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Optimizing the Particle Bombardment Method for Efficient Genetic Transformation

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Abstract

Particle bombardment method has evolved into a useful tool for biotechnologists, allowing direct gene transfer to a broad range of cells and tissues over the past several years. Some of the important applications of the process include the production of fertile transgenic crops including maize, soybean, rice, wheat, barley, sorghum, etc. Recent results have extended the range of gene transfer to animal and bacterial cells. In this article the method of particle bombardment is reviewed and discussed, and practical suggestions for improving transformation efficiency are presented.

Discipline: Biotechnology
Additional key words: biolistic, particle gun, direct gene transfer

Introduction to particle bombardment method

Particle bombardment method which is one of the technologies for introducing foreign genes into cells was developed by John Sanford and co-workers[23,37] at Cornell University in the United States. This technique involves accelerating DNA-coated particles (microprojectiles) directly into intact tissues or cells. The research was conducted with a view to avoiding the host-range restrictions of *Agrobacterium tumefaciens*, and the regeneration problems of protoplast transformation.

In the early system, DNA-coated tungsten powder (spherical particles 4 μm in diameter) was placed, as a suspension in a small aqueous volume, at the front end of a bullet-like plastic macroprojectile (Fig. 1). The macroprojectile was accelerated by a gunpowder charge. Upon impact with a plastic stopping plate at the end of the acceleration tube, the macroprojectile extruded through a small orifice. This extrusion further accelerated the microprojectiles. Although the gunpowder model was found to be successful for genetic transformation of various plant species in several laboratories, lack of control over the power of the bombardment as well as physical damage to target cells limited the number of stable transformations[22,37].

The current model, PDS-1000/He™, which is now marketed by BIO RAD Laboratories, represents a significant technical improvement over the gunpowder device. The basic design was developed by Sanford et al.[37]. The PDS-1000/He™ device is powered by a burst of helium gas that accelerates a macrocarrier, upon which millions of DNA-coated microcarriers have been dried (Figs. 2 and 3). Compared to the gunpowder device, it is cleaner and safer, allows better control over bombardment parameter, distributes microcarriers more uniformly over target cells, is more gentle to target cells, is more consistent from bombardment to bombardment, and yields several fold more transformations in the species tested[22].

Sanford et al.[38] suggested the term "biolistic" which is a coined word derived from "biological and ballistic", though the terms particle gun or particle bombardment are becoming generic terms. This method has also been called the microprojectile bombardment method, the gene gun method, the particle acceleration method, etc. Therefore, one must pay attention to the choice of key words when searching for literature.

Since the development of the first particle bombardment system, several different types of bombardment devices have been developed, including an electrically triggered discharge gun[29], pneumatic particle guns[32], helium, nitrogen and carbon dioxide-powered devices[13,43] and a micro-targeting gun[39].

