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Review

Physical methods for genetic plant transformation

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Abstract

Production of transgenic plants is a routine process for many crop species. Transgenes are introduced into plants to confer novel traits such as improved nutritional qualities, tolerance to pollutants, resistance to pathogens and for studies of plant metabolism. Nowadays, it is possible to insert genes from plants evolutionary distant from the host plant, as well as from fungi, viruses, bacteria and even animals. Genetic transformation requires penetration of the transgene through the plant cell wall, facilitated by biological or physical methods. The objective of this article is to review the state of the art of the physical methods used for genetic plant transformation and to describe the basic physics behind them.

Keywords: Genetic plant transformation; Electroporation; Biolistics; Silicon carbide fiber; Ultrasound; Shock waves

Contents

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1. Introduction

Owing to the need for improved plant cultivation there is an increasing interest in research of genetic transformations, i.e., transfer of deoxyribonucleic acid (DNA) molecules into plant cells [1–9]. Initial efforts for genetic plant transformation were performed almost half a century ago on maize but they were not successful [10]. The first production of recombinant DNA molecules was achieved at the beginning of 1970s with the use of biochemical scissors called restriction enzymes [11,12], and subsequently, in the 1980s, genetically stable transformed plants [13–16] like maize [17–19], tobacco [20,21], petunia [22], tomato [23], rice [24–30], celery [31], *Brassica napus* [32], wheat [33,34], grape [35], cassava [36], millets [37,38], and chrysanthemum [39] were obtained. Tomato was the first transgenic crop for food consumption approved by the Food and Drug Administration (FDA) to be distributed on the USA market in 1994 [5,40]. Nowadays, transgenic crops represent 10% of the cropland worldwide, and constitute one of the main sources of income for several countries [1,40–42]. Transgenic plants with special properties have displaced more than half of the varieties generated by standard breeding [43,44]. Furthermore, advances made in the use of transformed plants with genes for the production of novel recombinant proteins [45] opened a new future to the pharmaceutical industry [4,46–54] due to the lower production costs, its rapid scalability, the absence of human pathogens and the ability to fold and assemble complex proteins accurately. Moreover the genetic engineering of plants has already begun to play a crucial role in the production of biofuels [55–59], and has had an important biotechnological impact as shown by the numerous patents on the subject [60–71]. A search of the Web of Science literature database for citations of genetic transformation of plants by *Agrobacterium* reveals 1038 hits between 1985 and 1999, 3604 hits between 2000 and 2011, while direct methods show a moderate increase (see Fig. 1).

Genetic engineering provides the tools to introduce single components in more complex pathways and to regulate their expression spatially and temporally. Currently different technologies for gene transfer are available; however, low transformation efficiency and the randomness of integration sites are still limitations [1,2]. A fundamental tool to produce genetic improvements is the ability to introduce foreign genes (transgenes) not only from other non-related

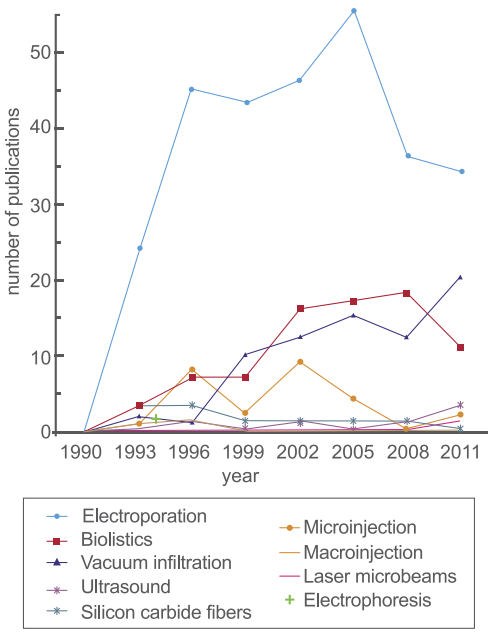


Fig. 1. Web of Science literature database searching for citations of genetic transformation of plants by direct methods.

plants but also from fungi, viruses, bacteria, and animals [4,72–76]. The transformed plant cells are regenerated into whole plants using tissue culture and the transgenes are stably inherited through generations.

The transitory expression of the transgene or its stable integration into the cellular nucleus has the goal of increasing crop productivity [77–81], provide improved nutritional qualities to plants [43,44,82–86], resistance mechanisms to herbicides [59,86–92], plague [92–94], drought [95–97], insects [98–102], viruses [12,103,104], antibiotics [20,27], salt tolerance [96,105,106], harvest damage [93,107], temperature changes [108–110], UV light [111–114], modifiers of color [113–116], biosynthetic processes [92,117–122], etc. Transgene integration has also been used to improve the expression of desired proteins to maximize the capacity of a particular metabolic process [49,50,123]. Moreover not only the introduction of exogenous genes has been exploited in genetic engineering, but also, the deletion of specific genes with the goal of reducing metabolic fluxes of alternate pathways and to redirect fluxes to the product-forming pathway have been attempted [107,120–122]. Antibodies, which are part of our immune system, may be produced in plants by transformation with the corresponding genes [1,47,83,124]. Furthermore, vaccines can be created to a huge number of pathogens, such as against bacteria that cause diarrhea [125]. Transformation can be carried out in a number of different ways depending on the species [1,2,126,127].

Transformation was discovered by F. Griffith in the late 1920s [124,128]. He reported that pneumococcal cells could convert from a harmless form to a disease-causing type. The term "transformation" is used to describe the insertion of foreign molecules into bacteria, plant cells and fungi, while the introduction of DNA into eukaryotic (animal) cells is referred to as "transfection". The major problem of transformation is that DNA is a macromolecule, highly charged, difficult to manipulate and cannot diffuse through the cell membrane, a protecting hydrophobic layer of about 10 nm that acts like a barrier. The membrane controls the entry of nutrients, ions and the exit of waste. The main constituents of the membrane are lipids, proteins and carbohydrates. In vitro culture facilitates rapid multiplication of clones and is a required technique for improvement of plants by genetic engineering, used together with the transformation techniques analyzed in this review.

In order to obtain genetic transformation with a reproducible methodology several requirements should be considered [41,77,123,129]:

- Low costs and easy procedures that lead to large numbers of transformations per event,
- operator safety avoiding dangerous procedures or substances,
- technical simplicity involving the minimum manipulations,
- capability to introduce in a stable way the desired DNA without vector sequences which are not required for gene integration or expression,
- small number of genetic copies introduced into each cell, and
- facility to regenerate transgenic plants from single transformed cells.

Common methods for genetic transformation are usually divided into indirect or direct transformation [82]. Biological methods using bacteria are referred to as indirect, while direct methods are physical; that is, based on the penetration of the cellular wall. Even if indirect methods are still more popular for plant transformation than direct techniques, recently there has been an increase in the application of physical methods. A comparison of these genetic transformation methods, showing their advantages and disadvantages, is given in Tables 1 and 2.

Indirect transformation methods introduce plasmids, that is, independent circular molecules of DNA that are found in bacteria, separate from the bacterial chromosome (Fig. 2), into the target cell by means of bacteria capable or transferring genes to higher plant species [130]. The most popular used microorganisms are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, two soil native bacteria [13–15,20,34,72,80,82,115,118,130–152]. The size of a plasmid employed for transformation may vary between 5 to 12 kilobase pairs (kbp) [153,154].

Plasmids carry several genes, are replicated in the same way as the bacterial chromosome, and are self-replicating, i.e., they are able to replicate autonomously within the host. A single cell may have up to 50 or more plasmids. *Agrobacterium* is a plant-pathogenic bacterium, capable of transferring a tumor-inducing plasmid to its host, promoting tumor formation [49]. This characteristic has been exploited to use the plasmid as a biological vector for genetic plant transformation, however the tumor-inducing genes have been removed from current (disarmed) vectors, and thus, they do not cause tumors anymore. Since the first successful gene insertion using *Agrobacterium* in the 1980s [14,15,20–22,133], this method has become popular in the industry [140,141], in spite of its problems with regeneration of some species. It is widely used for many applications, however it is limited by the low efficiency of transformation by

Table 1
Comparison of the most popular methods for genetic transformation of plants.

