marker and visual marker gene (Christou, 1997; Chen *et al.*, 1998b). This phenomenon has been exploited and extended to simultaneously insert 12 transgenes into soybean (Hadi *et al.*, 1996) and up to 13 genes into rice (Chen *et al.*, 1998b). In the latter case, an antibiotic selectable marker gene was mixed with thirteen different plasmids, each of which contained a different transgene expression cassette. The cocktail was precipitated onto gold particles and bombarded into embryogenic rice callus. Analysis of plants from 120 different independent transgenic events showed that 85% contained more than two transgenes, while 17% had co-integrated in excess of nine into their genome. Seventy-three percent of the soybean transgenic callus lines had integrated all 12 plasmids (Hadi *et al.*, 1996). Examination of the rice sexual progeny revealed that, as a rule, the transgenes co-segregated together, indicating that they had integrated at the same genetic locus. Co-expression of four markers genes in these plants was stable across three sexual generations.

Co-transformation is an attractive technology because it facilitates the introduction of multiple genes of interest in one simultaneous transformation step and requires the use of only one selectable marker gene to achieve this. (Francois *et al.*, 2002). Recently, Maqbool *et al.* (2001) reported the use of particle bombardment to co-integrate three insect resistance genes into two commercially important rice varieties. Co-expression of the three transgenes imparted high levels of resistance to pest species of yellow stemborer, brown leafhopper and rice leaf folder insects. Multiple transgene insertion and inheritance has also demonstrated in wheat (Campbell *et al.*, 2000). Co-integration and expression of multiple transgenes is thus an established capability in plants. The next major challenge is to achieve coordinated expression of multiple transgenes to direct complex biochemical pathways for enhanced agronomic traits and the accumulation of high value novel compounds in plants.

Integration of large DNA inserts

In most transformation systems, 5–15 kb of exogenous DNA is transferred to the plant genome by particle bombardment or *Agrobacterium* transformation. Examination of the transgenic rice described above revealed that in addition to simultaneously integrating up to different 13 transgenes, the transgenic plants contained more than one copy of each insert (Chen *et al.*, 1998b). A large amount of foreign DNA, estimated to have been up to 300 kb, had therefore been integrated into the plants genome as a result of the microbombardment process. Similar results are reported by Maqbool and Christou (1999), for the three *cry* genes.

The ability to insert large DNA fragments into the plant genome is an attractive proposition, most especially for use with map-based cloning of agriculturally important genes. In this manner, integration of yeast and bacterial artificial chromosomes (YACs and BACs), which can be up to 500 kb in size, becomes a possibility, allowing confirmation of the presence of a gene of interest within a given YAC/BAC clone. In addition, genes of interest within a YAC or BAC can be transformed directly into a target species along with their native DNA control sequences. Such a strategy, using particle bombardment of smaller, cosmid-based plasmids, proved effective for transferring a rice bacterial resistance gene (*Xa21*), along with its natural promoter and enhancer sequences, from wild rice into previously susceptible Chinese breeding lines (Song *et al.*, 1995). Integration of YACs into the plant genome by particle bombardment has been successful (Van Eck *et al.*, 1995; Adam *et al.*, 1997) with inserts of up to 150 kb achieved (Mullen *et al.*, 1998). Although clearly requiring further development, integration of large DNA fragments promises to be important tool in future plant research and crop biotechnology.

Plastid transformation

Plant cells contain plastid organelles that possess a circular, double-stranded DNA genome between 120 and 160 kb in size. Approximately 120 genes are encoded on the prokaryote-like genome. Genetic transformation of the chloroplast genome occurs via homologous recombination with the incoming transgene and can occur at high copy numbers (Francois *et al.*, 2001). After subsequent selection pressure for the transgenic events, and with each plant cell containing as many as 50 plastids, high expression levels for a desired protein is possible. Up to 45% of the total soluble cellular protein can be of transgenic origin from plastomic transformation (Maliga, 2002), making this technology attractive for the accumulation of pharmaceutical and industrial products within plant tissues. In addition, because chloroplasts are not transmitted through the pollen in many plant species, the spread of transgenes from transgenic crops of this type would not pose the same degree of concern as for plants in which the nuclear genome has been modified (Daniell *et al.*, 2001).