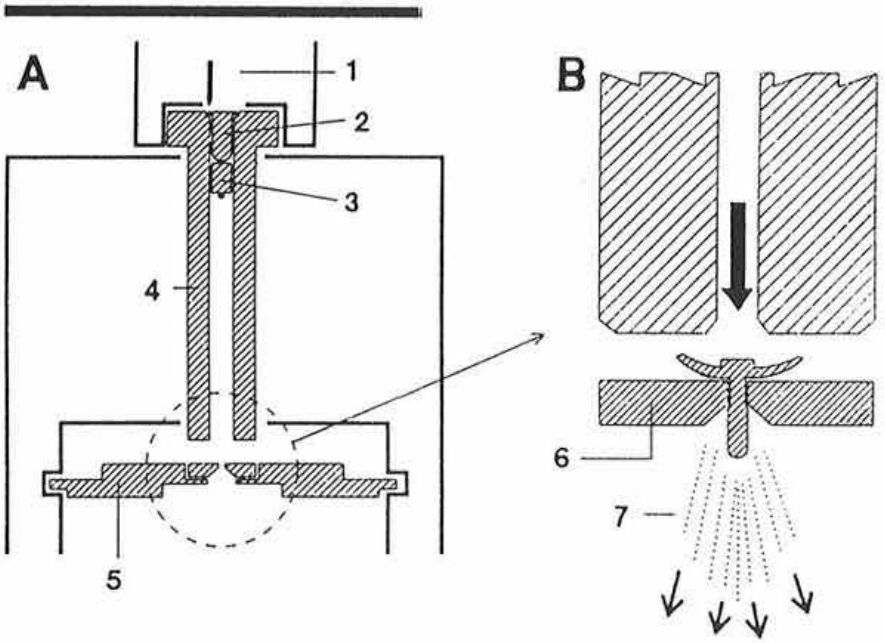

Fig. 1. Schematic diagrams of the gunpowder-driven particle gun developed by Klein et al.[25]
A: Before firing, B: After firing, 1: Firing pin, 2: Gunpowder charge, 3: Macrocarrier (plastic bullet) with microcarriers (tungsten particles), 4: Acceleration tube, 5: Stopping plate shelf with stopping plate, 6: Stopping plate with extruded macrocarrier (plastic bullet), 7: Launched microcarriers (tungsten particles).

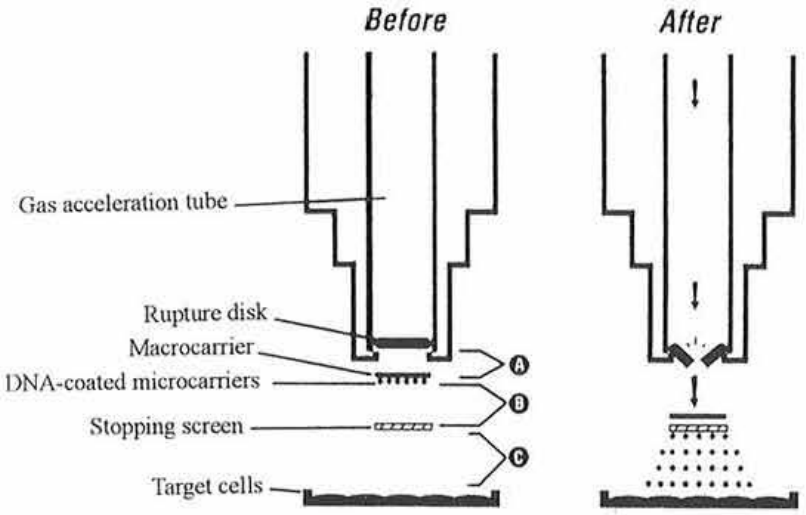

Fig. 2. Schematic diagrams of the helium-driven particle gun (from the catalog of BIO RAD PDS-1000/He™)
The distance of A, B and C can be changed.

These devices have been developed toward the same goals: more simplicity, safety, accuracy, and a lower cost for DNA delivery[5]. The basic principle of all these devices is the same as that originally developed by Sanford and co-workers[23,25,37].

This method was originally developed as a means of delivering foreign genes into the nuclear genome of higher plants and successful transformation of a wide range of tissues in a wide range of plant species was reported (Table 1). Tested plant tissues include cell suspensions, calli, immature embryos, microspores, etc. Transformed species include those for which transformation was otherwise impossible or very difficult.

Table 1. Production of transgenic plants by particle bombardment (focused mainly on cereals)

Plant	Target	Gene	Reference
Tobacco	Suspension cell	*Gus*[a], *npt II*[b]	24)
Soybean	Embryonic axes	*Gus*, *npt II*	29)
Papaya	Immature embryo, etc.	*Gus*, *npt II*	14)
Maize	Callus, Suspension cell	*Gus*, *bar*[c]	15)
Maize	Suspension cell	*Gus*, *bar*	16)
Populus	Protoplast-derived cell	*Gus*, BT[d]	30)
Cranberry	Stem section	*Gus*, *npt II*, BT	40)
Rice	Immature embryo	*Gus*, *bar*	10)
Rice	Suspension cell	*bar*	7)
Rice	Immature embryo	*Gus*, *hpt*[e]	28)
Sugarcane	Callus	*Gus*, *npt II*	2)
Dendrobium orchid	Protocorm	*npt II*, virus CP[f]	27)
Oat	Suspension cell	*Gus*, *bar*	41)
Wheat	Callus	*Gus*, *bar*	45)
Wheat	Immature embryo	*Gus*, *bar*	47)
Wheat	Scutellar tissue	*Gus*, *bar*	31)
Wheat	Immature embryo	*Gus*, *bar*	1)
Phaseolus vulgare	Seed meristem	*Gus*, *bar*	36)
Turfgrass	Callus	*Gus*	49)
Picea glauca	Somatic embryo	*Gus*, *npt II*	12)
Sorghum	Immature embryo	*Gus*, *bar*	8)
Sorghum	Immature embryo	*Gus*, *hpt*	18)
Peanut	Embryo axis	*Gus*, *bar*, virus CP	4)
Sunflower	Shoot apices	*Gus*, *npt II*	26)
Barley	Immature embryo	*Gus*, *bar*	46)
Barley	Immature embryo	*npt II*	35)
Barley	Microspore	*Gus*, *bar*	20)
Barley	Immature embryo	*Gus*, *hpt*	17)
Barley	Immature embryo	virus CP	19)
Alfalfa	Callus	*Gus*, *npt II*	33)
Italian ryegrass	Suspension cell	*Gus*, *hpt*	48)
Asparagus	Callus	*Gus*, *hpt*, *bar*	6)

