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Cryobiolistics : transformation of plant cells using frozen DNA as microprojectiles

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CRYOBIOLISTICS: TRANSFORMATION OF PLANT CELLS USING FROZEN DNA AS MICROPROJECTILES

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ABSTRACT

CRYOBIOLISTICS: TRANSFORMATION OF PLANT CELLS USING FROZEN DNA AS MICROPROJECTILES

BY
JAMES M. GILMORE

A new method of introducing DNA into cells, termed "cryobiolistics," is described. Cryobiolistics is related to biolistics, but instead of the biolistic method of accelerating DNA-coated tungsten particles into cells, the cryobiolistic technique uses accelerated frozen shards of DNA to pierce the cell. The delivery process is completed when the frozen shard melts, thus releasing the DNA for the cell to express.

To demonstrate this technique, three designs of air-powered accelerating apparati were constructed and evaluated. Plasmids containing the marker gene coding for the production of β-glucuronidase were frozen and accelerated by these apparati into tobacco leaf, callus, pith, and suspension cells. Twenty-four hours later, the bombarded cells were observed for a blue pigment that forms when β-glucuronidase cleaves an added substrate. Depending on acceleration parameters, type of plasmid used, and target tissue, up to 100 cells could be transformed per bombardment.

The results indicate that cryobiolistics is a practical technique and could be used to introduce not only DNA into the cell, but many other substances including proteins, antibodies, organelles, etc.

DEDICATION

In memory of my grandfather, Dee Beard, who did not understand the ruckus over the Tse-tse fly, but could spin a great yarn and taught me to shoot straight.

ACKNOWLEDGEMENTS

This thesis is the culmination of work from many people, to whom I am deeply indebted. First, to my mentor and friend Dr. Chris Brinegar; without his guidance, red pen, patience, and wit this whole crazy idea would have never got off the ground. I am also grateful to the NitroGenes gang of Michael Johnson (who engineered the accelerators and all the other nutty items that were not mentioned) and Nigel Walker (fellow brainstormer and colleague).
I would also like to thank the committee members, Dr. Pam Stacks and Dr. Susan Germeraad, for their creative insights and technical expertise.

Not all of the support was scientific--without the help of my family and friends (Carol, Mike, Chris, Beth, Kip, and the Oregon home-folk), I would have pulled out my hair, removed my cuticles, and called it quits long ago. Do you think you can stand it for four more years?

"A man with a mission....
A boy with a gun....
He's got a picture in his pocket of the lucky one"
DEVO--"A Big Mess"

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INTRODUCTION

While evolving, cells have developed a variety of mechanisms to protect themselves from taking up foreign DNA (methylation, DNases, etc.). These defense systems are needed, indeed required, because foreign DNA can be fatal to the cell. Extra pieces of DNA can insert into an important gene, thereby inactivating the gene. Even if the insertion is not fatal, and the foreign DNA manages to be expressed, it may code for a protein that is deadly to the cell. Finally, the foreign DNA (or its mRNA) can be complementary to the cell's own mRNA and hybridize to it, thereby preventing translation to a needed protein.

While these defenses are needed for survival, they tend to be headaches for plant molecular biologists who want to insert foreign DNA into cells. In order to defeat the cellular defenses, molecular biologists have developed a number of transformation (ie., gene insertion) methods, but only one works for all types of cells. This technique, termed biolistics, inserts foreign genes into cells by shooting DNA-coated microprojectiles into the target tissue. Unfortunately, biolistics suffers a number of shortcomings. This thesis introduces a modification of the biolistic transformation method that is intended to alleviate its inherent problems. In order to understand this modified technique, which we have termed "cryobiolistics," it is first necessary to review the strengths and

weaknesses of other transformation methods. Since there are so many methods, it is convenient to place them into one of three broad transformation categories: biological, chemical, or physical.

Biological Transformation

Agrobacterium Ti-mediated transformation is the most common biological gene delivery system for plant cells (Bevan *et al.*, 1983; Herrera-Estrella *et al.*, 1983; Chee *et al.*, 1986). In this system, the gene is introduced into *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* via bacterial conjugation (tri-parental mating) with *E. coli*. The *Agrobacterium* attacks a wounded plant and inserts the T-DNA of the Ti plasmid into damaged cells. Any foreign DNA that is included in the T-DNA region will also be transferred into the damaged plant cell.

Another biological delivery system, retroviral integration, while effective in animal cells (Hamer *et al.*, 1979; Mulligan *et al.*, 1979), has yet to be demonstrated in plant cells. In general, this system requires the cloning of a gene into a virus and the subsequent transfection of a host cell where the gene will be transcribed and translated.

Unfortunately, both *Agrobacterium* attack and retroviral integration are limited by the amount of DNA that can be transferred, difficult transformation protocols, and host specificity (*Agrobacterium* attacks dicots only, although the transient transformation of *Asparagus officinalis* with *Agrobacterium* has been

reported by Bytebier *et al.*, 1987). In addition, there are environmental concerns involved in using a "natural" organism (which may escape) for gene transfer.

Chemical Transformation

The most commonly used chemical transformation technique uses calcium chloride (Graham *et al.*, 1973). Calcium chloride probably works by making the cell membrane more permeable and adsorbing DNA onto the cell surface, thus allowing DNA to enter the cell. This works very well with animal cells and prokaryotes, but has two limitations:

1) Calcium chloride uptake into plant cells requires protoplasts (Lorz *et al.*, 1985). Protoplasts are plant cells that have had their cell walls enzymatically digested. This often results in somoclonal variation and sterility. Also, protoplasts are difficult to regenerate into plants (Strange, 1990) and may require a lengthy period of backcrossing to achieve stable transformation (Gasser and Fraley, 1989).
2) The size of DNA that can be transferred is limited to 20 kilobase pairs (kbp) or less (Maniatis *et al.*, 1982). Some desirable gene sequences, such as scaffold association regions which dramatically increase gene transcription (Stief *et al.*, 1989), are often greater than 20 kbp and could not be inserted into a host cell by this method.

Another chemical transformation process involves liposomes. Liposomes are spheres with an outer bilayer lipid coat which may surround a solution of DNA. Liposome-mediated delivery works by the lipid coat first fusing to the cell membrane and then rupturing to disgorge its DNA contents into the cell. Liposome transformation has been demonstrated in plant cells (Fraley *et al.*, 1982), but it, too, requires protoplasts.

Physical Transformation

The physical methods of gene delivery are diverse and hold the promise of becoming automated. There are four leading physical gene delivery methods: microinjection, electroporation, laser puncture, and microprojectile bombardment.

Microinjection (Crossway *et al.*, 1985; Graessman and Graessman, 1976) is the most direct transformation method. In general, micro-needles are manipulated under a microscope and inserted into target cells, where the contents of the needle are expelled. Stable transformation of tobacco, alfalfa, and rapeseed has been reported (Klein *et al.*, 1989a). Microinjection has the drawbacks of being costly and slow (approximately 50 microinjections can be performed in an hour). It also suffers from a low transformation rate (14 to 66 percent of injected protoplasts are stably transformed).

Electroporation (Fromm *et al.*, 1986; Ahokas, 1990; Cooper 1989) is a technique whereby foreign DNA is integrated into the cell

by electrical current. It is speculated that electroporation works by the current opening pores in the cell, thus allowing foreign DNA access. It allows incorporation of only limited DNA sizes and requires protoplasts (although Ahokas (1990) reported possible transformation of germinating barley seedlings). However, the monocots rice (Shimamoto *et al.*, 1989) and maize (Rhodes *et al.*, 1989) have had protoplasts transformed using electroporation.

Lasers have been used to puncture higher plant cells (Weber *et al.*, 1989), but entry of exogenous DNA and transformation has not been demonstrated. However, lasers have been used to stably transform animal cells (Tao *et al.*, 1987). Due to the cost of materials and the technology required, the laser method is expensive.

The last physical transformation method is known as biolistics (also called "microprojectile bombardment" or the "particle gun method"). Biolistics is rapidly becoming the transformation method of choice. In general, this method has three steps:

1) Ionic binding of foreign DNA to a carrier particle made of an inert substance (usually gold or tungsten).
2) Acceleration of the carrier particle and DNA into the cell.
3) Dissociation of the foreign DNA from the carrier (probably due to shear forces) and integration of the DNA into the cell genome.

Biolistics was first reported in *Particle Science Technology* in 1987 by Sanford *et al.*. In this paper, they demonstrated the ability of 5 *u*m tungsten particles, accelerated in a .22 caliber rifle, to

penetrate onion cells. Later, Klein and colleagues reported the first transformation of onion cells with DNA and RNA (Klein *et al.*, 1987). This report was confirmed by researchers from another lab using a different acceleration device (Christou *et al.*, 1988) to stably transform soybean. Christou *et al.* used DNA bound to gold particles, but accelerated them with a 30,000 volt electrical discharge (instead of gunpowder). Soon, Klein *et al.* followed up on their onion work and reported transient expression of a gene encoding β-glucuronidase in maize (Klein *et al.*, 1988a). Almost simultaneously they reported stable transformation in tobacco with a plasmid encoding the antibiotic resistance gene NPTII (neomycin phosphotransferase) (Klein *et al.*, 1988b). This was the first evidence that the biolistic process could produce stable transformation.

The biolistic process can not only transform the nuclear genome, it can be used to target yeast mitochondrial DNA (Johnston *et al.*, 1988; Fox *et al.*, 1988), chloroplast DNA of both *Chlamydomonas* (Boynton, 1988) and tobacco (Daniell *et al.*, 1990; Ye *et al.*, 1990), and plastids (Svab *et al.*, 1990). These studies have important implications as they demonstrate biolistics' capability to deliver foreign DNA to a specific organelle and assay putative organelle specific promoters.

Daniell *et al.* (1990) and Ye *et al.* (1990) used a new acceleration device that incorporated pressurized helium instead of gunpowder. This may result in higher acceleration rates and also reduce the "kill zone" (the center of the target where no

transformants are found since it receives too much tungsten and blast debris).

Biolistics has also been used to correct a gene deficiency in maize (Klein *et al.*, 1989b). Maize clones lacking aleurone tissue were transformed with a plasmid coding for anthocyanin synthesizing enzymes, changing unpigmented cells to cells containing a purple pigment.

