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Genetic Transformation of Cells using Physical Methods

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Abstract

The production of transgenic cells is a routinely process that allows to insert genes from plants, fungi, viruses, bacteria and even animals into cells. Genetic transformation requires penetration of the transgene through the cell wall. This process is facilitated by biological, chemical or physical methods. We present a short review of the state of the art of physical methods used for genetic transformation. A general panorama of the traditional physical genetic transformation methods like electroporation, biolistic, and agitation with glass beads, vacuum infiltration, silicon carbide whisker, laser microbeams, ultrasound, and the newly promising technique of shock wave-mediated genetic transformation of cells are described. These techniques have been applied to transform cells of bacteria, algae, plants, fungi, and animals. In human cells, genetic transformation is currently used for DNA vaccines, tissue engineering, and cancer therapy.

Keywords: Genetic transformation; Shock wave-mediated genetic transformation; Electroporation; Biolistic; Vacuum infiltration; Silicon carbide whisker; Laser microbeams

Introduction

Several protocols are used to transfer DNA into cells of plants, fungi and [1,2]. Genetic transformation, discovered by Griffith [3] has revolutionized molecular biology, but it was not until the first recombinant DNA was produced from Escherichia coli with the use of bio-chemical scissors called restriction enzymes [4] that genetic transformation of cells started.

Genetic transformation of cells requires the production of recombinant DNA fragments, transferring of the DNA into the cell by membrane permeabilization, the integration of the DNA into a chromosome and its maintenance and replication. It involves in vitro culture for multiplication of clones, to select suitable promoters for a specific gene, over-expressing activator genes, removing epigenetic silencing, introducing heterologous genes, generating strains with novel properties, improving bioinformatic programs of random mutagenesis, to identify sequences that confer resistance to antibiotics (selective markers), to produce enzymes that generate a specific property not observed in the wild type strain (reporter genes), to characterize genes involved in a metabolic route, etc. Novel methodologies for an efficient, reproducible and safe introduction of specific genes into cells as well as stronger promoters are still needed to increase production levels.

The cell membrane is a protecting hydrophobic layer of about 10 nm that acts like a barrier to the genetic transformation of cells. Another obstacle is that the DNA is a highly charged macromolecule, difficult to manipulate, that cannot diffuse through the cell membrane. To penetrate the cell membrane biological, chemical or physical methods can be used. Biological methods are based on Agrobacterium tumefaciens-mediated transformation [5-7], and protoplast transformation using cell wall degrading enzymes [8,9]. Chemical-based methods use calcium phosphate coprecipitation [10] and lipofection [11]. Even if biological and chemical methods are still more popular for genetic transformation, recently there has been an increase in the application of physical methods such as electroporation, biolistics, and agitation with glass beads, vacuum infiltration, silicon carbide whisker, laser microbeams, ultrasound, and shock-wave-mediated transformation. These physical methods will be discussed in this work and are compared in Table 1. The main goal of this short review on physical methods is to encourage young researchers, especially physicists, to start collaborations in the re-search field of genetic transformation.

Technique	Procedure	Most important parameters involved	Advantages	Drawbacks
Electroporation	DNA is inserted through pores due to permeabilization of the cell membrane induced by strong electrical pulses.	Pulse length, energy and duration of the electrical field, extent and duration of membrane permeation, mode and duration of molecular flow, DNA concentration, tolerance of cells to membrane permeation.	Simple, fast, low cost.	Low efficiency, requires laborious protocols, and transforms mainly protoplasts.
Biolistics	High density carrier particles covered with genes are accelerated through the cells	Kinetic energy of the bombarding particles, temperature, the amount of cells, their ability to regenerate,	Simple, no need to treat the cell wall, allows transformation of different	High cost, low efficiency Transformation parameters must be optimized to each