Method	Procedure	Advantages	Disadvantages
Agrobacterium	A pathogenic bacterium introduces a plasmid carrying gene.	Genome integration is precise, simple transgene insertions with defined ends and low copy number, stable integration and inheritance, and consistent gene expression over the generations. Different cell types can be used. Reproducible and efficient protocols have been developed for many dicotyledonous and some monocotyledonous crops. High efficiency.	Various parameters not easy to handle affect transformation efficiency and plant regeneration. Slow process. Introduction of unnecessary partner vectors that produce unknown genetic expressions into the plant. Requires sterile protocols.
Electroporation	Electric pulses induce membrane permeabilization providing a local driving force for ionic and molecular transport through the pores.	It can be applied to any plant protoplasts. Different cell types can be used. Simple, fast, and cheap.	Laborious protocols. Often requires protoplast formation. Depends on the electrophysiological characteristics of the plant. Low transformation efficiency.
Biolistics	Small particles covered with genes are accelerated to penetrate the cell wall.	Easy. No pretreatment of the cell wall required. Independent of the physiological properties of the cell. Transformation with multiple transgenes is possible.	Expensive. Requires continuous supply of consumables. DNA can be damaged. Produces multiple copies of introduced genes, which can lead to various unprofitable effects. Low transformation efficiency.

Table 2
Comparison of some direct methods for genetic transformation of plants.

Method	Procedure	Advantages	Disadvantages
Vacuum infiltration	Vacuum generates a negative atmospheric pressure that causes the air spaces between the cells in the plant tissue to decrease allowing the infiltration of bacteria like *Agrobacterium*.	Simple and fast. Medium efficiency. In vitro plant regeneration, many independent plants transformed.	Requires the use of bacteria that may have unwanted consequences.
Ultrasound and shock waves	Acoustic cavitation changes the permeability of the membrane facilitating the absorption of DNA.	Simple and safe. Medium efficiency. More effective on cellular plants containing gas–liquid interfaces.	May cause cell disruption. Expensive. Non-standard protocols.
Silicon carbide whisker-mediated transform	Silicon carbide fibers are mixed in a vortex with a suspension of tissue and DNA allowing its introduction by abrasion.	Easy, fast and cheap. Can be used in various plants without limitations.	Damage of cells affecting regeneration capabilities. Possible injuries due to inhalation of fibers. Very low efficiency (lower than biolistics).
Microinjection	Direct DNA delivery into the plant cell through an injection pipette.	Extremely high transformation efficiency. Allows introduction of plasmids, and whole chromosomes.	Expensive, tedious and slow.
Macroinjection	Injection of inheritable materials using a hypodermic syringe.	High stability and reproducibility.	Only a part of the plant is transformed. Very low efficiency.
Laser microbeams	Focused laser microbeam puncture holes into the cell wall allowing the introduction of DNA.	Efficient, precise.	Expensive, laborious.
Electrophoresis	Embryos are placed between two pipettes connected to electrodes. DNA flows through the cells from the cathode to the anode.	Cheap, simple.	Treated embryos have poor viability to survive.

Agrobacterium particularly in monocotyledonous plants like cereals [77,81,139,140]. Moreover, *Agrobacterium* may also introduce vector sequences, not necessary for the transformation, that may produce unknown effects in the plant. This has been a matter of discussion from a bioethical point of view [123,139,152]. *Agrobacterium* transformation

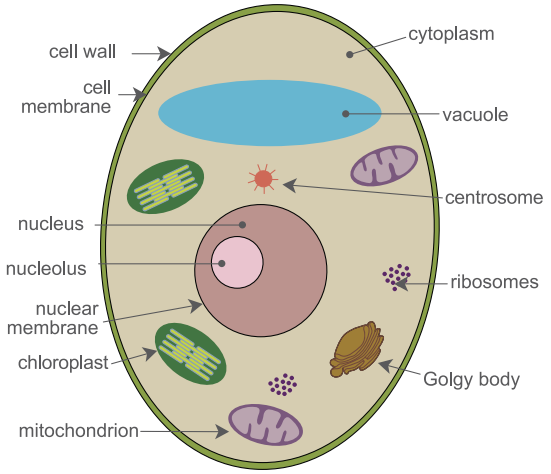


Fig. 2. Simplified sketch showing the main structure of a plant cell.

has been exhaustively reviewed in the literature [1,4,5,13,34,41,44,55,82,115,118,130,138–151]; however since it is not a physical method, it will not be discussed in this article.

The cellular wall is the natural barrier that all methods of genetic transformation have to overcome to achieve DNA penetration into the cell. Direct methods originated in the 1980s due to the big interest in modifying crops, almost impossible to be manipulated by *Agrobacterium* [155–158]. They offer an alternative for integrating multiple copies of a desired gene with minimal cellular toxicity at random sites into the genome [82]. Their disadvantages involve problems with plant regeneration and a low transient expression of transgenes. Direct methods in use or under research are electroporation [16,17,24–30,46,62,63,73–75,81,88,91,92,103,110,155–288], biolistics [37,38,57,64–67,76,77,89,90,92,97,99–102,106,118,126,144,180,289–504], vacuum infiltration [505–517], ultrasound [59,70,71,116,127,164,515–517,519–562], silicon carbide fibers [56,60,105,228,563–591], microinjection [92,592–604], macroinjection [16,69,509,605–613], laser microbeams [68,614–628], and electrophoresis [6,567,629,630]. In this paper only direct methods will be reviewed. A list of some of the transgenic plants transformed by direct methods is given in Tables 3, 4, 5 and 6.

2. Electroporation

Electroporation is a popular technique of genetic transformation because it is simple, quick and highly efficient for a wide variety of vegetable tissues [16,17,24–30,46,62,63,73–75,81,88,91,92,103,110,155–288]. It is commonly used to transport biochemical substances like lipids, proteins, ribonucleic acid (RNA) and DNA to the cell interior. The method enhances the formation of pores on the cell surface due to a polarity alteration on the membrane, caused by an electrical field [275,285,286]. This phenomenon can be observed with a microscope [162,288]. It has been mainly applied to transform plant protoplasts, i.e., cells without a wall, of various cellular types like corn [17,62,191] with an efficiency of 90 transgenic plants recovered from 1440 maize embryos (6.2%) [195], and wheat with an efficiency of 3 plants from 1080 embryos (0.3%) [194,222]. Other species have also been transformed by electroporation (see Table 3). Unfortunately, this technique has a low efficiency and can only be applied to protoplasts, using laborious protocols for the regeneration after genetic transformation [6,63,82,190].

It is a well established fact that an electrical field (alternate or pulsed) applied to a cellular suspension induces a dipolar moment inside the cells, and a potential difference through the plasmatic membrane [17,24,25,160,161,273,287]. This induced voltage can lead to cell permeabilization due to an electrical imbalance in the plasma membrane when the potential difference is bigger than 0.5 V at normal conditions of pressure and temperature (there is a membrane voltage threshold from 0.5 to 1 V). It has been shown that the pulse length, type and duration have a strong effect on the transformation efficiency [160,171,172,273]. The effects of this electrical imbalance are reversible only when the electrical pulse lasts less than 100 μs [256]. Under these circumstances, DNA can be introduced into the cells without changing the cellular functions or the membrane integrity [283]. It has been proposed [284–286]

Table 3
Selected transgenic plants produced by electroporation.