Particle bombardment remains the most efficient manner in which to genetically engineer plastids (Daniell, 1999). After early successes with tobacco (Svab *et al.*, 1990) progress was frustrated by difficulties in simplifying the transformation procedures and adjusting the technology to other plant species. In addition, inability to export transgenic proteins from the chloroplasts into the cytoplasm somewhat limits the impact of plastid transformation. However, recent advances in removing antibiotic marker genes from transgenic chloroplasts and adaptation to tomato and potato, indicate that this technology holds significant potential for applications in which the desired product can be compartmentalized and stored within the plastids (Daniell, 2001; Maliga *et al.*, 2002).

Controlling expression levels and sites of transgene integration

The fate of exogenous DNA introduced by particle bombardment and factors that affect expression of the resulting transgenes is discussed subsequently. Several strategies have been developed to enhance the frequency of stable transformants recovered after bombardment and levels of transgene expression.

Matrix attachment regions. Matrix attachment regions (MARs; also known as scaffold attachment or associated regions, SARs), are short A-T rich regions that anchor the 30 nm chromatin fiber to the chromosome scaffold, generating free loops of chromatin varying from 50–200 kb in length. (Homes-Davis and Comai, 1998). Many genes and gene loci are known to be flanked up and downstream by MARs. SAR based transformation vectors are commonly employed in animal studies and have been used as "boosters" for plant transformation (Galliano *et al.*, 1995). Several research groups have successfully exploited this phenomenon to generate transgenic tissues and plants with elevated transgene expression. Allen *et al.* (1996) transformed suspension cells, and Ulker *et al.* (1999) whole tobacco plants, with plasmids containing marker genes flanked by copies of the RB7 MAR, previously isolated from tobacco root tissues. Use of MARs in this way increased GUS expression in the suspension tissues by 140 times and in the whole plants by a factor of two compared with the controls. Elevated transgene expression and reduced frequency of silencing was also obtained in transgenic rice plants transformed with reporter genes flanked by MARs (Vain *et al.*, 1998). Recently, analysis of DNA regions flanking a highly expressing transgene was found to contain MAR-like sequences (Sawasaki *et al.*, 1998; Shimiz *et al.*, 2001). Excision and subsequent fusion of these sequences to flank marker genes, followed by reintroduction into plant cells by particle bombardment resulted in a significant increase in transgene expression and a reduction in silencing compared to the control tissues (Allen *et al.*, 2000; Shimizu *et al.*, 2001).

The use of MARs is, therefore, a potentially powerful technology for use in conjunction with particle bombardment to increase levels and stability of transgene expression in plants (Allen *et al.*, 2000).

Agrolistics. Agrolistics is a refinement to microbombardment-mediated transformation developed to counter the frequency with which broken fragments of the transgene and superfluous plasmid DNA are integrated into the plant genome. In this strategy virulence genes from *Agrobacterium*, which facilitate release of the T-DNA and contain nuclear targeting sequences, are co-bombarded into the target tissue along with the selectable marker and gene of interest (Smith *et al.*, 2001). Use of Agrolistics has been shown to increase the number of transgenic plants receiving "clean" or precisely defined transgene inserts and to reduce the frequency of degraded transgene integrations (Hansen and Chilton, 1996). This technology requires further development but has the potential to address one of the major drawbacks of particle bombardment technology.

INTEGRATION OF THE INTRODUCED DNA AND TRANSGENE EXPRESSION RESULTING FROM PARTICLE BOMBARDMENT

Particle bombardment in itself is only a physical means by which to deliver nucleic acids into the interior of a plant cell. For genetic transformation to be achieved, the foreign DNA must travel to and become integrated into the plant's native genetic material. Production of a desired phenotype then requires efficient transcription and translation of the introduced coding region. Significant research efforts have been directed at understanding the processes involved in each of these steps. Critical aspects concerning integration of exogenous DNA into the cell nuclear are outlined below. For details concerning plastid transformation the reader is referred to Maliga (2002) and Daniell (1999).