In another experiment, biolistics has been used to transform pollen (Twell *et al.*, 1989). This resulted in transient expression, although follow-up experiments need to be performed to determine if stable integration is obtained. Transformation of pollen may lead to a general procedure to transform all plants without the need to perform costly and time-consuming tissue culture.

Biolistics can transform a number of different species (other than those mentioned above). These include:

1) The transient transformation of barley (Mendell *et al.*, 1989; Kartha *et al.*, 1989), rice (Bruce and Quail, 1990), wheat (Oard *et al.*, 1990; Lonsdale *et al.*, 1990) and cotton (Finer *et al.*, 1990).
2) The stable transformation of papaya (Fitch *et al.*, 1990) and maize (Gordon *et al.*, 1990).
3) The transformation of a prokaryote, *Bacillus megaterium* (Shark *et al.*, 1991).
4) Gene transfer to mammalian somatic cells (Yang *et al.*, 1990).

Finally, biolistics may be able to deliver DNA to target cells *in vivo* for gene therapy. This has been demonstrated on the skin and liver of a living mouse (Williams *et al.*, 1991) and in differentiated myotubes (Williams and Johnston, 1991).

The benefits of the biolistic technique are numerous: It can transform many cells at once and is not restricted to protoplasts. It is quick, requiring no biological intermediary. It is also possible to target organelles for transformation. In addition, the biolistic process allows one to transform cells with either DNA or RNA. Finally, it is the one technique that does not appear to be limited by cell type or strain. This last advantage could prove to be the most important as biolistics is the single technique that can efficiently transform bacteria, plant, and animal cells.

There are some problems that are inherent in the biolistic technique. The tungsten microprojectiles often stick together, resulting in a large "zone of death" where few surviving cells are found. The amount of DNA that can adhere to a tungsten projectile is finite, resulting in a limited transformation capacity. Also, microprojectile bombardment is limited to only negatively charged molecules. There also may be adverse biological effects when a 5 um diameter tungsten particle lodges in a 10 um diameter cell. For these reasons, it is obvious that the most negative feature of the biolistic process is the microprojectile design.

While reflecting on possible design improvements, a novel idea came to mind. What if the microprojectile was a particle of frozen

DNA? If the particle could be made small enough and have enough structural rigidity to survive both acceleration and impact, then once the DNA ice particle lodged in the cell it would simply melt, thus freeing the introduced DNA to incorporate into the cell's genome. Since this would involve both freezing and biolistic acceleration of DNA, we dubbed this concept "cryobiolistics."

There are many potential advantages of the cryobiolistic technique over biolistics:

1) Increased cell viability. Tungsten is a toxic substance to most living organisms. Cryobiolistics replaces the tungsten particle with a naturally occurring component of cytoplasm; water.

2) Regulation of the amount of DNA per particle. The actual amount of DNA that coats a metal microprojectile is unknown and difficult to determine. However, by calculating the volume of an ice particle and knowing the initial DNA concentration, one can easily calculate the amount of DNA in one ice particle (Appendix A). Also, for a given microprojectile size, more DNA can be incorporated into the volume of an ice particle than on the surface of a metal microprojectile.

3) Reduction of microprojectile clumping. Since the DNA is no longer ionically bound to the carrier particle, the microprojectiles will have less tendency to "clump"

together. This may reduce the "zone of death" that is so prevalent to the biolistics technique.

4) Ability to incorporate material other than DNA. Since cryobiolistics would not be limited to negatively charged macromolecules, any biologically active molecule that can survive low temperatures could be introduced. This opens the possibility to introduce protein-DNA complexes, enzymes, organelles, and antibodies.

5) Potential of increasing transformation efficiency. If a specific restriction enzyme(s) could be included in an ice particle with the DNA, the enzyme could specifically splice the foreign gene out of the plasmid vector and then cut the host DNA in a similar fashion to facilitate the insertion of the foreign gene. Or, substances that aid in wound healing could be included to help the cell recover from the cell wall penetration. Both could increase the efficiency of stable transformation.

In theory, the potential of cryobiolistics was enormous, yet unproven. This thesis outlines the initial exploration of the cryobiolistic technique to determine its practicality. The following pages trace the development and rudimentary optimization of three different cryobiolistic devices.

MATERIALS AND METHODS

Plasmids

The plasmids pBI121 and pBI221 were used. Plasmid pBI221 (5.7 kbp) was purchased from CLONTECH (Palo Alto, CA) and contains a 3 kbp β-glucuronidase (GUS) gene construct: cauliflower mosaic virus (CaMV) 35S promoter, GUS gene coding sequence, and nopaline synthase (NOS) polyadenylation (terminator) region. The chimaeric gene is located on a HindIII-EcoR1 fragment of pUC19.

Plasmid pBI121 (also purchased from CLONTECH) is 13 kbp long and contains a gene (NPTII) that confers resistance to the antibiotic kanamycin. Kanamycin inhibits protein synthesis unless it is inactivated by the NPTII gene product neomycin phosphotransferase. The NPTII gene has a NOS promoter and terminator. Downstream of NPTII is a GUS sequence that is identical to the GUS cassette from pBI221. Plasmid maps are located in Appendix B.

Plasmid Isolation

E. coli strain DH5-α was transformed with pBI221 or pBI121 using calcium chloride induced uptake (Maniatis *et al.*, 1982). The transformed *E. coli* were grown overnight at 37° C in LB medium (Ausubel *et al.*, 1989) and the crude plasmid recovered by the alkaline lysis method of Ausubel *et al.* (1989). The plasmid was then purified by cesium chloride centrifugation (Maniatis *et al.*, 1982) or by polyethylene glycol precipitation (Ausubel *et al.*, 1989). The

purified plasmids were analyzed by electrophoresis in 1.5% agarose/Tris-acetate gels (Ausubel *et al.*, 1989) after restriction with HindIII and EcoR1 (Sigma Chemical Co., St. Louis, MO) to ensure the presence of the 3 kbp GUS cassette.

Target Material

Four different target tissues from tobacco, *Nicotiana tabacum* cvs. Wisconsin 38 or MSX (Monsanto Corporation, St. Louis, MO), were bombarded: leaves, suspension cells, callus, and pith. Callus was initiated by excising apical leaves and removing the mid-vein. The leaves were sterilized (5 min in 10% sodium hypochlorite + 0.1% sodium dodecyl sulfate, 30 sec in 95% ethanol, 5 min in sterile water) and cut into 1 cm squares. The explants were then placed basal side up on tobacco callus inducing media (TCIM) (Appendix C) and placed in the dark at room temperature for two weeks. Callus was maintained by transferring to tobacco maintenance media (TMM) (Appendix C) every four weeks.

Tobacco suspension cultures were prepared by mincing callus tissue with sterile forceps and transferring a small amount to a flask of tobacco suspension media (TSM) (Appendix C). The cultures were rotated at 140 rpm in the dark at room temperature. Cultures were transferred to fresh media every two weeks.

Pith tissue was obtained from the mid-stems of mature tobacco plants and cut into 1 cm lengths immediately prior to use. The tough epidermal tissues were removed before bombardment. Corn leaves

were obtained from one-week-old seedlings (seeds obtained from Carolina Biological Supply Co., Burlington, NC).

Design of the Acceleration Apparati

Three generations of acceleration apparati were developed. The name given to each accelerator refers to the model number of the air rifle used. The earliest accelerator, the Accelerator 766 (Fig. 1), incorporated a used Model 766 air rifle manufactured by Crossman (East Bloomfield, NY) which was rated at a maximum muzzle velocity of 230 m sec^{-1}. Since this air rifle was not new, its velocity needed to be determined. The Crossman 766 was pumped a fixed number of times, loaded with an intact Prometheus hunting pellet (Prometheus, Inc., London, England), and placed horizontally in a vise. A model 9206A photo-gate (Pasco Scientific, Hayward, CA) was placed 1 cm from the barrel, while another photo-gate was aligned on the projectile flight path 21 cm from the barrel. The Crossman was fired while a chronograph measured the time it took the pellet to pass between the photo-gates (See Results and Discussion for data).

The first 2.5 cm of the rifle sleeve were removed to allow the barrel to fit through the vacuum chamber and into the barrel hole of Plate 1 (Fig. 2). The rifle was held in place by an Allen screw threaded through one side of Plate 1. The interior structure (Figs. 3 and 4) was permanently attached to the vacuum chamber base and

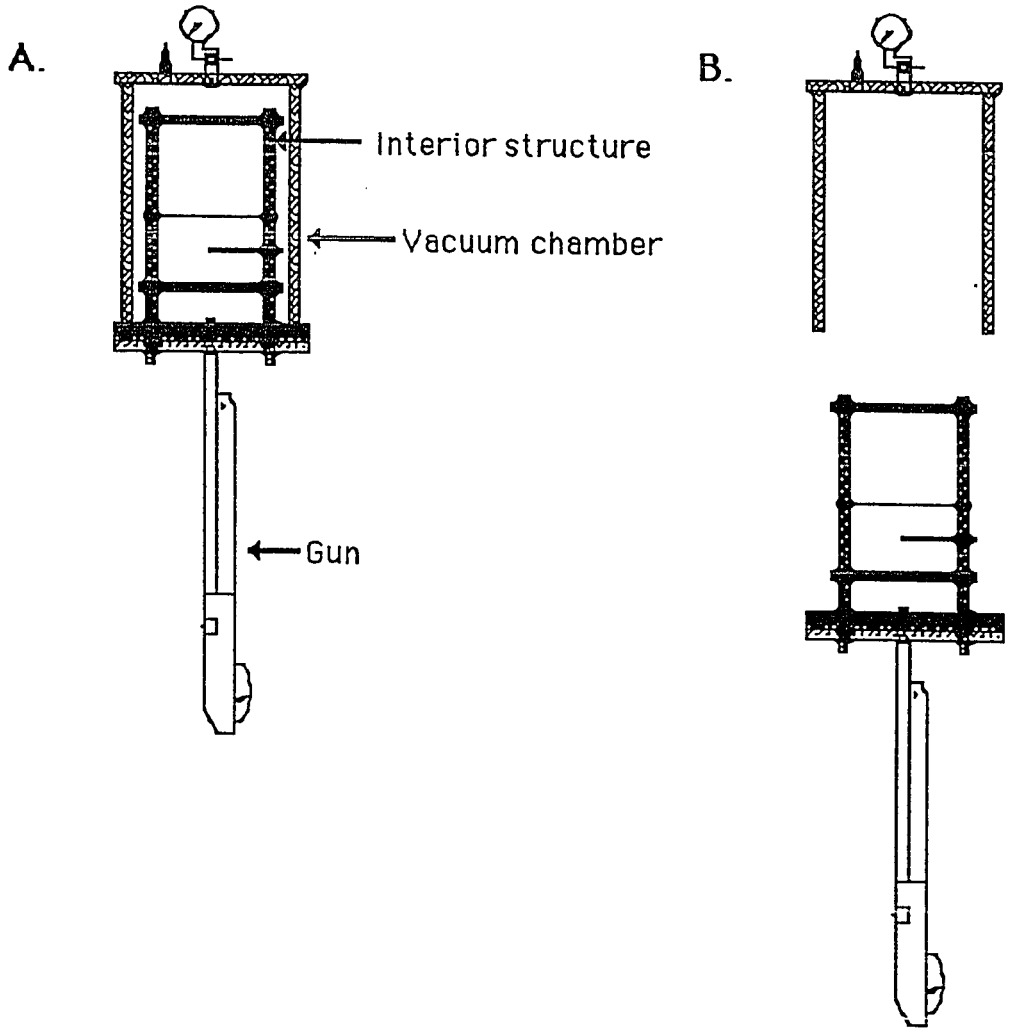

Figure 1. Side view of Accelerator 766. A: The assembled apparatus. B: The apparatus with the plexiglass vacuum chamber lifted off the chamber base.