Plant	Reference
maize	[17,62,110,179,186–205]
tobacco	[73–75,103,163–179,186]
rice	[16,24–30,86,178–185]
cucumber	[177,178]
carrot	[72,179,186,205–211]
petunia	[211–213]
sorghum	[213,214]
Brassica napus	[215]
potato	[73,94,216–219]
berry	[46]
wheat	[194,220–223]
cherry	[224,225]
pear	[225]
grass	[226–228]
meadow rue	[229]
red goose foot	[229]
Alnus incana	[230]
tomato	[231]
sugar beet	[172,232–234]
sugar cane	[235,236]
yeast	[237]
barley	[238,239]
pea	[239–243]
alfalfa	[244–246]
spruce	[247]
pine	[247,248]
conifer	[249,250]
soybean	[251,252]
legume	[253–256]
cotton	[257]
grape	[258,259]
strawberry	[260]
fescue	[261]
eucalyptus	[262]
cauliflower	[263]
geranium	[264]
algae	[265–267]
cereals	[81,85,157]
banana	[268]
lettuce	[269]
coffee	[270,271]
orange	[272]

that the membrane permeabilization is due to the transitory force of electro-deformation produced by the electrostatic interaction of the dipoles generated on the cells due to the applied electrical field. Fig. 3a shows the electrical field distribution, the induced interface due to the charge difference and the dipolar moments, μ_{lf}, for a cell under the action of frequency fields (E_{lf}) lower than 100 kHz. In this range, the cell is compressed by the field because its dipolar moment, μ_{lf}, is antiparallel to the electric field. In contrast, Fig. 3b represents a field (E_{hf}) with a frequency higher than 100 MHz. If the cell is exposed to a high frequency field, its cellular membrane suffers a short circuit. At the same time its dipolar moment, μ_{hf}, grows and rotates towards the direction of the field, producing a cellular stretching along this direction, leading to a temporal permeabilization of the membrane [277].

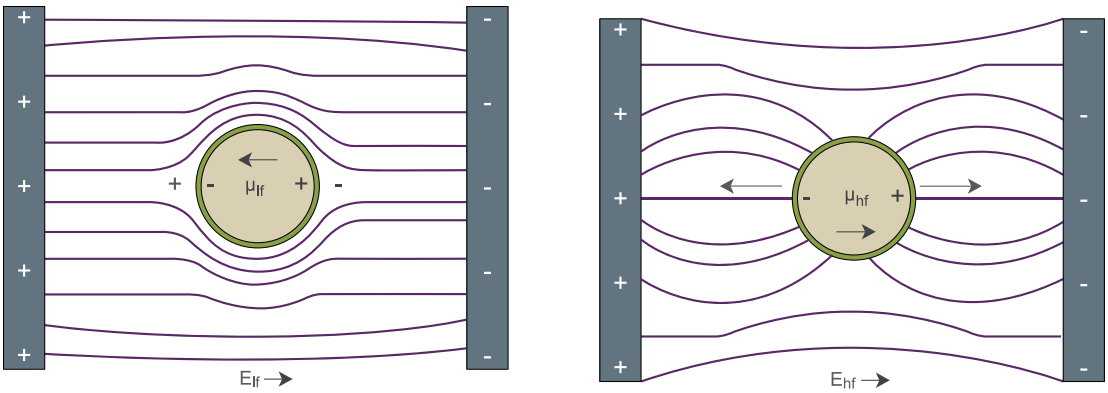
Several physical factors such as transmembrane potential created by the imposing pulsed electric field, extent of membrane permeation, duration of the permeated state, mode and duration of molecular flow, global and local (surface) concentrations of DNA, form of DNA, tolerance of cells to membrane permeation and the heterogeneity of the cell population may affect the electro-transfection efficiency [41,178,179,184,185,278,279,287].

Table 4
Selected transgenic plants produced by biolistics.

Plant	Reference
maize	[18,308–324]
tobacco	[99,291–307]
rice	[76,102,180,322–349]
carrot	[106,290,291]
petunia	[289]
sorghum	[332,380,381]
Brassica napus	[382]
potato	[291,383–388]
wheat	[321,322,349–369]
grass	[89,369–379]
tomato	[389–391]
sugar beet	[65,392]
sugar cane	[393]
barley	[364,365,394–400]
cow pea	[401,402]
peanut	[403,404]
chickpeas	[405]
alfalfa	[295]
spruce	[406–411]
conifer	[412–415]
pine	[414–420]
eucalyptus	[421,422]
fescue	[423]
soybean	[291,308,343,424–431]
legume	[432]
arabidopsis	[314,338,357,432–434]
cotton	[90,343,431,435–439]
strawberry	[66,440]
algae	[298,441–447]
cereals	[81,448]
banana	[449–451]
papaya	[452,453]
onion	[295,454–456]
garlic	[457]
bean	[431,458–462]
nut	[460]
grape	[463–466]
cassava	[36,467]
triticale	[364]
oat	[395,468,469]
millets	[37,38]
chrysanthemum	[39]
rose	[470–474]
orchid	[64,475,476]
jute	[477]
linum	[478]
rape	[479]
rye	[480]
lesquerella	[481]
betalain	[482]
lettuce	[483]
lemon fruit tree	[484]
citrus	[485]
palm	[486,487]
silver birch	[488]
coffee	[489,490]
pepper	[491,492]
moss	[493]
paulownia	[57]

Table 5
Selected transgenic plants produced by bacteria mediated methods.

Method	Plant	Reference
Vacuum infiltration	*Arabidopsis*	[505–507]
	petunia	[509]
	pines	[510]
	cotton	[511]
	banana	[514,515]
	lettuce	[512]
	coffee	[513]
	bean	[516]
	citrus	[517]
Ultrasound	tobacco	[164,527,528]
	rice	[558]
	sugar beet	[554]
	petunia	[116,549]
	potato	[529]
	soybean	[531,535–538]
	buckeye	[539]
	wheat	[524,525]
	black locust	[59]
	eucalyptus	[71,534]
	sunflower	[533]
	pine	[546–548]
	woody tree	[71]
	papaya	[540]
	sorghum	[541]
	red goose foot	[542]
	flax	[543]
	chickpeas	[544]
	bean	[516]
	citrus	[517]
	banana	[515,545]
	orchids	[116]



(a) (b)

Fig. 3. Electric field distribution (E) inside an electroporator, showing the charge difference and the dipolar moments (μ) for a cell exposed to (a) a low frequency (lf) and (b) a high frequency (hf) electric field.

The device to perform genetic transformation by electroporation is called electroporator (Fig. 4) [280,281]. It consists of an 80 to 800 μl container with a 4 mm slot having two parallel plane electrodes of aluminum inside. The electrodes are in contact with an aqueous electrolyte, containing intact cells in suspension and the DNA desired to

Table 6
Transgenic plants produced by other direct methods.

Method	Plant	Reference
Silicon carbide whisker-mediated transformation	maize	[18,563,564,566,567,570–574]
	tobacco	[563–565]
	rice	[575–579]
	soybean	[580,581]
	grass	[56,228,563,589,590]
	algae	[582,583]
	wheat	[584–587]
	nut	[588]
	cotton	[105]
Microinjection	tobacco	[594,600,601]
	petunia	[595]
	oilseed rape	[596]
	soybean	[598]
	barley	[603]
Macroinjection	rye	[16]
	cotton	[606]
	rice	[607,608]
	watermelon	[610]
	wheat	[611]
	soybean	[612,613]
	petunia	[509]
Laser microbeams	tobacco	[616,617,621]
	wheat	[622]
	rice	[624]
	Brassica napus	[615–618]
	vinca	[619]
	lilium	[620]
	rapeseed	[621]
	algae	[623]
Electrophoresis	orchid	[630]

be incorporated into the cell. Each treatment consists on the application of one or more electrical pulses lasting 10^{-6} to 10^{-2} s with a voltage between 1.6 and 2.0 kV. The recovery of the cellular membranes and the isolation of the transformed cells are promoted immediately after application of the electric field.