a): *Gus*; β-Glucuronidase, b): *npt II*; Neomycin phosphotransferase II, c): *bar*; (PAT) Phosphinothricin acetyl transferase, d): BT; *Bacillus thuringiensis*, e): *hpt*; Hygromycin phosphotransferase, f): CP; Coat protein.

This method has also been found to be effective in microbial species, including *Bacillus megaterium, Pseudomonas syringae, Agrobacterium tumefaciens, Erwinia amylovora, Escherichia coli*, etc.[38]. It first made possible the transformation of organelles. Chloroplasts of *Chlamydomonas*[3] and mitochondria of yeast and *Chlamydomonas*[21] can be transformed. In 1990, Svab et al.[42] reported the transformation of animal cells using particle bombardment.

In 1996, the advanced design of the hand-held particle gun was released in the U.S.A. It has become commercially available in Japan since March 1997. This device called "Helios™ Gene Gun System" is marketed by BIO RAD (Fig. 4). In contrast to the conventional particle guns where the overall size of the target to be transformed is limited by the size of the chamber and the target tissue is subject to a vacuum during bombardment, the new device does not require vacuum and any target accessible to the barrel can be transformed. It may be used in a much wider variety of gene transfer applications and provides a tool for both *in vitro* and *in vivo* transformations.

In this article the method of particle bombardment is reviewed and discussed, and practical suggestions for improving transformation efficiency are presented. Most of the discussion will relate to BIO RAD PDS-1000/He™ which was developed by Sanford et al.[23,37] and is most widely used in the world. Basic principle of the particle bombardment method is the same regardless of the device used.

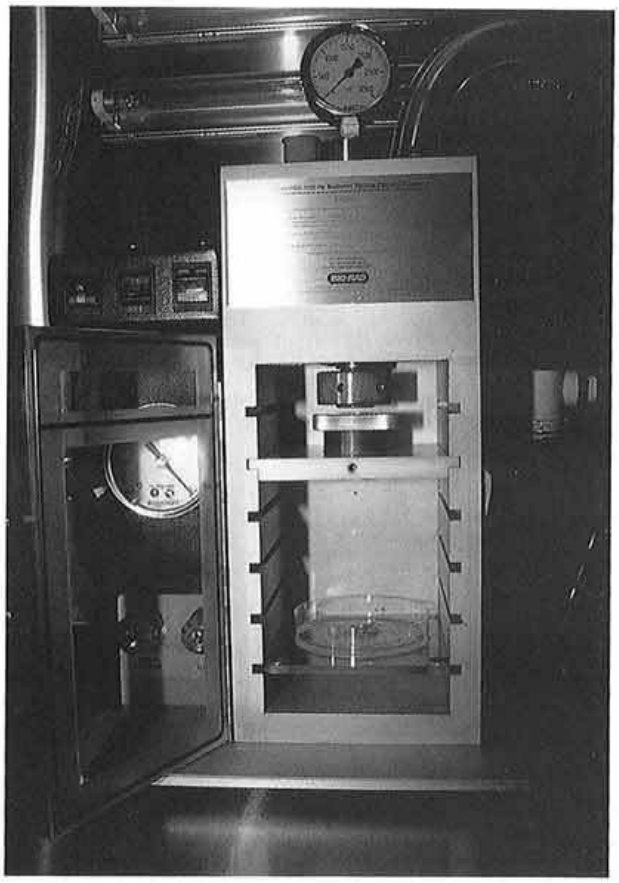

Fig. 3. Helium-driven particle gun (BIO RAD PDS-1000/He™)

Characteristics of the method

This method has become the second most widely used vehicle for plant genetic transformation after *Agrobacterium*-mediated transformation[9]. The number of researchers using particle gun has been increasing in spite of the availability of protoplast transformation because the protoplast-to-plant method of obtaining transgenic plants is laborious and time-consuming[9].