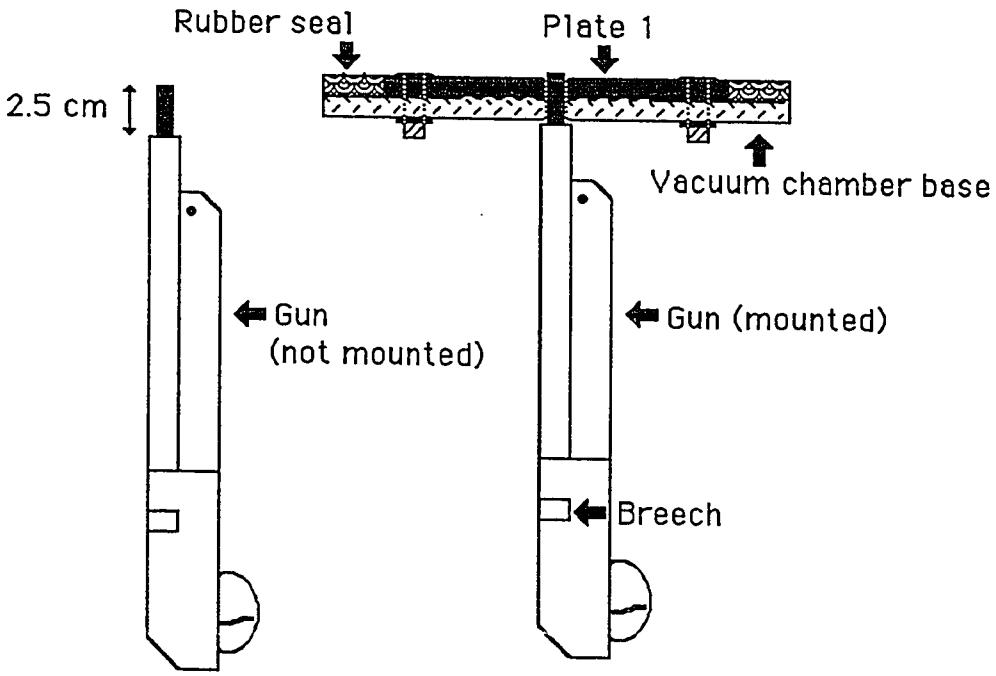

Figure 2. Side view of the vacuum chamber base and gun attachment for Accelerator 766.

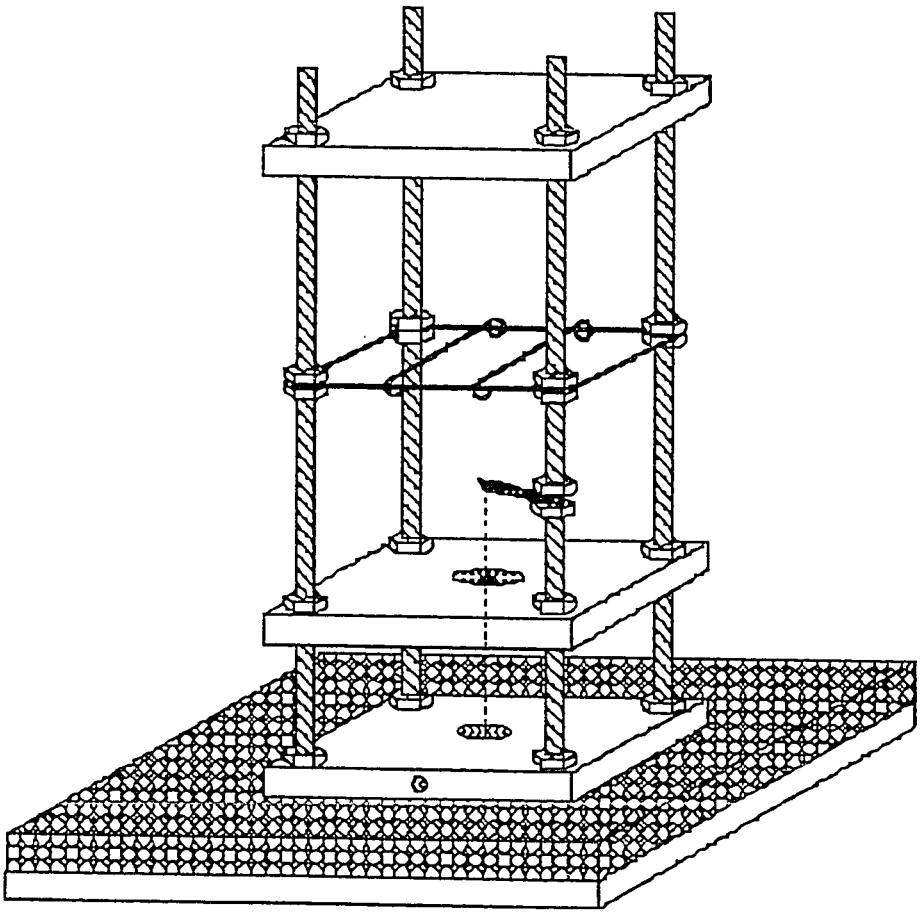

Figure 3. Perspective of the interior structure of Accelerator 766. Dashed lines indicate the path of the projectile. See Fig. 4 for details.

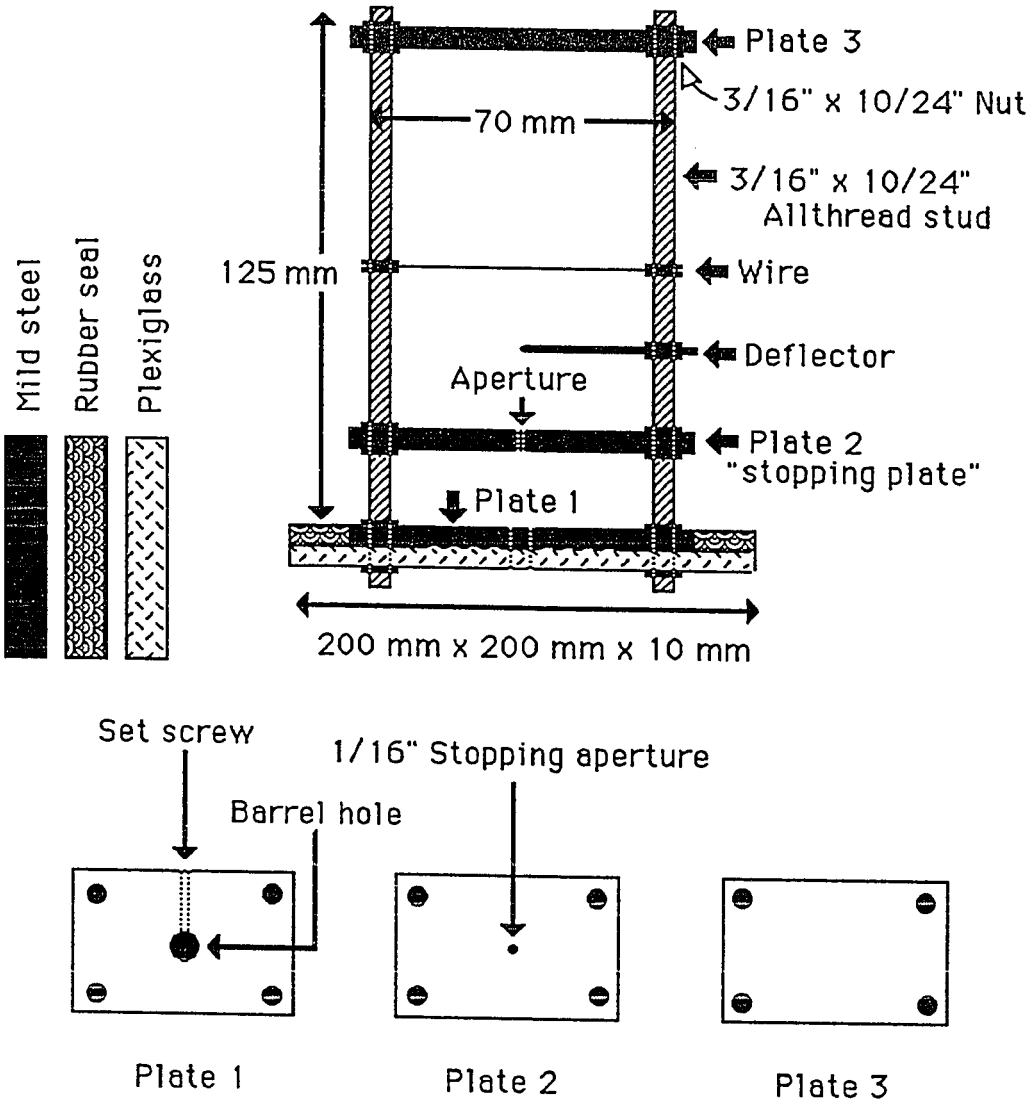

Figure 4. Details of the interior structure of Accelerator 766.
Top: Side view of the interior structure.
Bottom: Plate details.