It is possible to model the frequency spectrum of the field generated in the membrane due to the voltage difference (V_g) and the electro-deformation force (F_{def}) as a function of time, in response to the electrical pulse generated by a direct electric field [282]. As a first approximation, the treated cells are considered as conducting spheres of radius a containing a fluid (cytosol), with a specific conductivity σ_i, and a dielectric constant ϵ_i, inside an isolated layer (plasmatic membrane) of thickness $d \ll a$, specific conductivity $\sigma_m \ll \sigma_i$, dielectric constant ϵ_m, specific conductance per area unit $G_m = \frac{\sigma_m}{d}$, and capacitance $C_m = \frac{\epsilon_m}{d}$, suspended in an aqueous medium with permittivity $\epsilon_e = 80\epsilon_0$, and specific conductance σ_e, exposed to a uniform electric field [282]. The electrical properties of the plasmatic membrane, the cytosol, i.e., the intracellular fluid, and the external medium are considered to be independent of the frequency, i.e., the σs and ϵs are real constants. Nevertheless, due to the structural layer of the cell, the induced dipolar moment, μ_C, and the membrane generated voltage, V_g, change with the field frequency and the time in a complicated way.

In the frequency domain, the induced dipole, μ_C, is a function of the cellular polarizability (U^*) described by the function of Clausius–Mossoti [631]:

$$U^* = \frac{\epsilon_C^* - \epsilon_e^*}{\epsilon_C^* + 2\epsilon_e^*},$$

this is,

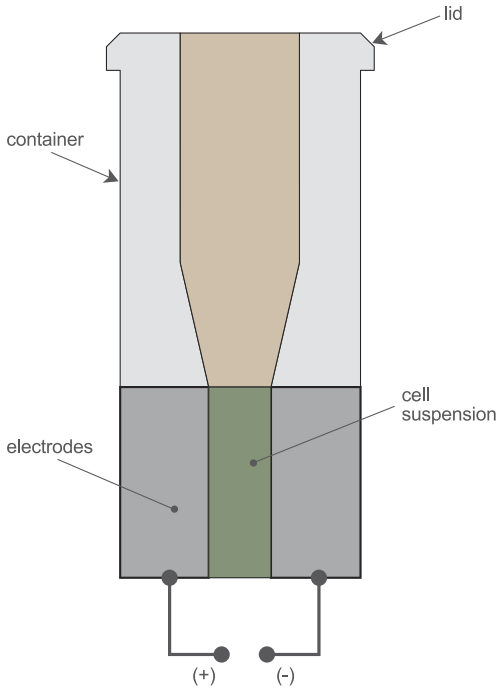


Fig. 4. Simplified sketch of the main components of an electroporator as used for transformation of plant cells.

$$\mu_C = 4\pi\epsilon_e a^3 E\left(\frac{\epsilon_C^* - \epsilon_e^*}{\epsilon_C^* + 2\epsilon_e^*}\right), \tag{1}$$

where ϵ_C^* is the cell effective permittivity, that can be approximated by [632]:

$$\epsilon_C^* = \frac{aC_m^*\epsilon_i^*}{aC_m^* + \epsilon_i^*}.$$

Eq. (1) indicates that the effective polarizability U^* determines the induced dipolar moment, and from this, the mechanical forces and pressures in the cell due to the interactions of the induce dipole μ_C with the external electrical field E, i.e., the deformation force.

The polarizability U^* of a spherical cell, and the induced voltage V_g through the membrane can be calculated solving the Laplace equation with the appropriate electrostatic contour conditions replacing ϵ_m, ϵ_e, and ϵ_i by their complex equivalents [631]:

$$\epsilon_m^* = \epsilon_m - i\frac{\sigma_m}{\omega},$$

where $\omega = 2\pi f$. The induced voltage V_g in the membrane of the cell exposed to the electrodes can be approximated by [633]:

$$V_g = \frac{3aE}{2\sqrt{1 + (\omega\tau_m)^2}}, \tag{2}$$

where f_m is the dispersion frequency of the membrane, and the charging time, τ_m, of the membrane is given by:

$$\tau_m = \frac{1}{2\pi f_m} = aC_m\left(\frac{1}{\sigma_i} + \frac{2}{\sigma_e}\right).$$

Eq. (2) establishes that the generated transmembrane potential at a given frequency is proportional to the cell radius and the external field force. This phenomenon has been simulated, showing that the membrane modification depends not only on the applied field, but also on the local mechanical stress and the bilayer edge energy [274].

3. Biolistics: particle bombardment

Biolistics, also known as "particle bombardment" or "gene gun technique" consists on the acceleration of high density carrier particles, approximately two microns in diameter (which is smaller than a plant cell), covered with genes that pass through the cells, leaving the DNA inside [37,38,57,64–67,76,77,89,90,92,97,99–102,106,118,126,144,289–504]. It was designed at Cornell University in 1987 to handle the genetic transformation of cereals [494]; however, it can be used on many species. The technique can be employed for nuclear and chloroplast transformation [441]. Cells, protoplasts, organized tissues like meristems (a group of non-differentiated cells with active mitosis), embryos or callus (vegetable tissue with disorganized growing) can be used as a target [500]. Originally the biolistic method was developed with the aim to transform monocotyledons (a group of flowering plants), which are recalcitrant to transformation with *Agrobacterium*. Comparison of *Agrobacterium* and biolistics in terms of transformation efficiency, transgene copy number, expression, inheritance and physical structure of the transgenic loci using fluorescence in situ hybridization shows that, in general, *Agrobacterium* offers significant advantages over biolistics [392,394,497,499]. Nevertheless, biolistics is the most accepted direct technique for genetic transformation of plants because it can be used for many species, subcellular organelles, bacteria, fungi and even animal cells [6], because it has a short processing time, low costs involved in the production of transgenic plants and due to its simplicity for introduction of multiple genes or chimeric DNA (DNA from two different species). Furthermore, it does not need a vector of a specific sequence, and does not depend on the electrophysiological properties of the cell, like the electrical potential and the structural components of the cellular membrane [500]. However, the transformation parameters must be optimized to each biological target employed [500].

In plant research, the major applications of biolistics include transient gene expression studies, production of transgenic plants and inoculation of plants with viral pathogens [497–499]. Only 50% of the tissue under bombardment survives to obtain a transformed plant. The method has a transformation efficiency of 0.002 with a genetic translational degree from 17 to 36% of the relative activity during events in one bombardment, while there is up to 70% of activity in the genetic expression during events in multiple bombardments [322]. Particle bombardment has been used to genetically transform several plants (see Table 4).

A comparison of transgenic rice obtained by *Agrobacterium* and biolistic techniques [346] shows a higher percentage of transgenic plants containing intact copies of foreign genes, especially non-selection genes, more stable transformation and better fertility for *Agrobacterium*-mediated transformation, while biolistics has higher efficiency with a wide range of gene expression. The factor limiting the use of the gene gun is the presence of multiple copies of introduced genes, which can lead to various undesirable side effects such as gene silencing or altered gene expression [6]. The high costs of gene gun accessories should also be considered.