Fate of the microprojectiles and entry of exogenous DNA into the plant nucleus

In direct gene transfer by particle bombardment, DNA is transported into the plant cell bound to metal microcarriers in the double stranded, supercoiled, or occasionally linearized, forms (Smith *et al.*, 2001). Localization of the microcarriers after particle bombarded has been determined by sectioning the target tissues. It was found that 90% of transiently expressing cells received delivery of a microprojecticle directly into their nucleus (Yamashita *et al.*, 1991; Hunold *et al.*, 1994), and that this was 45 times more likely to result in expression of the transgene than when the microcarrier became arrested within the cytoplasm. Delivery to the vacuole rarely resulted in expression of the marker gene. Embryogenic and meristematic cells, the two target tissues most commonly used for particle bombardment, are characterized by their rapid division, small vacuoles and large nuclear to total volume ratio, and are thus well suited to receiving and integrating incoming DNA.

Delivery of a gold particle to the cytoplasm requires movement of the foreign DNA into the nucleus, if transgene integration and expression is to be achieved. Through the use of fluorescent probes, Gisel *et al.* (1998) have shown that DNA fragments of 1.5 kb were able to pass through the nuclear pore complex and enter the nucleus but that fragments of 2.5 kb were rarely able to achieve this. It is apparent, therefore, that the majority of successful transformation events require delivery of the exogenous DNA directly into the nucleus of the plant cell, and that this is most likely the only manner in which larger segments of DNA such as cosmids, YACs and BACs can become integrated through particle bombardment.

Process of DNA integration

Integration of exogenous DNA into the plant's genetic material following particle bombardment can result in simple, single-copy insertions. However, analysis of transgenic events across a range of plant species have proved that in the majority of cases complex multicopy integrations take place (Christou, 1997; Finer *et al.*, 1999; Smith *et al.*, 2001). Transgene insertion is considered to be a two-step process. First, the exogenous DNA can undergo homologous recombinations and ligations prior to insertion into the plant genome, to create head to head and/or head to tail rearrangements of the introduced DNA. Fragments of the plasmid and broken copies of the expression cassette can also be spliced together within these concantamers. Such extrachromosomal recombinations take place within 30 min of the transformation process and are followed by a second phase in which the rearranged DNA is inserted into the genome. Integration is thought to take place with involvement of native DNA repair mechanisms and by nonhomologous recombination at locations of double stranded breaks in the plant's chromosome (Kohli *et al.*, 1998). A combination of homologous and illegitimate recombination is considered a possibility by some workers (Putcha, 1998), along with the involvement of plant histone genes (Mysore *et al.*, 2000). Matrix attachment regions have been reported as favored locations for insertions with topisomerase I catalyzing the integration process (Sawasaki *et al.*, 1998). Once established, the site of initial integration is thought to become a "hot spot" for further insertion events. As a result, transgenic material tends to become localized at one or a few genetic loci, in which the foreign DNA is interspersed with stretches of native genomic material, the latter ranging from less than three, to several hundred kb in length (Kholi *et al.*, 1998; Stoger *et al.*, 1998; Smith *et al.*, 2001; Svitashev and Somers, 2001).

Evidence presented by these workers supports the observed incidence of multicopy insertions resulting from particle bombardment-mediated gene transfer and explains how 12 or more transgenes were successfully transferred to rice and soybean (Hadi *et al.*, 1996; Chen *et al.*, 1998b). Generation of integration hot spots can be beneficial, as transgenes will be integrated at a discreet genetic locus and will be inherited as an intact unit. Such plants can then be easily incorporated into conventional breeding programs. Conversely, there is often a desire to remove selectable marker genes from transgenic crop plants or to select for single transgene insertions by segregation through the sexual cycle. In such cases tight linkage of the transgenic material is a disadvantage. Enhanced ability to control the sites of multiple transgene integrations by placing the selectable markers at discreet locations from the gene(s) of interests is therefore a desirable avenue of research for plant biotechnology.