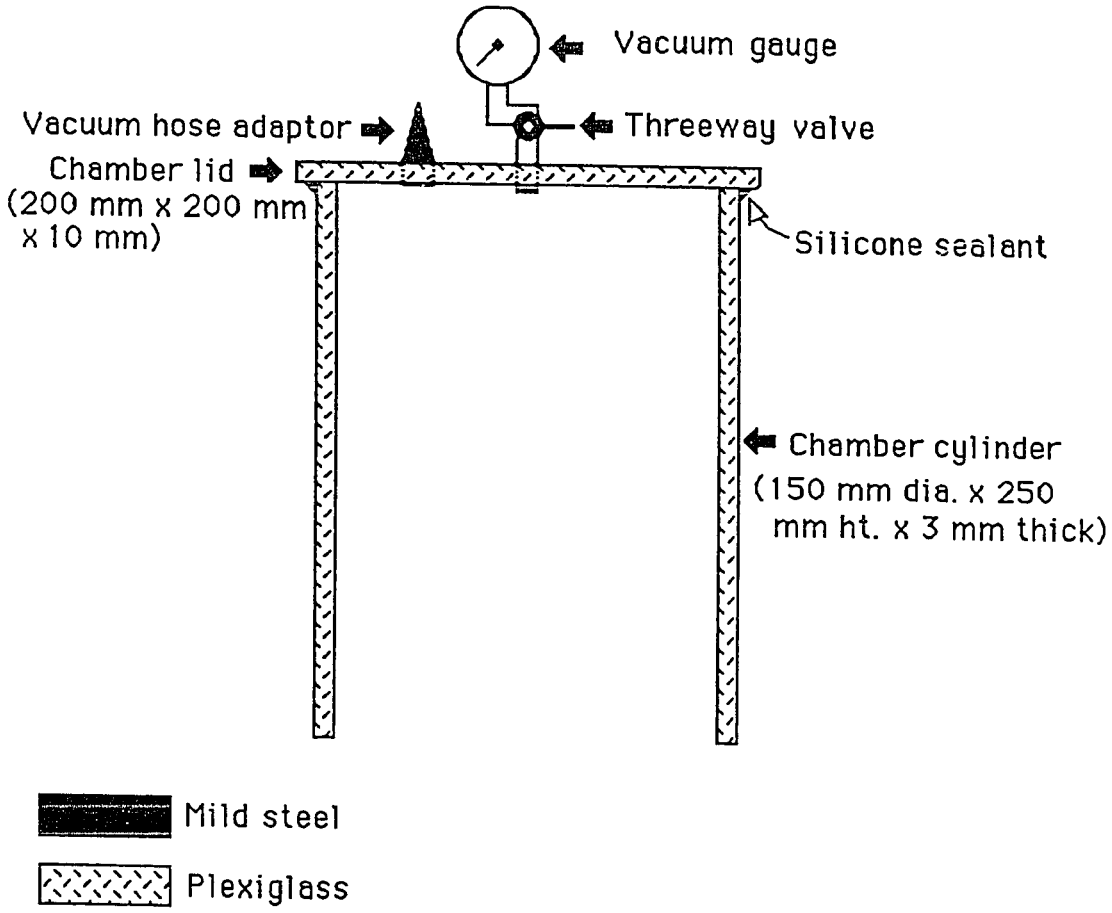

Figure 5. Side view of the vacuum chamber for Accelerator 766.

stabilized by Plate 3. Target material was held in place by wire attached to the four interior studs. This allowed the target height to be easily adjusted. Between the target material and the barrel was a metal deflector and Plate 2 (with an aperture slightly smaller than the bore diameter). This plate, known as the "stopping plate," was responsible for retarding the flight of the macroprojectile (a carrier that held the frozen DNA intact as it was accelerated). After the macroprojectile hit the stopping plate, the frozen DNA passed through the aperture and fractured upon impact with the deflector to create microprojectile shards of frozen DNA. The microprojectiles continued to travel into the target cells.

A vacuum chamber (Fig. 5) was placed over the interior structure and rested on the rubber seal of the chamber base. A vacuum hose was fitted to the adaptor on the chamber lid and the vacuum chamber pressure measured and regulated by a gauge mounted onto a threeway valve.

The next generation of accelerator, the Accelerator 760 (not shown), used the same interior structure and vacuum chamber as the Accelerator 766. The Model 760 air rifle was rated at a slightly lower muzzle velocity (213 m sec^{-1}) than the Model 766 (230 m sec^{-1}).

The latest generation of accelerators, the Accelerator 36, has significant improvements over earlier models. It consists of a high muzzle velocity (280 m sec^{-1}) Diana Model 36 air rifle (Dianawerk GmbH & Co. KG, Rastatt, Germany) attached to a ring stand which

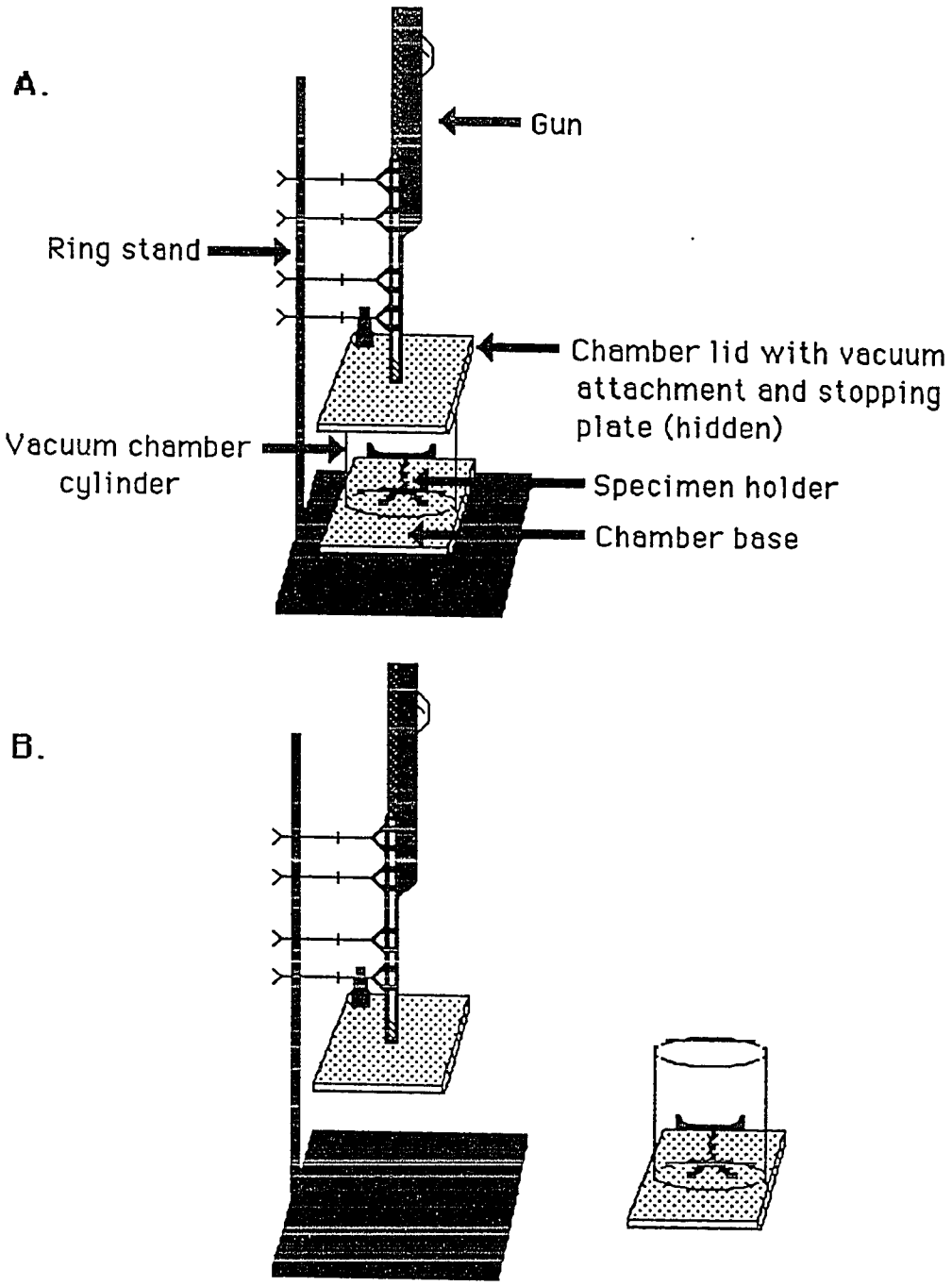

Figure 6. Perspective of Accelerator 36.
A: The assembled apparatus.
B: Vacuum chamber removed from the accelerator.

Figure 7. Photograph of Accelerator 36.

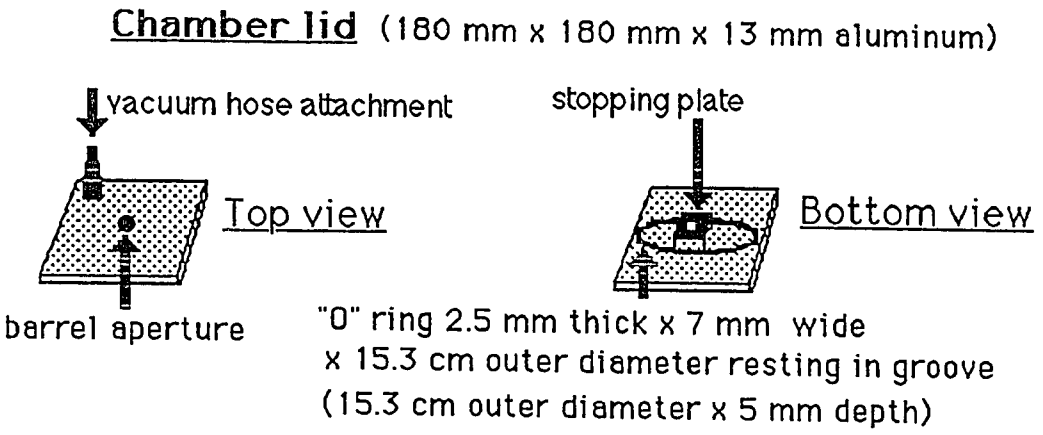

Figure 8. Components of the vacuum chamber for Accelerator 36.