The gene gun (Fig. 5) consists of a high-pressure and a low-pressure chamber with a diaphragm in the middle [292,323]. When the diaphragm is ruptured because of a pressure excess, the pressure difference accelerates a projectile along a barrel until it hits a porous screen. The projectile is previously covered on its tip with DNA-coated microparticles. After impact, the microparticles are launched toward the target tissue placed on a petri plate [500]. As the microparticles hit the cells, some transgenes are released and may incorporate into the chromosomal DNA. It has been proved that He is better for particle acceleration than N_2 or compressed air because it is lighter, inert, has high diffusivity and expands faster, allowing the particles to reach a higher velocity [400,502]. In most applications the optimum He pressure is 1100 psi, however pressures from 600 to 2400 psi have been reported. The transformation efficiency with this technique depends on several parameters, some of which are the temperature, the amount of cells, their ability to regenerate, and the number of DNA-coated particles, as well as the amount of DNA that covers each particle. Another issue that should be considered is that the friction on the particles diminishes at higher vacuum [455,496,500]. Use of mannitol or sorbitol as osmotica in the bombardment medium is effective and causes higher rates of stable transformants for all suspension cultured cells, as does brief air-drying [501]. Furthermore, it has been published that the probability to introduce particles inside the cell is proportional to their kinetic energy [455]. Due to this, particles are normally made out of gold, tungsten or platinum [313,322,455,496–499]. These particles which act like DNA transporters (by an adsorption mechanism), are used due to their availability, non-cellular toxicity and high density. For each transformation event, 50 μg of microprojectiles covered with DNA are accelerated to speeds of 430 m/s or higher in a partial vacuum of about 30 mm Hg. Gold particles are used more often, because they are biologically inert and can be fabricated in smaller sizes (about 0.7 to 1.0 μm of diameter) [496]. Because of the low

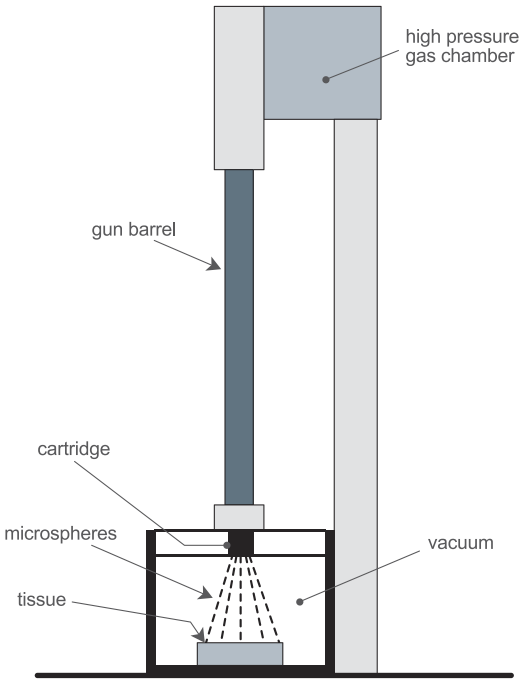


Fig. 5. Simplified sketch of the main components of a gene gun as used for transformation of plant cells.

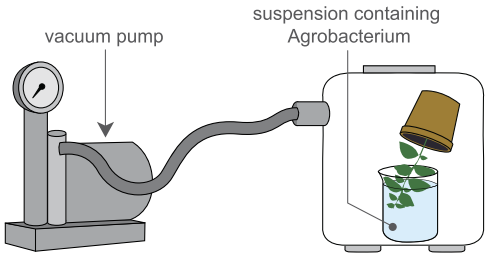


Fig. 6. Simplified sketch showing *Agrobacterium* infiltration mediated by vacuum.

cost, tungsten particles are also widely accepted, but transformation efficiency is smaller than with gold particles [292,313,499,500].

4. Vacuum infiltration

Another way to mediate the incorporation of *Agrobacterium* for plant transformation, is to apply a vacuum for a certain time period (see Fig. 6). Physically, vacuum generates a negative atmospheric pressure that causes the air spaces between the cells in the plant tissue to decrease allowing the penetration of pathogenic bacteria into the inter cell spaces. The longer the duration and the lower the pressure of the vacuum, the less air space there is within the plant tissue. An increase in the pressure allows the infiltration medium, including the infective transformation vector, to relocate into the plant tissue. The time that a plant part or tissue is exposed to vacuum is critical as prolonged exposure causes hyperhydricity. The use of *Agrobacterium*-mediated transformation assisted by vacuum infiltration was first reported in 1993 for transforming Arabidopsis (small flowering plants related to cabbage and mustard) [505]. Since then, many improvements have been made to establish protocols and make transformation in different plants (see Table 5) [506–517]. The utility of this method has been recently demonstrated in a successful production of a plant-derived vaccine under the current Good Manufacture Practice (cGMP) regulations for human clinical trials [518].

Some advantages of vacuum infiltration-facilitated transformation are the generation of many independently transformed plants from a single plant, a reduction in somaclonal variation because there is no tissue culture involved, and the possibility of high throughput testing because the process is fast. The method is also potentially useful for transformation of plants recalcitrant to plant tissue culture and regeneration.

5. Ultrasound-mediated transformation

The limitations of the previously described methods for transformation motivated the search for more efficient, easier and safer techniques for DNA exogenous incorporation to vegetable cells [6,70,71,116,127,137,164,515–517,519–559]. Ultrasonic waves, also known as sonication, are among these potential techniques.

Sonoporation, i.e., the rupture of cellular membranes by acoustic waves opens the possibility to non-invasive introduce molecules like DNA to the interior of cells for therapeutic applications [555,570] because it increases membrane permeability [522], thereby facilitating the entrance of macromolecules into cells [523]. Sonoporation can be induced by acoustic cavitation bubbles in a wide range of conditions from low frequency sonication (kilohertz), to medium frequencies (clinical shock waves), and diagnostic ultrasound (megahertz) [526]. Ultrasound may produce bioeffects via acoustic cavitation that temporarily change the permeability of the cell membrane [70,521,526,550,551]. This phenomena generates microscopic channels that favor the exposure of the internal plant tissue to *Agrobacterium* increasing the level of transient expression of the transfered DNA [530] even to the double [544].

Moreover, ultrasonic waves increase the transfection efficiency of animal cells, in vitro tissues and protoplasts [164,521,526,549,550,552,553] with spatial and temporal specificity, this is, restriction of the gene transfection to the desired area at a given time. Nevertheless, it has been reported that ultrasound can damage the cell, completely breaking its membrane [521]. It may be due to this that there is little research on ultrasound-enhanced transformation of plant cells or tissues [41,521].

Ultrasonic waves, as used for transformation, propagate through aqueous media as longitudinal pressure waves with frequencies higher than 20 kHz. An important mechanism producing biological effects on cells is heating due to tissue absorption through conduction, convection and radiation [526]. Heating by few degrees above the average biological temperature may enhance cell metabolism and induce perfusion of the tissue [559]. Acoustic cavitation, i.e., the growth and collapse of microscopic gas bubbles produced by the fast pressure change is another crucial phenomenon [551]. Cavitation may open holes in the membrane or even fragment it. The parameters that affect this phenomenon are not only the intensity, exposure time, and central frequency, but also the type of application (continuous or pulsed), the pulse repetition frequency, and the duty cycle [535]. Because cavitation depends on the presence of gas bodies, it is more effective on cellular plants, i.e., flowerless plants which have no ducts or fiber in their tissue, as mosses, fungi, lichens, and algae. DNA has been introduced into protoplasts of tobacco and beetroot by 20 kHz ultrasound at 0.5 to 1.5 W/cm^2 during 500 to 900 ms, obtaining a better efficiency in transitory genetic expression than using electroporation [554].

Through explants are suspended in a few milliliters of sonication medium in a microcentrifuge tube. Plasmid DNA (and possibly carrier DNA) is then added and after rapid mixing, the samples are ready for sonication. The cells are finally transferred to fresh growth medium. Sound, frequency and exposure time determine the uptake efficiency [521]. Early papers report transient expression of chloramphenicol acetyltransferase (cat) gene in sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana tabacum* L.) via a brief exposure of the protoplasts to 20 kHz ultrasound in the presence of plasmid DNA [554]. Stable transformation of tobacco by sonication of leaf pieces required 1500 to 2000 longer ultrasound exposure time than using protoplasts sonication [527]. Intact tissue transformation mediated by ultrasound has also been tested on potato tuber disks [529].