This method of genetic transformation offers both advantages and disadvantages over *Agrobacterium* or protoplast-mediated transformation as follows.

1) Advantages

(1) Almost any kinds of cells or tissues can be treated.

(2) Device operation is easy.

Transformation protocols are simplified. A large number of samples can be treated within a short time by technicians once the method has been routinized by researchers.

(3) Plasmid construction is simplified.

DNA sequences essential for T-DNA replication and transfer in *Agrobacterium* are not required. Furthermore, the introduction of multiple plasmids (co-transformation) is routinely accomplished.

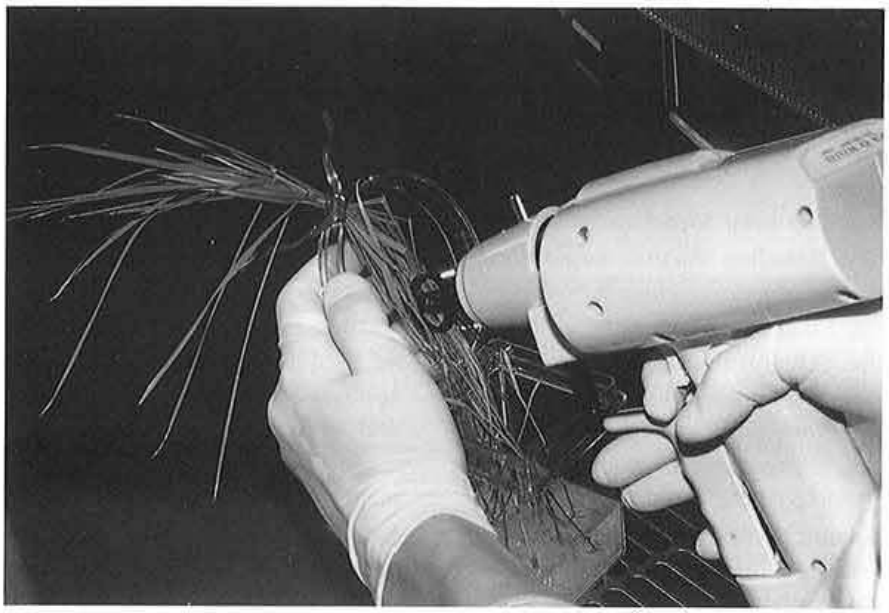

Fig. 4. Hand-held particle gun (BIO RAD Helios™ Gene Gun System)
Rice leaves are bombarded. The lid of the plastic petri dish is placed behind the leaves to prevent them from being blown.

(4) False positive results arising from the growth of *Agrobacterium* in host tissues are eliminated.
(5) Small amount of plasmid DNA is required.
Only 0.8 μg DNA is required for one bombardment in BIO RAD PDS-1000/He™.
(6) Transient gene expression can be examined within a few days.
It is conveniently used for evaluating transient expression of different gene constructs in intact tissues.

2) Disadvantages

(1) Generally, transformation efficiency is still low.
Even though this method has become one of the most widely used vehicles for plant genetic transformation, transformation frequency is still low compared with *Agrobacterium*-mediated or protoplast transformation.
(2) Consumable items are expensive in some models.
The cost of one bombardment is 260 yen in the case of BIO RAD PDS-1000/He™ using standard pressure kit (130,000 yen for 500 bombardments).
(3) One must consider patent royalty in commercial use.
This is a very delicate and complicated issue. One should consult with a specialist in patent issues.

Basic operation of the device

BIO RAD PDS-1000/He™ is routinely used in our laboratory. The protocol we currently use to prepare DNA-coated microcarrier (tungsten or gold particles) is as follows. The method is generally applied by following the instruction manual but some modifications were made by the author to enhance the transformation efficiency. This is one of the most important sources of variation affecting the transformation efficiency[38]. One should strive to make the precipitation reaction mixture as homogeneous and reproducible as possible.