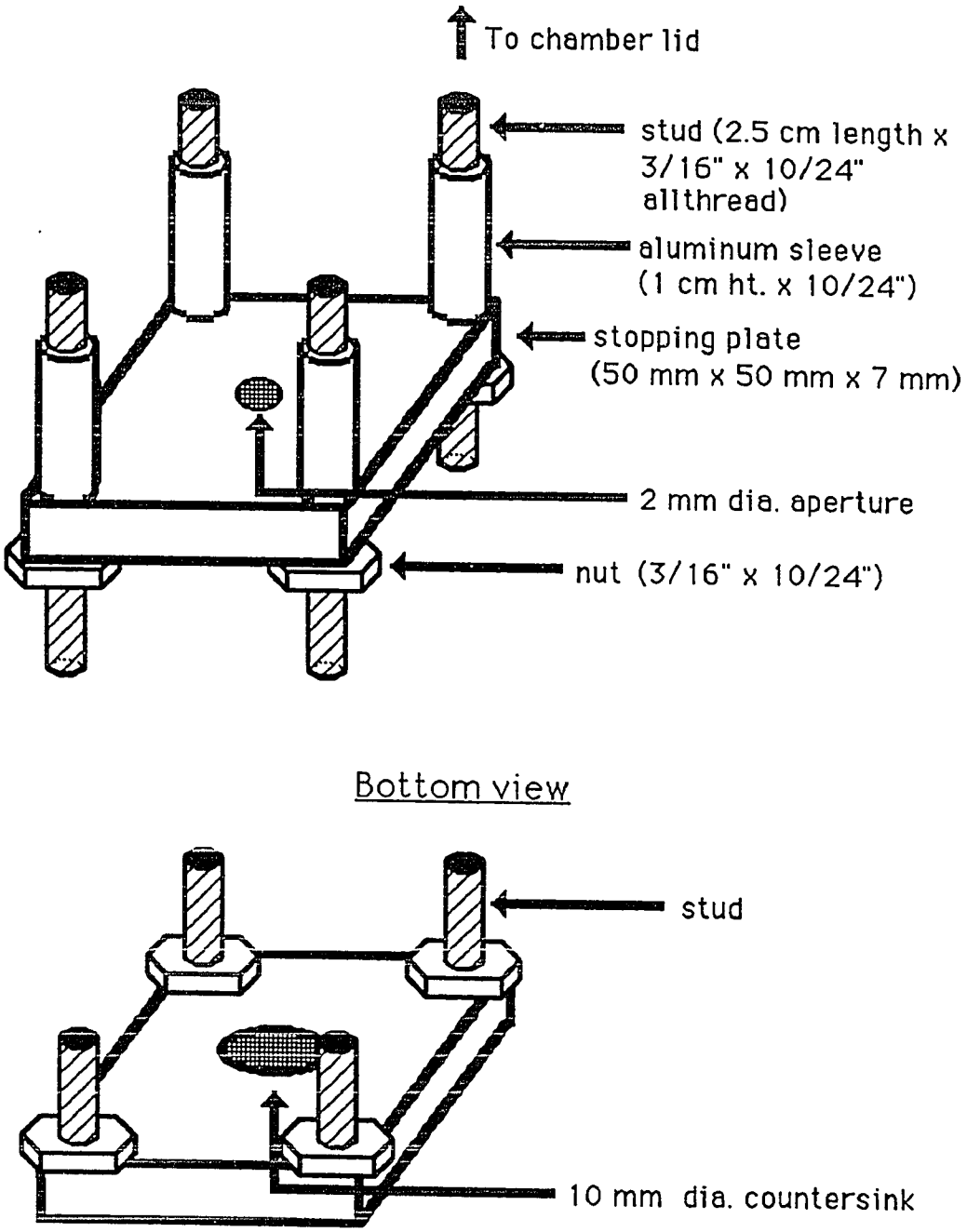

Figure 9. Perspectives of the Accelerator 36 stopping plate.

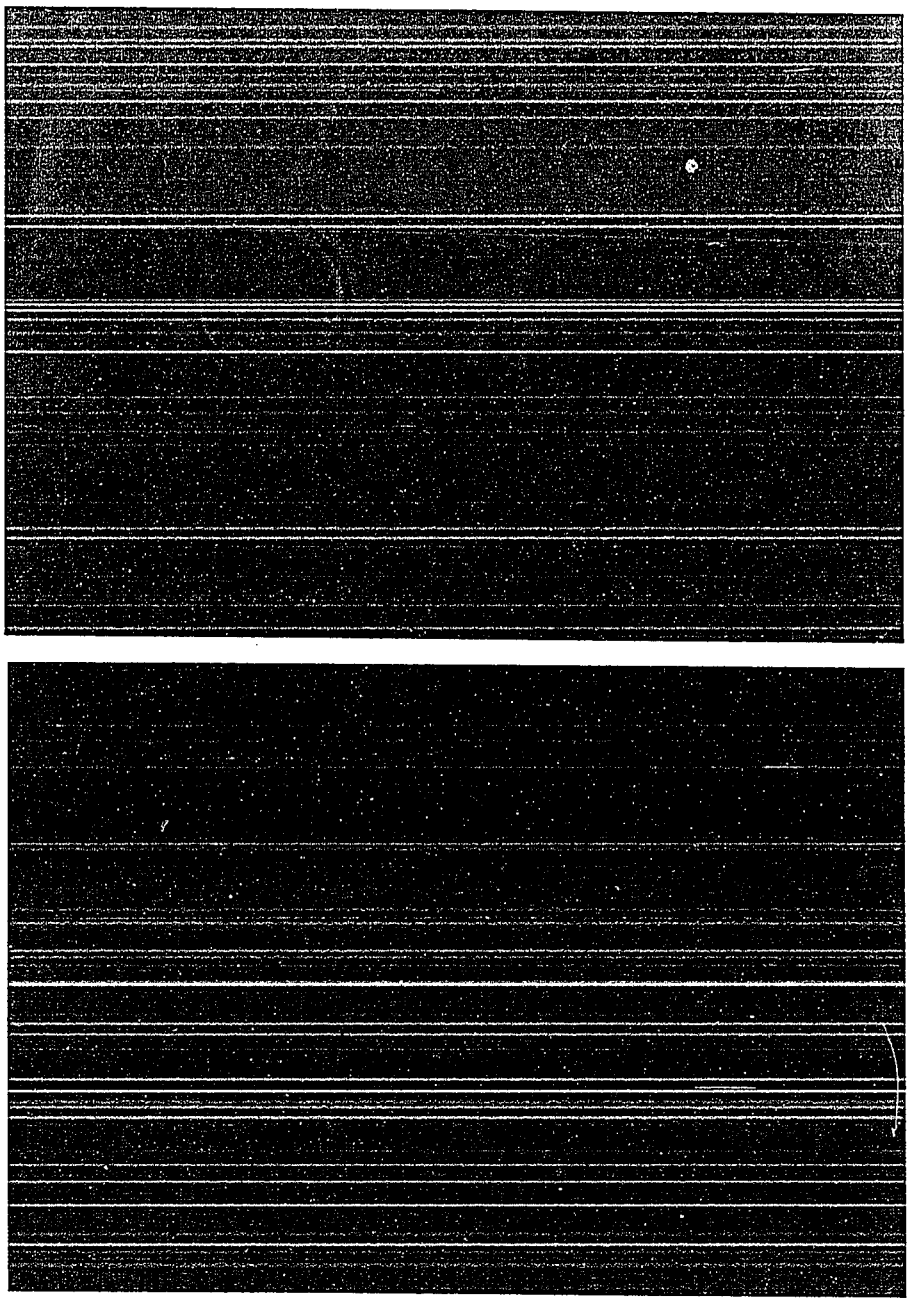

Figure 10. Photographs of Accelerator 36 vacuum chamber components. Top (left to right): Chamber base, cylinder, and specimen holder. Bottom: Underside of chamber lid showing the stopping plate.

allows the gun to fire down instead of up (see Fig. 6 diagram and Fig. 7 photograph). The vacuum chamber components are shown in Fig. 8. The barrel aperture in the vacuum chamber lid and the first 20 mm of the rifle barrel are threaded to ensure a tight fit. A plexiglass cylinder fits snugly onto the "O" rings grooved into the chamber lid and base resulting in an air-tight seal when vacuum is applied. An adjustable specimen holder for the target material is placed inside the vacuum chamber. Attached 1 cm below the chamber lid is the stopping plate (Fig. 9). Photographs of the vacuum chamber components are shown in Fig. 10.

Bombardment Procedure

Three hours prior to bombardment, the accelerator was moved to a 4° C walk-in cold room. After rinsing the interior of the vacuum chamber with 95% ethanol, a vacuum hose was attached to the chamber lid and the vacuum pump turned on to warm the vacuum oil.

In initial experiments, inverted intact Prometheus hunting pellets were used. Up to 10 *u*l of plasmid could be loaded into the hollow end of the pellet. However, it was later discovered that removal of the aluminum core from the cylindrical plastic skirt allowed a larger volume of DNA to be frozen within the skirt. After removal of the cores, the interiors of the skirts were lightly scored with a sharp probe to help the ice adhere better to the plastic surface. The skirts were then cleaned in 95% ethanol for 90 minutes,

dried, and placed on double-stick tape in a Petri dish. Plasmid solution (up to 40 *u*l) was loaded into each skirt and frozen at various temperatures for at least 30 min (See Table footnotes in Results and Discussion). Whole pellets or pellet skirts containing frozen plasmid solution are hereafter referred to as "macroprojectiles."

If the target material was suspension cells, a Whatman filter paper circle (54 mm diameter) was placed on a Buchner funnel and wetted with TSM. If needed, the suspension cultures were diluted with TSM then 2 ml pipetted slowly onto the filter paper. A slight vacuum was applied to achieve an even coating of cells. The filter paper was then transferred to the surface of 3% agar in a Petri dish which served as a "shock absorbing" backing during bombardment of the cells. Callus tissue required no preparation.

The firing sequences for Accelerator 766 and 760 were identical. Initially, the gun pump was warmed by pumping five times and firing it empty. This also served to clean the barrel of any debris. The gun was then pumped ten times and the target material placed onto the wires strung across the interior studs (cells facing the stopping plate). The vacuum chamber was placed onto the chamber base, a frozen macroprojectile quickly loaded into the accelerator breech, and a vacuum drawn from the interior of the vacuum chamber. At 15 inches vacuum, the accelerator was fired and the vacuum released.