Much of the ultrasound technique is aimed at Sonication-Assisted Agro bacterium-mediated Transformation (SAAT) in plant cells or tissues [530,532,533], a technique that subjects the tissue to brief periods of ultrasound in the presence of *Agrobacterium*. Experiments demonstrated that SAAT tremendously improved the efficiency of *Agrobacterium* infection by introducing large numbers of micro-wounds into the target plant cells or tissues [59,515–517,519,528,530,531,534–548]. SAAT could also be useful for transformation of woody trees [71]. Some of the plants transformed by sonication are listed in Table 5.

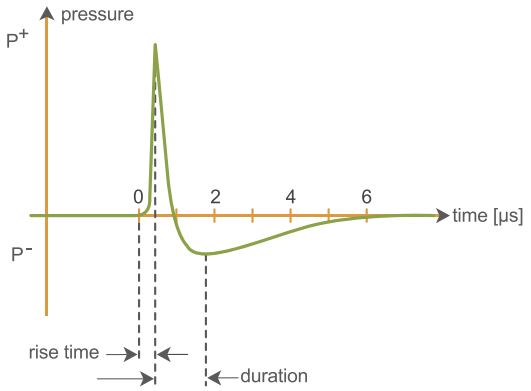


Fig. 7. Sketch of the pressure profile of a typical underwater shock wave as used in medicine.

6. Shock wave-mediated transformation

Shock wave generators designed for extracorporeal shock wave lithotripsy (SWL), orthopedics and other fields of medicine [635–637], have been used successfully for cell transfection and transformation [561,634,638–645]. These systems produce microsecond pulses with a peak positive pressure in the range of 30 to 150 MPa, lasting between 0.5 and 3 μs, followed by a tensile pulse of up to −20 MPa and duration of 2 to 20 μs (Fig. 7). To produce underwater shock waves for biomedical applications, electrohydraulic, piezoelectric, or electromagnetic devices have been designed [637,645].

Electrohydraulic shock wave generators induce shock waves by electrical breakdown (15 to 30 kV) of water between two electrodes located at the focus (F1) closest to a paraellipsoidal reflector (Fig. 8a). Shock waves are created at F1, partially reflected, and concentrated at the second focus F2. The underwater high-voltage discharge has a continuum in the ultraviolet (UV) spectrum with a peak between approximately 55 and 150 nm. Intense visible light is also produced. This radiation could affect certain type of cells placed at F2 during in vitro shock wave-enhanced transformation in an open water tub. Placing the vial in a water-filled black polypropylene bag to protect the cells from the spark generated radiation is advisable.

Piezoelectric systems (Fig. 8b) produce shock waves by a high-voltage (5 to 10 kV) discharge applied across an array of piezoelectric crystals mounted on a hemispherical bowl-shaped aluminum backing. Each electric pulse causes sudden expansion of the crystals, producing a pressure wave. The shock wave generator is placed inside a cavity filled with degassed water. Piezoelectric crystals are insulated from the water with a flexible polymer. The shock wave arriving at the center of the sphere is generated by superposition of the pressure waves formed by all crystals.

There are three different electromagnetic shock wave generating systems on the market. The first type generates shock waves by the movement of a metal diaphragm, about 120 mm in diameter, located at the base of a water-filled shock tube (Fig. 8c). A short electrical pulse (16 to 22 kV) sent through the coil, produces a magnetic field, which induces eddy currents in the metallic membrane. The magnetic field produced by these eddy currents causes the membrane to be repelled, transmitting mechanical energy to the water. The sudden movement of the membrane produces a pressure wave that propagates through the water. A special foil insulates the membrane from the water and a polystyrene lens focuses the pressure wave. During its path through water, the pulse steepens and forms a shock wave after passing through the lens. In the second type of electromagnetic shock wave generator, a cylindrically diverging wave is generated by an electromagnetically driven cylindrical membrane. It consists of a hollow acoustical backing supporting a coil-membrane system (Fig. 8d). A foil insulates the metallic membrane from the water. The acoustic pulses radiate perpendicular to the cylinder axis, after application of short electrical pulses to the coil. Focusing of the cylindrical wave is achieved by reflection off a parabolic reflector (about 300 mm in diameter). The reflector transforms the cylindrical wave into a spherical wave that is focused concentrically onto the focal point of the generator. The displacement of the cylindrical coil generates a high-intensity ultrasonic wave that undergoes nonlinear distortion during propagation. The pressure wave converts into a shock wave on its way to the focus. The third type of electromagnetic shock wave generator uses a spherical one-layer spiral coil to generate and focus shock waves on a relatively large focal volume. A copper diaphragm is repelled by the spiral coil, producing a compression wave (Fig. 8e). Due