(1) Vortex the microcarrier suspension prepared in 50% glycerol (60 mg/mL) for at least 5 min on a platform vortex (Fig. 5) to resuspend and disrupt agglomerated particles.
(2) Remove 5 μL (3 mg) of microcarrier suspension and put it into a 1.5 mL microfuge tube.
It is important to vortex the tube containing the microcarriers continuously in order to maximize uniform sampling. In the previous manual, it was recommended that particles be sonicated to maximize uniform sampling. From the author's experience this is not beneficial and under certain conditions can make particle agglomeration worse rather than better, especially when gold particles are used.

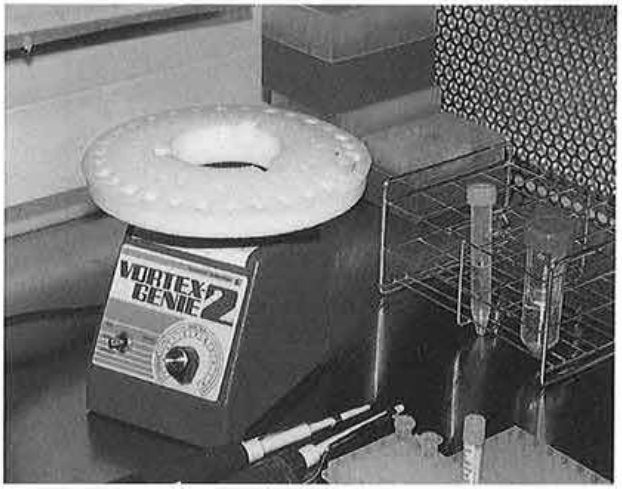

Fig. 5. Platform vortex™
Tubes must be shaken on the platform vortex™ while adding microcarriers (tungsten or gold particles), DNA, and other solutions.

(3) While vortexing vigorously, add in order:
5 μL DNA (1 μg/μL),
50 μL $CaCl_2$ (2.5 M),
20 μL spermidine (free-base, 0.1 M).
(4) Continue vortexing for 3 min.
(5) Allow the microcarriers to settle for 3 min.
(6) Pellet microcarriers by spinning 2 sec in a microcentrifuge. (approx. 5,000 rpm)
(7) Remove liquid and discard.
(8) Add 150 μL of 70% ethanol without disturbing the pellet.
(9) Remove liquid and discard.
(10) Add 150 μL of 100% ethanol without disturbing the pellet.
99.5% ethanol can also be used.
(11) Remove liquid and discard.
(12) Add 55–60 μL of 100% ethanol.
99.5% ethanol can also be used.
(13) Resuspend the pellet by tapping the side of the tube.
(14) Remove 6μL aliquots of microcarriers and transfer them to the center of a macrocarrier.
Spread microcarriers over the central 1 cm of the macrocarrier (Fig. 6).
Wait until microcarriers dry.
If fewer bombardments are needed, prepare enough microcarriers for 3 bombardments by

Fig. 6. Coating microcarriers (DNA-coated particles) over a macrocarrier
Six μL aliquots of microcarriers are being transferred to the center of the macrocarrier. One needs some practice to obtain uniform coating.

reducing all volumes by one-half.

The operation of the device is as follows. The method is generally applied by following the instruction manual but some modifications were also made by the author. Regardless of the apparatus used the basic principle of how to bombard the target is the same.

(1) Turn on the vacuum pump and the power switch.
(2) Set the helium regulator roughly at 200 psi above the selected rupture disk.
(3) Load the rupture disk.
(4) Load the stopping screen.
(5) Load the microcarrier assembly.
(6) Place the target cells or tissue.
(7) Press the VAC switch.
(8) When the vacuum gauge registers the desired vacuum level (above 27.5), put the VAC switch on the HOLD position.
(9) Turn off the vacuum pump to prevent overheating.
(10) Keep pressing the FIRE button until the rupture disk automatically bursts.
(11) After the bombardment is completed, put the VAC switch on the VENT position.
(12) Remove the sample.
(13) Remove the microcarrier assembly. Stopping screen may be used a few more times.
(14) Remove the rupture disk. If you continue the work, repeat the process from (3).
(15) Clean the chamber with 70% ethanol.
(16) Close the valve of helium tank.
(17) Release the helium from the device.
(18) Turn off the power switch.