The firing sequence for Accelerator 36 was similar. First, the target tissue was placed into the specimen holder which was centered within the vacuum cylinder on the chamber base (refer to Fig. 7). The gun was then cocked and the breech left exposed. A macroprojectile was withdrawn from the liquid nitrogen, rapidly loaded into the breech, and the gun closed. The vacuum chamber cylinder was lifted and aligned into the chamber lid grooves and a vacuum drawn. At 24-27 inches vacuum (later experiments determined 24 inches of vacuum to be optimal), the gun was fired and the vacuum released. In one experiment, in an attempt to create more microprojectiles (i.e., ice shards), a 2.5 cm diameter brass screen (0.5 mm x 0.5 mm squares, 16 squares per cm) was clipped over the target side of the stopping plate aperture. A new screen was used for each shot.

Following bombardment, suspension cells and pith tissue were placed on CRM (Appendix C). If callus was bombarded, it was placed on TCIM. Bombarded leaves were placed on a moist paper towel inside a Petri dish. After parafilming the Petri plates, the tissue was left in the dark overnight at room temperature to allow expression of the introduced genes.

Analysis of GUS Expression

Following overnight incubation, the production of β-glucuronidase was checked with the synthetic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc [Molecular Probes,

Eugene, OR]) by a modification of the assay described by Jefferson (1987). Five ml of substrate consisted of 2.5 ml 0.2 M sodium phosphate buffer, pH 7.0 (62.0 ml 0.2 M dibasic sodium phosphate + 38.0 ml 0.2 M monobasic sodium phosphate), 2.3 ml distilled water, 0.025 ml 0.1 M potassium ferricyanide, 0.025 ml 0.1 M potassium ferrocyanide, 0.10 ml 0.5 M disodium ethylenediaminetetraacetic acid (EDTA), 0.10 ml 10% Triton X-100, and 0.02 ml X-gluc dissolved in dimethylformamide at 75 mg/ml. (The original method does not include ferricyanide, ferrocyanide, EDTA, and Triton X-100 in the substrate solution.) The tissue was then incubated overnight at 21° C. If β-glucuronidase was present, cleavage of the substrate formed a blue precipitate. The number of blue cells expressing the precipitate was counted using a dissecting microscope. Diffuse blue staining was counted as one cell. If the target tissue pigments hindered observation, the tissue was destained in Farmer's Fixative (25% glacial acetic acid, 75% ethanol).

RESULTS AND DISCUSSION

An investigator is often led to the next logical experiment based on results from a prior experiment. However, in developing a new technique a point may be reached whereby the next logical experiment cannot be performed due to technical limitations. This predicament was reached twice, resulting in three different cryobiolistic devices: Accelerators 766, 760, and 36. Testing of the first accelerator, the 766, resulted in a body of knowledge that allowed the building of what was hoped would be a better accelerator, the 760. Similarly, while testing the 760, it was apparent that the next generation of cryobiolistic apparatus, the Accelerator 36, needed to be constructed. To illustrate the development of the cryobiolistic apparati, key experiments from each accelerator will be described.

The first cryobiolistic apparatus incorporated a Crossman 766 air rifle. The results of the photo-gate experiment to determine its actual velocity are shown in Fig. 11. The maximum velocity at 10 pumps, 215 m sec^{-1}, compared well with its rated velocity of 230 m sec^{-1}. The photo-gates were unavailable subsequent to this experiment, so the velocity of an ice-filled macroprojectile was not determined.

Assured that the Crossman 766 was working properly, the Accelerator 766 was assembled. A series of experiments were run to determine optimal firing parameters. These involved accelerating

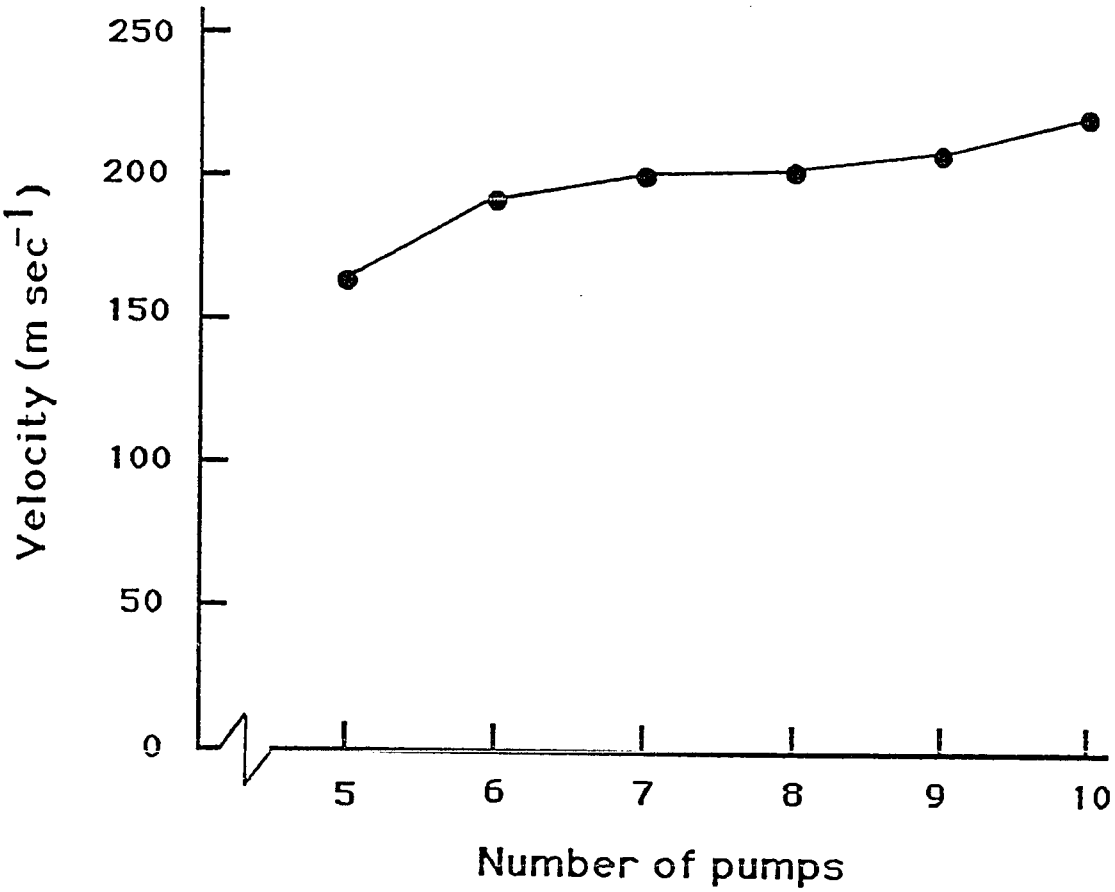

Figure 11. Velocity of a Prometheus pellet fired from a Crossman 766 at different pumps.

dye into filter paper under a variety of conditions (different vacuum pressures, target heights, freezing conditions, volumes, and velocities) to observe the pattern formed and damage. Satisfied that base line parameters had been generated, the decision was made to attempt cell transformation.

The first target tissues bombarded were leaves from tobacco and corn. Three leaves of the target tissue were stacked on top of each other 1 cm from the target side of the stopping plate (target height). The results of bombarding the leaves with different volumes of pBI121 are shown in Table 1. The most important observation was that acceleration of frozen plasmid resulted in significantly more transformants than acceleration of an equal amount of liquid plasmid. Also, a trend was detected that indicated larger volumes of frozen plasmid resulted in more transformants. Tissue damage of bombarded corn was more severe than bombarded tobacco. As would be the case in further experiments, the majority of the transform-ants for both types of tissue were located within 1 cm of the blast hole. Controls of unshot tissue had no transformants.

To increase the volume of plasmid accelerated, and hopefully the transformation rate, subsequent experiments were performed using modified Prometheus pellets (see Materials and Methods). By removing the aluminum core it was possible to load 40 *u*l of plasmid into a skirt. Firing the new skirt with 40 *u*l of frozen dye into filter paper resulted in a hole with an average diameter of 1.5 cm--

Table 1. Transient GUS expression in corn and tobacco leaves bombarded with pBI121[a] using Accelerator 766.

Plasmid[b] volume	Leaf	Average number of positives[c,d] Lower leaf	Middle leaf	Top leaf
10 *u* l (Liquid)	corn	1.5	4.0	0
10 *u* l (Frozen)[e]	corn	10.5	7.0	1.5
3 *u* l (Frozen)	corn	4.0	0.5	1.5
1 *u* l (Frozen)	corn	4.0	2.0	0.5
10 *u* l (Frozen)	tobacco[f]	5.0	0	7.0

[a] 0.1 mg/ml in 10 mM NaCl.

[b] Plasmid was placed in the hollow end of an inverted, intact Prometheus pellet.

[c] Average number of blue cells observed from a minimum of three shots.

[d] In the stack of three leaves, the lower leaf was closest to the stopping plate.

[e] Frozen at -20° C for 30 minutes, then stored on dry ice (-60° C).

[f] Data from one shot.

significantly larger than the 0.5 cm diameter holes caused by 10 ul of dye. Also, shards of ice were detected in the vacuum chamber after acceleration of the macroprojectile.

Since the larger volume caused more damage to the filter paper it was suspected that fragile leaves would be destroyed. Therefore, the next experiment used tobacco suspension cells as targets. (Suspension cells were also thought to be a more homogeneous and reproducible target.) The plasmid volume (20 ul or 40 ul) and target height (20 mm or 30 mm) were varied. The results (Table 2) demonstrate that acceleration of larger volumes of plasmid results in higher transformation rates (although the number of transformed cells can vary widely between shots). A photomicrograph of GUS-positive cells is shown in Fig. 12. Unshot filter paper had a background of 0-3 positive cells per paper. According to Jefferson (1987) a possible source of background is chemical hydrolysis of the substrate during prolonged incubations.

Even though reasonable transformation rates were being obtained, it was feared that extensive use had decreased the velocity of Accelerator 766. Therefore, it was decided to replace the Crossman 766 rifle with a new Crossman 760 rifle. This cryobiolistic apparatus was called Accelerator 760. Unfortunately, while the Accelerator 760 was being built, a series of mishaps occurred. First, a power failure caused the loss of all suspension cultures, then an incubator thermostat broke,

Table 2. Transient GUS expression in Wisconsin 38 tobacco suspension cells bombarded with pBI121[a] using Accelerator 766.