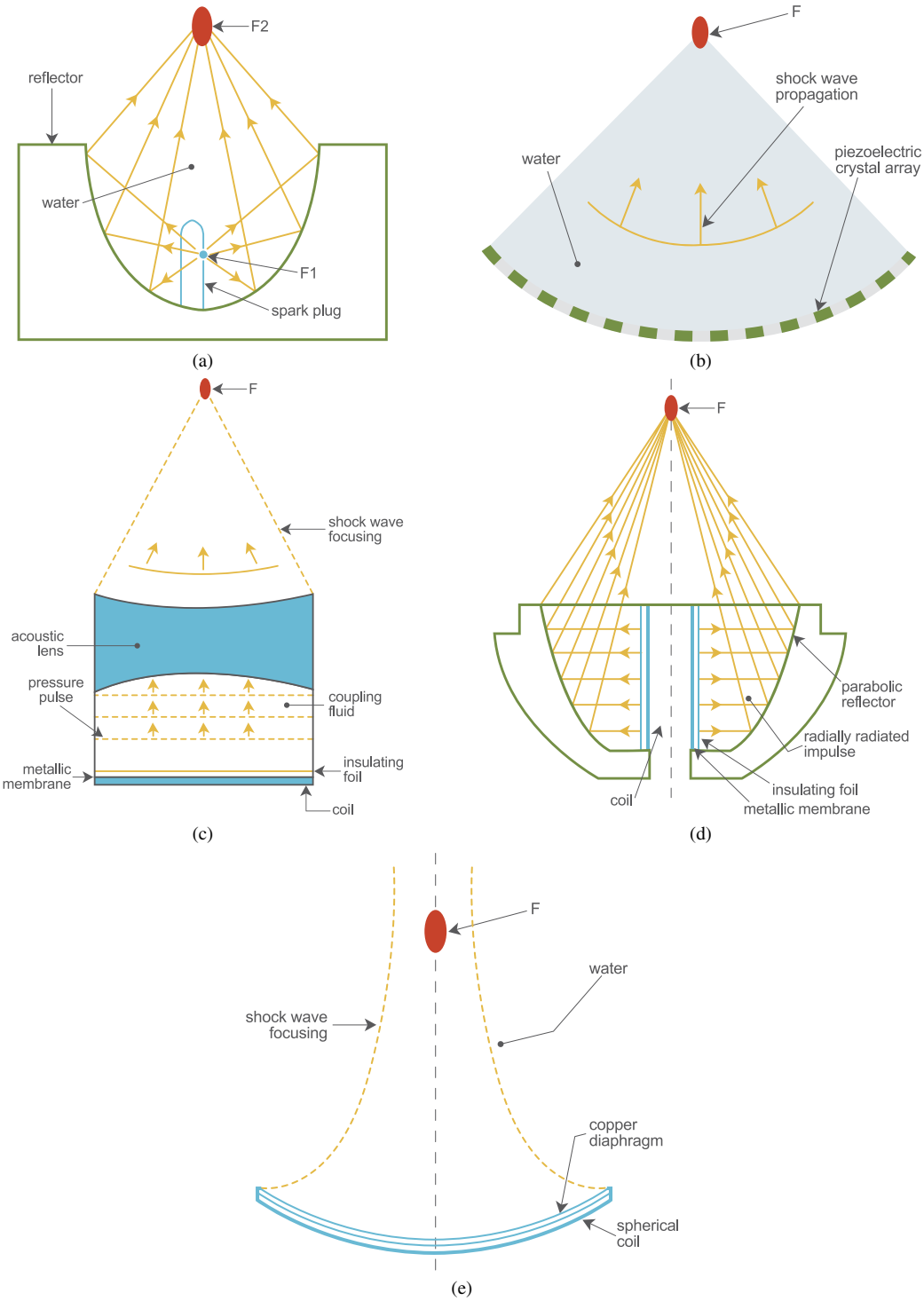


Fig. 8. (a) Simplified sketch of an electrohydraulic underwater shock wave generator, as manufactured by Dornier Medizintechnik GmbH, Wessling, Germany. (b) Simplified sketch of a piezoelectric underwater shock wave generator, as manufactured by Richard Wolf GmbH, Knittlingen, Germany. (c) Simplified sketch of an electromagnetic underwater shock wave generator as manufactured by Siemens AG, Erlangen, Germany. (d) Simplified sketch of an electromagnetic underwater shock wave generator as manufactured by Storz Medical AG, Kreuzlingen, Switzerland. (e) Simplified sketch of an electromagnetic underwater shock wave generator as manufactured by Xixin Medical Instruments Co. Ltd., Wuxian-Suzhou, China.

to the spherical shape of the spiral coil, no lens is needed. The diameter of the coil (aperture of the generator) is about 120 mm, and the distance to the focus, i.e., the distance to the center of the spherical arrangement, is about 200 mm.

The inactivation of bacteria and the transformation of bacteria, as well as of animal and human cells by underwater shock waves have been studied by several authors [561,634,638–645]. The mechanisms involved in shock wave-assisted cell permeabilization are still a research topic; however, there is evidence that shock wave-induced cavitation is the most important phenomenon for cell transformation [643,644]. During shock wave exposure of a cell suspension, the microbubbles contained in the vial collapse due to the positive peak of the incoming wave. Almost immediately after this compression, the bubbles expand rapidly, triggered by the negative phase. Their radius increase and remain almost stable during a "quiet phase" until a second, more violent collapse. Shock wave-generated bubbles generally expand in about 50 to 100 μs after shock wave passage, and collapse after approximately 250 to 500 μs [646]. It is known that the internal bubble pressure is highest at the second compression (collapse), not at the initial squeeze of the bubble [647]. In general, the collapse is not symmetric. Due to this, the bubble collapses inward, leading to a fluid jet that pierces its way through the bubble, exiting at the other side at up to 400 m/s [648]. Microjet emission occurs in the direction of shock wave propagation. The collision between the inward-moving wall of the bubble and the microjet produces a secondary shock wave with a pressure of up to 300 MPa that may strike adjacent cavitation bubbles, causing a more rapid collapse of the neighboring bubbles. Cavitation depends on the content of dissolved gases, viscosity, surface tension, temperature of the liquid, the applied pressure profile, bubble radius and other factors. At a shock wave rate of 1 Hz, cavitation bubbles do not last long enough to interfere with the next shock wave, but nuclei seeded by cavitation may still exist as the next shock wave arrives.

It is believed that shock wave-induced microjets act as micro syringes, promoting cell transformation. Ohl and Ikink [648] estimated that the amount of liquid that can be injected into a cell by a microjet is about 0.1 R_0^3, where R_0 is the initial bubble radius. Shock wave-mediated gene transfer has certain similarities with the biolistic technique; however, in this case the projectiles are fluid jets instead of miniature gene-covered projectiles.

The dynamics of bubble collapse after shock wave passage is complicated. Nevertheless, bubble growth and collapse in the field generated by extracorporeal lithotripters has been reported by several authors [649–654]. A shock wave as used for cell transformation may be modeled using the description reported by Church [649]:

$$P(t) = 2P^+ \exp(-\alpha t)\cos(\omega t - \pi/3). \tag{3}$$

P^+ is the peak positive pressure (Fig. 7), α is the decay constant (9.1×10^5 s^{-1}), and ω is the angular frequency ($2\pi \times 83.3$ kHz). Even if the rise time of the shock wave is between 2 and 20 ns, for simplicity, it may be chosen to be zero. The positive pulse duration equals 0.338 μs.

A popular equation to study the dynamics of a bubble immersed in water and subjected to a shock wave is the Gilmore–Akulichev formulation [649–652]:

$$R\left(1 - \frac{U}{c}\right)\frac{dU}{dt} + \frac{3}{2}\left(1 - \frac{U}{3c}\right)U^2 = H\left(1 - \frac{U}{c}\right) + \frac{RU}{c}\left(1 - \frac{U}{c}\right)\frac{dH}{dR} \tag{4}$$

where R is the bubble radius at the time t, c is the speed of sound in water, H is the enthalpy difference between the liquid at the undisturbed pressure and the pressure P, and $U = \frac{dR}{dt}$ the velocity of the bubble wall. H and c are determined using the equation of Tait. An assumption of the Gilmore formulation is that the initial bubble radius is much smaller than the length of the driving pulse (about 2.5 mm). This is valid for microbubbles immersed inside fluid-filled vials containing cells [649–652].

The typical response of a pre-existing air bubble (initial radius $R_0 = 0.05$ mm) in tap water to a shock wave is shown in Fig. 9. The radius of the bubble varies by several orders of magnitude. Before shock wave arrival, the energy of the bubble is zero. As the positive pressure peak arrives, the bubble suffers a forced collapse, gaining kinetic and potential energy. After this first compression, the bubble grows until all kinetic energy has been transformed into potential energy. At this instant, a second, so-called "inertial collapse" starts and the size of the bubble becomes even smaller than during the forced collapse. In a numerical simulation, the bubble elastically bounces several times and finally reaches equilibrium; however, in a more realistic situation, the microbubble inside the vial loses its spherical symmetry and fragments after its inertial collapse. It is important to keep in mind that the numerical simulation mentioned here refers to a single spherical bubble immersed in an infinite liquid. In reality, a bubble cloud is formed at the focal point of the shock wave generator after passage of each shock wave and the collapse of each bubble is

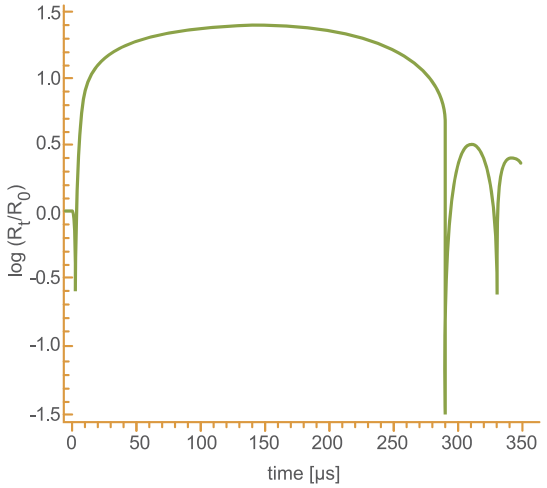


Fig. 9. Base 10 logarithm of the bubble radius R_t normalized by the initial radius R_0, plotted as a function of time for an air bubble exposed to an underwater shock wave. The first and second bubble collapses occur at about 0.2 μs and 290 μs, respectively. Initial bubble radius $R_0 = 0.05$ mm.

influenced by several factors, like the presence of neighboring bubbles, the vial, and the suspension-air interface inside the vial.

Several authors have reported that cavitation can be enhanced if a second shock wave arrives tenths of microseconds before the inertial bubble collapse, producing more powerful microjets [655–660]. This was already demonstrated transforming *Escherichia coli* [644] and opens the possibility for improved vegetable cell transformation. Actually there is only little evidence of the applicability of shock waves for plant transformation; nevertheless it can be considered as an alternative technique for eukaryotic cells.