Multiple copy insertions and superfluous DNA integration

As we have seen, direct gene transfer systems are prone to integrating multiple copies of the desired transgene, in addition to superfluous DNA sequences associated with the plasmid vector (Smith *et al.*, 2001). This occurs across all species studied, with between 1 and 20, and occasionally more, copies of the desired transgene being integrated. In addition, several different insertion sites can be generated in the plant's genome by the bombardment and subsequent integration processes (Register *et al.*, 1994; Finer *et al.*, 1999; Maqbool and Christou, 1999; Smith *et al.*, 2001). Multicopy and superfluous DNA insertions are disadvantageous for a number of reasons and are recognized drawbacks associated with the use of particle bombardment technology. "Clean gene" technology, by which non-essential plasmid DNA is removed by enzymic digestion prior to insertion by particle bombardment has been developed to address the problem of integrating superfluous DNA. In this manner, only the desired coding region with its control elements are "shot" into the target cells.

Genetically transformed plants recovered from tissue culture sometimes show phenotypic and genotype variations from the mother plant, in addition to the intended effects of the inserted transgene(s). These can be due to tissue culture-induced mutagenesis (somaclonal variation), insertional mutagenesis, pleiotropic effects of the transgene, or a combination of these phenomena. In all cases, detrimental effects might be imparted to the plant's metabolism, potentially leading to changes in a range of characteristics, such as development, response to stress and nutritional qualities. These variants are of little value either for laboratory experimental purposes or as part of a transgenic crop improvement program. The probability of insertional mutagenesis and pleiotropy increases with the number of transgene insertions and the total amount of DNA integrated into the plant's genome (Arencibia *et al.*, 1998; Smith *et al.*, 2001). In addition, there is evidence for correlation between increasing transgene copy number and transgene silencing (see below). Strict biosaftey regulations for transgenic crops favor the acceptance of genetically modified plants with single copy insertions, as these are easier to fully analyze and are statistically less likely to suffer from the problems outlined above.

Development of Agrolistics, redesigning transgenes and vectors to reduce the incidence of recombination, and reduction in the amount to DNA bombarded into each cell, are examples of efforts being made to alleviate the problem of multicopy insertions resulting from particle bombardment (Repellin *et al.*, 2001; Smith *et al.*, 2001).

Expression and silencing of transgenes

In most cases, high levels of constitutive, tissue specific or inducible transgene expression are required over the life of a genetically transformed plant. Reports of significant variation in the levels of transgene expression between independent genetic transformation events are common in the literature, and are an accepted aspect of transgenic plant production. As a result hundreds, if not thousands, of genetically transformed plants must be produced in the initial stages of a transgenic crop improvement program. Plant lines with stable transgene expression and otherwise normal phenotype are then selected for further study and development toward the desired end product. Better understanding and control of transgene expression is therefore desirable to increase the efficiency by which useful transgenic plants are produced.

Agrobacterium mediated transformation is considered to favor insertion of transgenic material into transcriptionally active regions of the plant's genetic material (Komari *et al.*, 1998; Finer *et al.*, 1999). Conversely, particle bombardment appears to cause integrations at random locations throughout the genome, even if homologous regions are included in the transferred sequence (Puchta, 1998), leading to what is known as "position effect." In this way, transgenes that are inserted at positions of highly condensed chromatin or which become methylated will be not be expressed, or will be expressed at a lower level that those integrated into sites of active transcription. Efforts to direct transgene integration met with little success until recently. Despite the use of a 22–kb region of homologous sequence, Thyjaer *et al.*, (1997), were unable to achieve site directed integration from almost 19 000 transformation events. The use of Cre-*lox* recombinase-mediated site-specific integration is proving more promising, with the recovery of plants possessing single-copy transgene sequences inserted into a preexisting *lox* site in tobacco (Day *et al.*, 2000). Similar results were reported recently for particle bombarded rice tissues (Srivastava and Ow, 2001). Such developments hold potential for achieving greater control of transformation events and have implications for the study of gene expression and more efficient production of transgenic crops plants.