Plasmid volume[b] (*u*l)	Target height[c] (mm)	Number of positives[d]
20	20	17
20	20	29
20	20	08
20	20	05
	mean ± s.d. = 15±11	
40	30	25
40	30	61
40	30	66
40	30	103
40	30	22
40	30	52
	mean ± s.d. = 55±30	

[a] 0.1 mg/ml in 10 mM NaCl.

[b] Plasmid solutions were frozen in modified Prometheus pellets on dry ice (-60° C).

[c] Distance between stopping plate and target cells.

[d] Number of blue cells observed per bombarded filter paper.

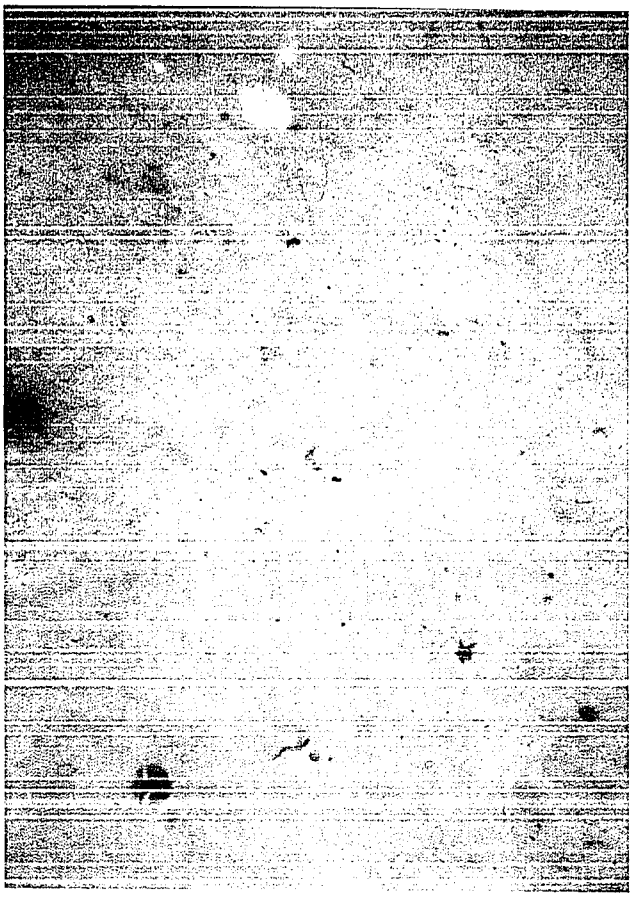

Figure 12. Micrograph of bombarded tobacco suspension cells.

resulting in the loss of all callus cultures. Also, a decision was made to switch from pBI121 to pBI221 since the GUS constructs were identical but the plasmid yield was greater with pBI221. Because of these accidents and modifications, when Accelerator 760 was finally completed it would be tested with entirely different materials than were used with Accelerator 766.

Firing frozen dye in Accelerator 760 demonstrated that it had the same basic parameters as Accelerator 766. However, when 40 ul of dye were frozen and accelerated into filter paper, the hole that resulted had a smaller diameter than with the Accelerator 766, suggesting that the actual muzzle velocity of the new Crossman 760 was not as high as the worn Crossman 766.

Although Accelerator 760 may have had a lower velocity than the previous apparatus, it appeared to be functional so transformation of Wisconsin 38 suspension cells with pBI221 was attempted at two different plasmid concentrations (1.0 mg/ml or 0.1 mg/ml) and two different target heights (20 mm or 30 mm). The results are shown in Table 3. This experiment resulted in much lower transformation rates regardless of the target height. There were a number of possible reasons for these lower transformation values. The velocity of the Crossman 760 may have been too low to allow the ice microprojectiles to penetrate the target cells. Maybe pBI221 did not promote transformation as effectively as pBI121 due to some feature of the vector, e.g., its origin of replication or the lack of appropriate border regions flanking the GUS cassette. Finally,

Table 3. Transient GUS expression in Wisconsin 38 tobacco suspension cells bombarded with pBI221[a] using Accelerator 760.

Plasmid concentration[b] (mg/ml)	Target height[c] (mm)	Average number of positives[d]
0.1	20	0
0.1	30	0.3
1.0	20	7.5
1.0	30	3.0

[a] in 10 mM NaCl.

[b] 40 *ul* of plasmid were frozen in modified Prometheus pellets on liquid nitrogen (-195° C).

[c] Distance between stopping plate and target cells.

[d] Average number of blue cells observed from four shots (at 0.1 mg/ml) or three shots (1.0 mg/ml).

there was the possibility that the new Wisconsin 38 cell line was recalcitrant to any transformation. An experiment was performed to check this possibility by using a second tobacco cell line, MSX, for comparison (Table 4). This showed there was little, if any, transformation in either two cell lines. An experiment was also performed to determine if increasing macroprojectile velocity (by decreasing the volume of plasmid) would increase transformation rates in either cell line. Again, regardless of plasmid volume, transformation was marginal (data not shown).

Accelerator 760 was clearly not transforming cells as efficiently as Accelerator 766. Therefore, Accelerator 766 was reassembled and tested, but little transformation was seen using pBI121 or pBI221. Obviously, some parameter or parameters had changed since the positive results shown in Tables 1 and 2. From the observations just described, it was concluded that either a threshold velocity was not being achieved or that the cell lines, for whatever reasons, were no longer capable of being transformed.

In order to continue the cryobiolistic project it was decided to build Accelerator 36 which incorporated an air rifle with a greater muzzle velocity (280 m sec^{-1}), a thicker cylinder wall to allow a higher vacuum to be drawn, and a precision-bored stopping plate. Initial firings resulted in greater skirt destruction than any previous accelerator and a hole with the largest diameter observed to date. Due to the extensive tissue damage, brass screens (see Materials and Methods) were sometimes placed on the target side of the stopping

plate to further fracture the ice pellet. Yet another new line of Wisconsin 38 suspension cells was prepared and a new tissue type (callus) was bombarded for comparison. Results from the first experiment with Accelerator 36 are shown in Table 5.

There was still essentially no transformation of the tobacco suspension cells. However, some transformation was observed in the callus tissue. The callus received heavy damage, indicating a high velocity had been achieved (this was also evidenced by large dents in the screens when they were used). These observations provided some important clues. First, many of the transformed cells were probably not being recovered. Also, the fact that callus tissue was transformed, but not suspension cells indicated that the suspension cells were probably recalcitrant to transformation.

The next experiment used pith tissue instead of callus or suspension cells. Also, in order to recover more tissue, the pith was placed into a small plastic cup while it was shot. While the target height and tissue type were fixed, the plasmid concentration was varied. The results are shown in Table 6. A reasonable transformation efficiency was achieved. Interestingly, increasing the plasmid concentration did not increase the transformation rate. In fact, the opposite effect occurred--lower plasmid concentrations resulted in more transformants. This suggested that receiving too many plasmid copies may be lethal to a cell.

It appears that after the initial successes using Accelerator 766, the loss of competent cells and a decreased velocity were

Table 5. Transient GUS expression in Wisconsin 38 tobacco suspension cells and callus bombarded with pBI121[a] using Accelerator 36.

Target height[b] (mm)	Screen	Tissue	Average number of positives[c]
15	-	suspension cells	0
15	+	suspension cells	0
30	-	suspension cells	0.3
30	+	suspension cells	0
15	-	callus	4.0

[a] 0.1 mg/ml in 10 mM NaCl; 40 *u*l of plasmid were frozen in modified Prometheus pellets on liquid nitrogen (-195° C).

[b] Distance between stopping plate and target cells.

[c] Average number of blue cells observed from a minimum of three shots (suspension cells) or one shot (callus).

Table 6. Transient GUS expression in tobacco pith[a] bombarded with pBI121 using Accelerator 36.

Plasmid concentration[b] (mg/ml)	Average number of positives[c,d]
0.03	27.3
0.10	4.3
0.30	6.3

[a] Target height was 1-2 mm.

[b] in 10 mM NaCl; 40 *u*l of plasmid were frozen in modified Prometheus pellets on liquid nitrogen (-195° C).

[c] Average number of blue cells observed from three shots.

[d] Controls of unshot pith soaked in 40 *u*l of 0.1 mg/ml plasmid had an average of 1.5 GUS-positive cells.

responsible for diminished transformation rates. But groundwork experiments using Accelerator 36 have resulted in a transformation rate comparable to those early successes. Further optimizing of Accelerator 36 will hopefully result in even higher transformation frequencies.

CONCLUSION

This research was designed to assess the practicality of using accelerated frozen DNA to penetrate and transform plant cells. The questions that needed to be addressed were:

1) Can frozen DNA survive both acceleration and impact on the stopping plate?
2) Are shards of frozen DNA created that have enough momentum to penetrate the cells?
3) Can the DNA contained in the frozen shards effect transformation?

There is a great deal of evidence to support the conclusion that accelerated frozen DNA does not thaw before it reaches the target cells. First, following acceleration, ice particles were observed in the vacuum chamber of each accelerator model. Secondly, skirts containing liquid DNA suffered less damage upon hitting the stopping plate than skirts with frozen DNA. Damage to skirts containing liquid DNA was comparable to empty skirts that had been accelerated. Damage to target cells was significantly heavier when the cells were bombarded with frozen DNA rather than liquid DNA. When Accelerator 36 was used to propel different volumes of frozen DNA into cells shielded by brass screens, the screens suffered damage that was proportional to the volume. In contrast, accelerated liquid DNA caused very little damage to the brass screens. Finally, the number

of GUS-positive cells generated by bombardment with liquid DNA was never higher than non-bombarded control cells.

Direct evidence that microprojectile shards of frozen DNA are created is difficult to obtain; however, indirect evidence is available. The strongest evidence is based on the results of the different accelerators. The rate of acceleration and the impact of the frozen DNA on the stopping plate must be related to the number of microprojectiles created. Accelerator 760, with the lowest muzzle velocity, never achieved transformation rates above the background count. It may be argued that this was due to recalcitrant suspension cells, but few GUS-positive cells were found, even in bombarded tobacco leaves. Also, cell and skirt damage with Accelerator 760 was consistently lower than damage caused by other accelerators. Lastly, accelerating liquid DNA did not cause transformation rates above background, but acceleration of frozen DNA resulted in transformation rates significantly higher than background. However, a note of caution must be sounded: It is possible that some transformation is the result of DNA binding to small pieces of the plastic skirt that manage to squeeze through the stopping plate aperture and lodge into the target cells.