7. Silicon carbide whisker-mediated transformation

Physical and chemical characteristics of silicon carbide fibers make them capable of puncturing cells without killing them. Using this property, the silicon carbide (SiC) mediated transformation (SCMT) method was proposed in 1990 to transform maize and tobacco [563]. SCMT is an easy, cheap, and quick procedure that can be effectively implemented for various species [56,60,105,228,563–591]. Silicon carbide fibers are added to a suspension of tissue (cell clusters, immature embryos, and callus) and plasmid DNA using a vortex, shaker or blender. DNA coated fibers penetrate the cell membrane through small holes created by collisions between the plant cells and the fibers [563]. SCMT efficiency depends on the fiber size, vortex parameters (type, duration and speed of agitation), vessel shape, the plant species and their cell characteristics, specially the thickness of the cell wall [6,578]. Since SCMT does not require sophisticated equipment, costly materials or skilled technicians, it is considered as a promising method for large scale transformations [82]. Moreover, SCMT through established protocols [589] allows the stable transformation of different plants (see Table 6). However, it has low transformation efficiency, and may damage the cells influencing their regeneration capability. Furthermore, extreme care should be taken to prevent the laboratory staff from breathing the fibers, because it can produce a respiratory hazard and should be treated as hazardous waste [567–569].

The exact transformation mechanism by SCMT is unknown [6,566]. It is believed that the strong and sharp edges of the silicon carbide fibers cut the cellular wall when they collide, acting as needles allowing the delivery of DNA into the target cells. Most whisker preparations are highly heterogeneous, ranging in length from 5 to 500 μm with an approximate diameter of 0.5 to 1 μm [566,573]. Scanning electron microscopy analysis of maize cells transformed by SCMT suggests that the fibers effectively penetrate the cellular walls [563]. The surface of silicon carbide fibers is negatively charged [591], producing a small rejection between the DNA molecules (also negatively charged) and the fiber at neutral pH. It has been proven that previous shaking of the fibers with a DNA suspension does not increase the transformation efficiency [426], which suggests that the fibers do not transport the DNA into the interior of the cells, but facilitate their transfer by perforation and abrasion mechanisms [566]. Other materials with similar characteristics

than silicon carbide fibers, like carborundum, silicon nitrate, and glass, can also introduce DNA to cells of plants; nevertheless their transformation efficiency is lower [589].

8. Microinjection

The most effective method for genetic transformation of animal cells is microinjection, a technique also applied for plants [92,592–604]. Several plant species have been transformed using microinjection (see Table 6). The method consists on the direct and precise delivery of DNA into the plant cell through a glass microcapillary-injection pipette [593,594]. The technique is very slow, requires an expensive micromanipulator and tedious procedures to immobilize the cells with a holding pipette and gentle suction. However, it is extremely efficient, very precise from the delivery point of view and allows the introduction not only of plasmids but also of whole chromosomes into plant cells [595,602]. The final transformation efficiency was about 10 times lower than that of biolistics. Nevertheless this approach has been suggested as a potential method for stable plant transformation applied to several types (see Table 6).

9. Macroinjection

The injection of inheritable materials like immature embryos, meristems, immature pollen, germinating pollen, etc. using a hypodermic syringe is called macroinjection [16,69,509,605–613]. The main disadvantage of this technique is the likelihood for the production of chimeric plants with only a part of the plant transformed. However, from this chimeric plant, transformed plants of single cell origin can be subsequently obtained. This procedure has been applied to different species (see Table 6). Moreover, the final transformation efficiency was about 10 times lower than with biolistics. However, this approach has been suggested as a potential method for stable plant transformation [69].

10. Laser microbeams

A problem with the above mentioned direct methods for plant transformation is the fragility of the protoplasts of many species that cannot be regenerated into plants [661]. Introduction of DNA into organelles like chloroplasts is even more difficult [616]. To avoid these difficulties it is possible to use laser microbeams to introduce genetic materials into cells [68,614–628].

Laser-mediated transformation works by a focused laser microbeam to puncture self-healing holes (≈0.5 μm) into the cell wall. These holes close again in less than five seconds. Through the temporary opening in the membrane, the buffer together with DNA enter the cell. Membrane perforation can also be performed using laser pulses (laserporation) and can be combined with laser-facilitated partial removal of the cell wall [620]. Therefore, exogenous DNA could simply be taken up by cells. Complete manipulation by laser light allows precise and gentle treatment of plant cells, subcellular structures, and even individual DNA molecules. For this it is necessary to have an adequate laser system (like nitrogen lasers, excimer pumped dye lasers, or titanium–sapphire lasers) that can be used as an optical tweezer with the appropriate microscope [623]. An optical tweezer consist of a continuous IR laser like a diode or diode pumped Nd-YAG laser. UV laser microbeam cell fusion has been induced selectively and DNA was introduced into isolated chloroplasts [615–617,627].

This method is not popular because it requires expensive equipment to allow focusing a laser beam on dimensions of the order of 100 nm [628], even when a large number of cells can be irradiated and the cells recover completely after the DNA incorporation. It also has to be conducted with a lot of care because laser radiation can damage biological material, so it is necessary to restrict the beam through a channel and control the energy and pulse duration with high precision and reproducibility [625]. The method requires further assessing for different experimental conditions and plant species. The different plants transformed by this method are listed in Table 6.

11. Electrophoresis

Electrophoresis is an alternative cheap and simple transformation method [6,567,629,630]. Embryos to be transformed are placed between the tips of two pipettes connected to electrodes. The pipette connected to the anode is filled in its narrow part with agar, followed by an electrophoresis buffer. The pipette connected to the cathode contains

agar mixed with DNA and an electrophoresis buffer and is in contact with the meristems of the embryo. Switching the current on causes a slow flowing of DNA from the cathode to the anode through the embryo. The transformation depends on the electrical field applied, the duration of electrophoresis, the concentration of the buffers and the physico-chemical properties of the embryo [6]. Typical parameters used for electrophoresis are a voltage of 25 mV and a current of 0.5 mA during 15 minutes [567]. The principal disadvantages of this technique is that treated embryos have a poor viability to survive. The first attempts on barley have not succeeded [629]. In an exhaustive bibliographic search orchid was found to be the only plant transformed by electrophoresis [630].

12. Conclusions

Growing interest on biotechnological research demands the development of novel strategies to manipulate and incorporate specific genetic sequences into plants to improve their characteristics in agreement with the society needs in an easy, safe, trusty and reproducible form. Genetic plant transformation whether performed by physical or other methods, currently faces major challenges. Random integration of the transgenes continues to be a major issue; however methods to overcome this have been developed, such as the ones that utilize Zinc-finger nucleases which can be used to generate high-frequency homologous recombination to modify specific plant genes. Transgene silencing is also a major challenge and to address it, several virus-derived proteins (such as the ones mentioned in Section 5) have been employed. To suppress specific transgenes, methods such as antisense and RNAi have been developed. The RNAi method is more powerful and its ability to suppress, or silence, expression of specific genes has made it a major new tool for functional genomics and genetic engineering of many organisms. However, little is known about efficiency and stability of RNAi-induced gene suppression in the diversity of organisms where it has been applied. So far, most of the methods employed have relied on the use of *Agrobacterium*, but due to the limitations described above, direct, physical methods represent an interesting alternative to overcome some of these obstacles. They may seem the method of choice if one wishes to exclude vector sequences and for species recalcitrant to *Agrobacterium* transformation. Nevertheless, for a proper implementation, it is important to understand the physics behind many of these methods to make a better use of the technique and eventually to enhance penetration of the cellular wall and integration of the transgene. Some techniques have been successfully established for few plant types, but there is still a lot of research to be done in order to effectively exploit them in a wide variety of species and to increase the efficiency and reproducibility of the genetic transformations. A better understanding of the physics involved will help to make more rigorous protocols and may open new strategies for genetic plant transformation.

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