Improvement of the method for more efficient gene transfer

In some research articles the authors stated that "Particle bombardment is an efficient method for delivery of DNA into plant cells. This method is especially beneficial for those plants which appear to be a poor host for *Agrobacterium*."[5], "Particle bombardment offers a rapid method for delivery of DNA to plant cells for both transient gene expression and stable transformation studies."[13], or "These advances have given us the opportunity to create, characterize and select plant cultivars which could not be obtained by traditional breeding methods."[11]

Although such optimism is understandable and gives a reader hopes for the future, my experience in working towards genetic transformation using particle gun convinces me that we still have problems to overcome. Success requires more than occasional gene transfer into experimentally well-suited varieties of some species. It requires routine and efficient gene transfer into any desired variety of any species[34].

Some suggestions for improving transformation efficiency which are presented below are based on our accumulated experience and are still more empirical than scientific, in the author's laboratory.

(1) Water:

Use autoclaved ultra pure water when preparing buffers and solutions.

(2) Spermidine:

After adjusting the concentration to 0.1 M, pour the solution into 1.5 mL microcentrifuge tubes. Keep them in a deep freezer (−80°C). At the start, when the solution is being used, keep the tube in an ordinary freezer (−20°C) and finish using it within 2 weeks. Discard it even if some solution is left after 2 weeks.

(3) Ethanol:

Use fresh ethanol. When using 70% ethanol,

prepare it just before the experiment. Absolute ethanol is better than commercially available 99.5% ethanol. Molecular sieve (SIGMA, M-9882) is recommended to absorb water in ethanol.

(4) Particle wash:

Remove the supernatant as much as possible. After the second wash with absolute ethanol, one more ethanol wash is recommended to remove the residues of spermidine and water. Once particles have been coated with DNA they should be used as soon as possible.

(5) Centrifuge:

The particles must be gently pelleted. Lower speed is recommended (3,000–5,000 rpm for 1–2 sec is enough). Higher speed enhances particle agglomeration.

(6) Plasmid DNA:

Plasmid DNA must be very pure. Do not use the sample containing RNA sometimes derived from miniprep extraction method.

(7) Carrier DNA:

Do not use carrier DNA. Carrier DNA is often used in electroporation of protoplasts but carrier DNA is also absorbed by the particles.

(8) Microcarrier (particle) coating on the macrocarrier:

Uniform coating is very important. For more reproducible coating procedures one needs some practice to master the uniform coating method. DNA-coated macrocarrier should be used within 2 h.

(9) Osmoticum (Osmotic treatment):

Addition of an *osmoticum* (mannitol or sorbitol) to the bombardment medium increases the rates of transient and stable transformation[44]. The author has observed that this holds true for some plant species, although the optimum concentration for each species varies. Increased *osmoticum* concentrations may enable to protect the cells from leakage and bursting, and may also improve particle penetration itself. The optimum osmotic concentration for tobacco BY-2 cell is approximately 0.4–0.5 M mannitol, however, the cells grow slowly.

(10) Routinize:

Operation of the particle gun device itself is easy. The method can be routinized once the researcher sets up the bombardment parameters.

(11) Bombardment of the sample in which there are no references about the bombardment parameters:

The following conditions are applied in the author's laboratory.

Microcarrier: 1.6 μm gold particles.
Target position: 9 cm from the stopping screen to the target cells or tissues.
Helium gas pressure: 1,100 psi and 1,300 psi.
Bombardment time: 2 times per sample.
Promoter gene: Dicot; 35 S, Monocot; 35 S, Adh, or rice actin.
Reporter gene: *Gus*.

If there are no blue spots, another method of gene transfer or construction of the new promoter suited to the sample is strongly recommended. If there are one or more blue spots, it is suggested that the experiment should be continued to identify optimum conditions.

Conclusion

It is not necessary to rely on the particle bombardment method if *Agrobacterium* or a protoplast transformation system is available because the transformation frequency of particle bombardment is still low. The method is sometimes too labor-intensive and rather expensive to obtain large numbers of independently transformed plants.

The author considers that the protoplast transformation system is most appropriate. The advantage of isolated protoplasts is that they represent a true single cell system because each protoplast is completely separated from other cells and gene transfer can be performed by a relatively simple method without using specialized equipment like a particle gun. An electroporator device is easily constructed and is also commercially available. The PEG method does not require any special device.

Particle bombardment is certainly not a panacea. There are still major technical and scientific obstacles that need to be overcome in order to bring the technology to its full potential[11]. However, in the past few years the author learned a great deal about how to make the process more effective. Some research groups including ours are still trying to determine how to optimize the process with its diverse fields of application.

In the near future this method will become a tool for wider application of molecular and genetic approaches to crop improvement.

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