	leaving the DNA inside by an adsorption mechanism.	susceptibility of the tissue, the number of DNA-coated particles, as well as the amount of DNA that covers each particle.	cells, independent of the physiological properties of the cell, allows the use of multiple transgenes.	biological target employed, there is a risk of multiple copies of the introduced genes, DNA and cells can be damage.
Agitation with glass beads	Rapid agitation with glass beads allows the penetration of the plasmid DNA.	DNA and concentration, sensitivity of cells to membrane permeation, amount of cells and their ability to regenerate.	Fast, simple, low cost. Does not need sophisticated devices, chemical treatments or enzymatic cocktails.	Low efficiency because DNA get damaged.
Vacuum infiltration	Vacuum application generates a negative pressure that increases inter-cell spaces allowing the infiltration of Agrobacterium.	Duration and intensity of the vacuum, temperature, pH and time of induction of virulence genes.	Simple, fast, medium efficiency, with low somaclonal variation and many independent cells transformed.	Some strains of Agrobacterium are unable to infect certain cell types, risk of multiple copies of the introduced genes.
Silicon carbide whisker	Silicon carbide fibers are mixed in a vortex with a suspension of tissue and DNA allowing introduction by abrasion.	Fiber size, vortex parameters (type, duration and speed of agitation), vessel shape, thickness of the cell wall and cell's ability to regenerate.	Simple, fast, low cost and can be used in different cell types.	Very low efficiency. Cells can be damaged affecting regeneration capabilities. Could be hazardous to technicians due to fibers' inhalation.
Laser microbeams	A laser microbeam punctures self-healing holes into the cell wall allowing DNA penetration.	Laser characteristics to be used as optical tweezers coupled to the appropriate microscope.	Allows precise and gentle treatment of cells, subcellular structures, and even individual DNA molecules.	High cost (expensive equipment required), and laborious.
Ultrasound	Introduces DNA molecules into cells via acoustic cavitation that temporarily changes the permeability of the cell membrane.	Intensity, exposure time, central frequency, type of application (continuous or pulsed), pulse repetition frequency, and duty cycle.	High efficiency, medium cost and can be used in different cell's types.	May damage the cells by breaking their membrane.
Shock waves	Cell permeabilization occurs due to shock wave-induced cavitation.	Frequency, energy, voltage, shock wave profile and number of shock waves.	Fast, easy to perform, reproducible with high efficiency, no need of enzymatic cocktails, can be used to transform several cell types.	Shock wave generators for this purpose are not on the market yet and experimental devices are relatively expensive.

Table 1: Comparison of physical methods for genetic transformation of cells

Electroporation

The most popular physical genetic transformation method is electroporation. This is due to its quick-ness, low cost, and simplicity even when it has a low efficiency, requires laborious protocols for regeneration after genetic transformation, and can only be applied to protoplasts [1,2,12-15]. Electroporation is based on the application of a strong electrical field to enhance the formation of pores on the cell membrane due to a polarity alteration, caused by the electrical field (alternated or pulsed) that induces a dipolar moment inside the cells, and a potential difference through the plasmatic membrane [15-18]. If the cell is exposed to a high frequency field, its cellular membrane suffers a short circuit and its dipolar moment grows and rotates towards the direction of the field, producing a cellular stretching along this direction, leading to a temporal permeabilization of the membrane [17].

Several physical factors such as pulse length, type and duration of the electrical field, transmembrane potential created, 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 have a strong effect on the transformation efficiency [19].

Initially electroporation has been mainly applied to transform plant protoplasts, i.e., cells without a wall, of various cellular types like corn [15]. Experiments of electroporation of animal cells started little after [20-23]. The first transformed fungus was Trichoderma harzanium [24] and the first transformed bacterium was Actinobacillus actinomycetemcomitans [25]. Other applications of electroporation include the insertion of enzymes, metabolites, lipids and pharmaceutical compounds [26], the study of gene function [27], the elaboration of DNA vaccines [28], and cancer treatment [29].

Electroporation is extremely used to transform yeast, especially Saccharomyces cerevisiae, but the frequency of transformation (up to 1x105 trans-formants per microgram of DNA) is low in comparison with that obtained for bacteria. Due to this, the cell wall of Saccharomyces cerevisiae has been manipulated by using chemical treatments or employing enzymatic cocktails to produce protoplasts before electroporation [30,31].When using protoplasts of plants, fungi or animals, the uptake of DNA can be achieved by electrofusion, i.e., two membranes located very close to each other can be fused by application of an electric field and the DNA present in the cell suspension is trapped in the cytoplasm of the joined cells [13,32].

A very interesting method based on electroporation is Nucleofection™, developed in 1998 and introduced to the research

market in 2001 [33,34]. It has been successful in cancer studies and tissue engineering. Nucleofection™ is a patented commercial electroporation system developed by Amaxa, and owned by Lonza. The voltage, frequency, and pulse duration for each cell type are not disclosed to the user. The methodology permits to transfect many difficult-to-transfect primary cells, cell lines, and stem cells. It would be interesting to apply this method to plant and fungal cells.