With respect to the last question, the bombardment of tissue with frozen pBI121 followed by an assay for β–glucuronidase activity resulted in more blue cells than assayed control tissue. This indicated that the frozen plasmid survived acceleration, was able to enter the target cell, and could be expressed into a functioning

protein. It is interesting to note that acceleration of pBI121 resulted in consistently higher transformation rates than did pBI221. This may be due to the Ti-derived flanking border regions of pBI121 that are required for transformation using *Agrobacterium*.

Although cryobiolistics is easier and faster than biolistics, it has a lower transformation rate (Klein *et al.* (1988a) reported over 500 GUS-positive cells per shot). But, all of the cryobiolistic results to date are based on crude optimizations of parameters. As such, the reported transformation rates must be viewed as minimum values. Future experiments that refine parameters will undoubtedly result in higher transformation rates. Such work would define the optimal concentration and type of plasmid, target height, and plasmid suspension buffer. Also, the correct size and number of microprojectile ice shards need to be controlled. This may be achieved by using different sizes of screens or sonicating the frozen skirt before bombardment to induce shattering.

With few host limitations, the potential uses of cryobiolistics are both many and varied. Once optimized, the cryobiolistics system could be used to transform cells with protein/DNA complexes. It should also be possible to efficiently deliver proteins (such as antibodies) into target cells. Finally, including a segment of DNA with a restriction enzyme may allow the DNA to target a specific area of the host's genome instead of relying on random integration as do current transformation methods.

In summary, a new technique has been described, called "cryobiolistics," whereby frozen DNA is accelerated into plant cells causing transient transformation.

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Appendix A. Calculation of the number of plasmid molecules in a hypothetical spherical ice particle of 5μm diameter made from pBI121 at 0.1 mg/ml.

Volume of particle: $(4/3)\pi r^3 = (4/3)\pi(2.5\mu m)^3$

$= 65.4\,\mu m^3$

$= (65.4\,\mu m^3)\,(10^{-12}\,cm^3/\mu m^3)$

$= 6.54 \times 10^{-11}\,cm^3$

$= 6.54 \times 10^{-11}\,ml$

$= 6.54 \times 10^{-8}\,\mu l$

Mass of plasmid in particle: $(0.1\mu g/\mu l)\,(6.54 \times 10^{-8}\,\mu l) = 6.54 \times 10^{-9}\,\mu g$

$= 6.54\,fg$

Moles of plasmid in particle: $$\frac{6.54\,fg}{(13{,}000\,bp)\,(660\,fg \cdot fmol^{-1} \cdot bp^{-1})}$$

$= 7.62 \times 10^{-7}\,fmol$

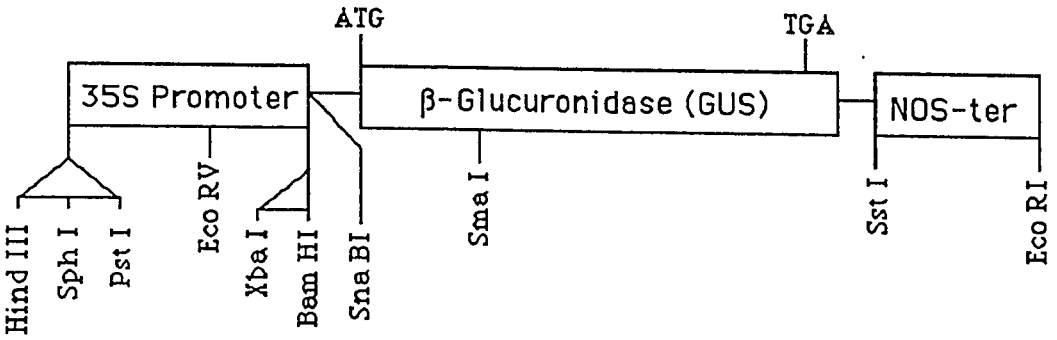
$= 7.62 \times 10^{-19}\,mol$

Number of plasmids in particle: $(7.62 \times 10^{-19}\,mol) \times (6.02 \times 10^{23}\,molecules/mol)$

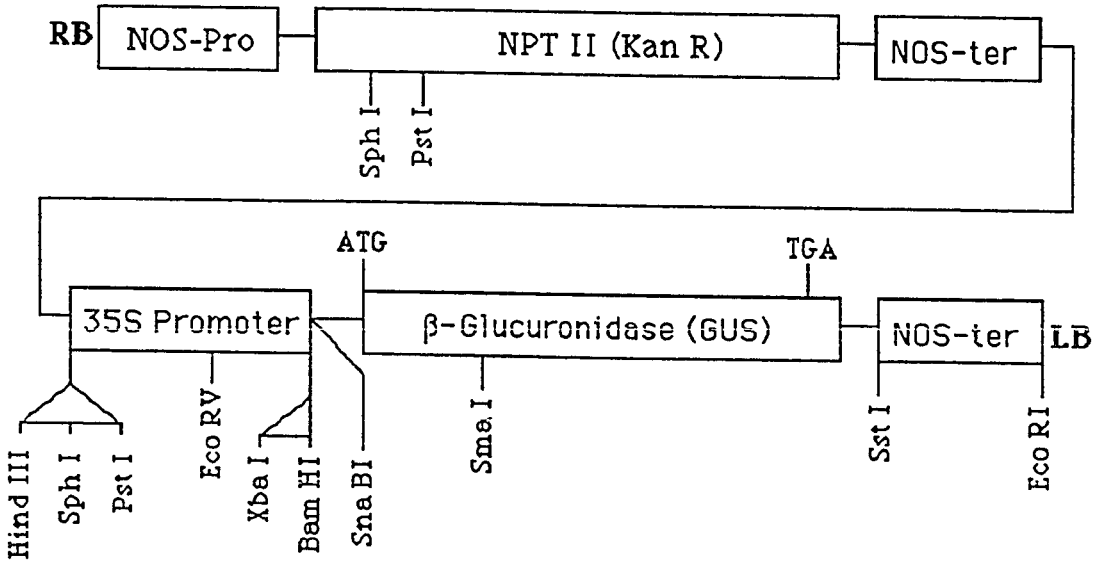
$= 4.59 \times 10^{5}$

$= 459{,}000$

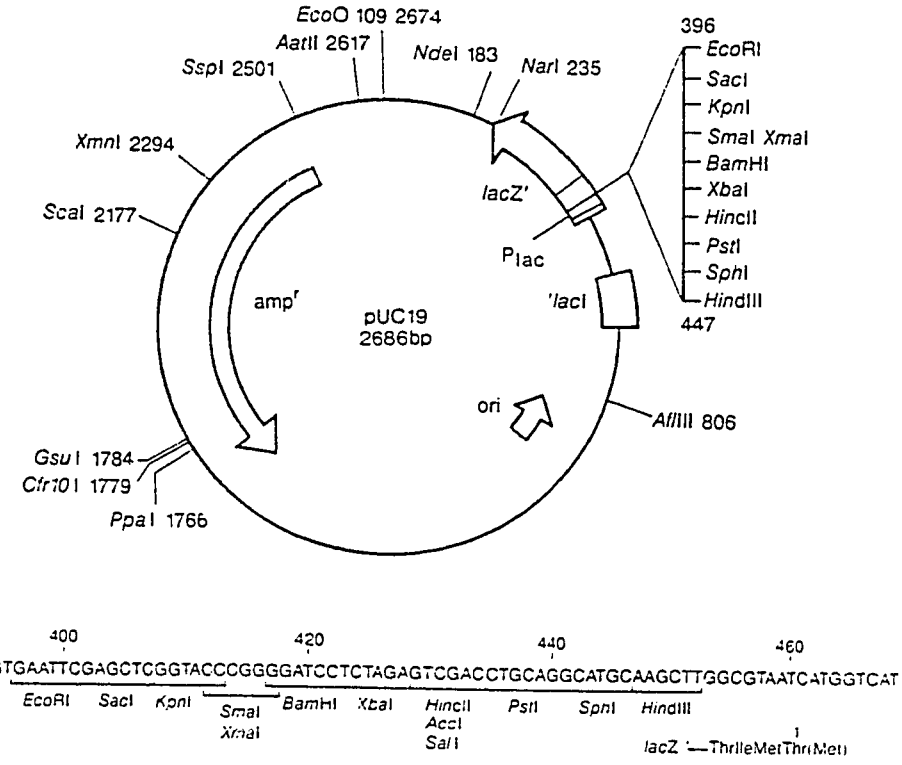
Appendix B. Plasmid maps for pBI221, pBI121, and pUC19.



pBI221



pBI121



pUC19
(Reproduced from Ausubel *et al.* 1989)

Appendix C. Media recipes for tobacco tissue cultures.

Tobacco Callus Inducing Media (TCIM)

Dissolve 4.4 g M&S Basal Salts in 900 ml water. Add 0.1 ml of 1.0 mg/ml 2,4 Dichlorophenoxyacetic Acid, 3.0 ml of 1.0 mg/ml α–Naphthaleneacetic Acid, 0.4 ml of 1.0 mg/ml 6-Benzylaminopurine, then adjust pH to 6.0. Add 30 g sucrose and 7 g agar. Bring volume to 1000 ml and autoclave 15 minutes.

Tobacco Maintenance Media (TMM)

Dissolve 4.4 g M&S Basal Salts in 900 ml water. Add 3.0 ml of 1.0 mg/ml Indole-3-Acetic Acid, 0.3 ml of 1.0 mg/ml Kinetin, then adjust pH to 6.0. Add 30 g sucrose and 7 g agar. Bring volume to 1000 ml and autoclave 15 minutes.

Tobacco Suspension Cell Media (TSM)

Dissolve 4.4 g M&S Basal Salts in 900 ml water. Add 0.5 ml of 1.0 mg/ml 2,4 Dichlorophenoxyacetic Acid, then adjust pH to 6.0. Add 30 g sucrose. Bring volume to 1000 ml, dispense 25 ml per 125 ml Erlenmeyer flask, and autoclave 12 minutes.

Cell Recovery Media (CRM)

Dissolve 4.4 g M&S Basal Salts in 900 ml water. Add 0.5 ml of 1.0 mg/ml 2,4 Dichlorophenoxyacetic Acid, then adjust pH to 6.5. Add 20 g sucrose, 7 g agar, and 135.5 g mannitol. Bring volume to 1000 ml and autoclave 15 minutes.