Suppression of transgene expression through silencing is a relatively common phenomenon in genetically transformed plants and is exasperated by the presence of multiple copies within the plant's genome. Both cytosine methylation and co-suppression are known to operate, resulting in down-regulation of transgene expression, sometimes in an inconsistent and unpredictable manner (Komari *et al.*, 1998; Finer *et al.*, 1999; Iyer *et al.*, 2000). Silencing at the transcriptional level is thought to occur primarily by methylation of promoter sequences, thereby interfering with assembly of the transcription factors and/or by attracting chromatin re-modeling proteins to these sites (Meyer, 2000; Wang and Waterhouse, 2002). Co-supression operates at the RNA level, and involves the production of double stranded RNA which acts as a trigger to initiate degradation of a target RNA, thereby resulting in gene silencing (Vance and Vaucheret, 2001). Gene silencing and its implications for transgene expression is an area of intense research at this time, and the reader is directed to recent reviews on this large subject (Meyer, 2000; Vance and Vaucheret, 2001; Wang and Waterhouse, 2002).

Until recently, gene silencing was seen as a problem for plant genetic transformation, as it prevented reliable expression of a desired phenotype within transgenic plants. However, with increasing knowledge of the mechanisms underlying this phenomena, and realization that it can be utilized to down-regulate native genes within the plants, there is no doubt that it will become a powerful tool in future transgenic applications (Vance and Vaucheret, 2001).

PARTICLE BOMBARDMENT VERSUS *Agrobacterium*-MEDIATED GENE TRANSFER

Much debate has revolved around the various merits of particle bombardment and *Agrobacterium*, and which is the preferred method for the production of genetically transformed plants. Bombardment technologies were developed to circumvent the problems of incompatibility between tissues of many plant species and the *Agrobacterium* vector. However, since the mid-1990s the use of super virulent strains of the bacteria has resulted in the successful recovery of transgenic plants in all the major cereals and in a range of other crops which were previously transformed by particle bombardment (Ishaida *et al.*, 1996, Hiei *et al.*, 1997; Gonzales *et al*,. 1998). Progress has also been made in techniques to insert large DNA fragments and multiple genes via *Agrobacterium*, capabilities previously achieved only by direct gene transfer. The ability of *Agrobacterium* to achieve single-copy insertions compared with the prevalence for multiple copy rearrangements and broken transgene integration when using particle bombardment, is also an attractive feature of the former technology. These developments are persuading many researchers to move away from particle bombardment when production of stably transformed plants is the main aim. For example, early work in the well-known Golden Rice used particle bombardment technology (Burkhardt *et al.*, 1997), but switched to use of *Agrobacterium* for production of the final product (Ye *et al.*, 2000). Lack of capital outlay and operating costs required for *Agrobacterium*-based transformation systems are also attractive, and are an important factor in the ability to transfer capacity for plant genetic engineering to developing countries (Taylor *et al.*, 2002).

Nevertheless, some of the perceived advantages of *Agrobacterium*-mediated transformation are being challenged. These include evidence that DNA insertion by the bacterium is not as elegant as previously thought and that multiple copy insertions and superfluous DNA from outside the T-DNA borders can be integrated into the plant's genome when using this transformation technology (Repellin *et al.*, 2001; Smith *et al.*, 2001). Presence of viable *Agrobacterium* within the tissues of regenerated plants is also a potential concern (Christou, 1996). Limited host range still presents a barrier in some species, meaning that for them, particle bombardment remains the only reliable method for the production of transgenic plants. At the time of writing, although there is a movement away from particle bombardment for the production of transgenic plants, conflicting reports concerning evidence for the advantages and drawbacks of each system across different species, mean that both technologies remain central to plant biotechnology.

CONCLUSION

The present review has attempted to describe particle bombardment and the manner in which is has impacted plant research over the last 15 years. Certain drawbacks and limitations to its application have been discussed to illustrate the constraints or challenges that remain in fully exploiting its potential. It should be realized, however, that many of these represent fundamental questions in plant science were brought to light as a direct result of using bombardment technology, and as such illustrate the important contribution it has made to advancing our knowledge of plant biology.

There is no doubt that the development and application of particle bombardment technologies also represented a significant breakthrough in plant biotechnology. As a result, it became possible to produce genetically engineered plants in a wide range of crop species in an increasingly efficient manner. To what extent the use of particle bombardment will change over the next 5–10 years as enhanced, *Agrobacterium* transformation systems are developed for the cereals and other crops, remains to be seen, but it is certain that this direct gene transfer technology will continue to be an important tool in a large number of plant research programs for many years to come.

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