Biolistics

Genetic transformation of different types of cells from subcellular organelles, bacteria, algae, fungi and even animal cells can be done by biolistics, also known as "particle bombardment" or "gene gun technique". Biolistics consists on the acceleration of high density carrier particles (usually made of gold, tungsten or platinum) covered with genes that pass through the cells, leaving the DNA inside by an ad-sorption mechanism [1,2,35,36]. Cells, protoplasts, organized tissues like meristems, embryos or callus can be used as a target [37], and the introduction of multiple genes or chimeric DNA (DNA from two different species) is simple.

Biolistic protocols were developed for genetic transformation of cereals [38], algae (Boynton et al.) [39], and yeast (Johnston et al.) [40]. For human cells, biolistics has been used as DNA vaccines [41,42], for tissue engineering [43,44], and cancer treatment [27,45,46].

Biolistics is an expensive technique, transformation parameters must be optimized to each bio-logical target employed and there is a risk of multiple copies of the introduced genes, which can lead to various undesirable side effects such as gene silencing or altered gene expression [37]. Transformation efficiency depends on the kinetic energy of the bombarding particles, 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 [35,37].

Agitation with Glass Beads

Cells can be genetically transformed by rapid agitation with glass beads in the presence of carrier and plasmid DNA. It can be performed without sophisticated devices and does not require chemical treatments or enzymatic cocktails. This technique is easy, cheap, and rivals electroporation for being the least time consuming methodology, but it is also one of the least efficient, because high quantities of DNA are damaged in the process and the viability of the cells is drastically reduced [2,47].

This methodology was first used with the yeast Saccharomyces cerevisiae [48], and little later with Rhizobacteria [49], other bacteria [50], and also algae [51]. Cytogenetic studies have also been performed through agitation with glass beads [52].

Vacuum Infiltration

Another way to mediate the incorporation of Agrobacterium for genetic transformation is to apply a vacuum for a certain time period [1,2]. Physically, vacuum generates a negative atmospheric pressure that causes the air spaces between the cells in the membrane to decrease allowing the penetration of Agrobacterium into the inter cell spaces. The longer the duration and the lower the pressure, the less air space there is within the plant tissue. The temperature, pH and time of induction of virulence genes have a dramatic effect on the frequency of transformation [53]. It has the advantage of being a fast procedure with a low somaclonal variation because there is no tissue culture involved. Its main limitation is that some strains of Agrobacterium are unable to infect certain cell types.

Vacuum infiltration initially was used for transforming Arabidopsis [54], and apples [55]. The first fungus transformed was Saccharomyces cerevisiae [56]. The method has been used to produce a plant-derived vaccine under the current Good Manufacture Practice regulations for human clinical trials [57]. However, it has not been applied for transformation of bacteria or animal cells due to the tumorous characteristic of Agrobacterium.

Silicon carbide whisker

Silicon carbide fibers are capable of puncturing cells without killing them. Using this property, the silicon carbide (SiC) mediated transformation (SCMT) method was proposed to transform maize and tobacco [58]. SCMT is an easy, cheap, and quick procedure that can be effectively implemented for various cells [2]; however, it has low transformation efficiency, and may damage the cells influencing their regeneration capability. It could also cause injury to the laboratory staff [59].

Silicon carbide fibers are added to a suspension of tissue (cell clusters, immature embryos, or 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. The exact transformation mechanism by SCMT is unknown, but it has been proposed 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 [60]. SCMT efficiency depends on the fiber size, vortex parameters (type, duration and speed of agitation), vessel shape, and the thickness of the cell wall [36].

There exist other materials with similar characteristics to those of silicon carbide fibers, like carborundum, silicon nitrate, and glass, that can also introduce DNA to cells; nevertheless their transformation efficiency is lower [61].

Laser microbeams

Laser microbeams have been used to introduce genetic materials into cells puncturing self-healing holes into the cell wall [2,62-65]. Complete manipulation by laser light allows precise and gentle treatment of cells, subcellular structures, and even individual DNA molecules; in particular it has been used in animal cells [66]. It requires an adequate laser system (like nitrogen lasers, excimer pumped dye lasers, or titanium-sapphire lasers) that can be used as optical tweezers, coupled to the appropriate microscope. This is an expensive and laborious technique [67].

Ultrasound

Ultrasonic wave-mediated transformation, also known as sonication, is based on sonoporation (the rupture of cellular membranes by acoustic waves). It is a non-invasive way to introduce DNA molecules into cells via acoustic cavitation that temporarily changes the permeability of the cell membrane [1,68-70]. Ultrasound increases the transfection efficiency of animal cells, in vitro tissues and protoplasts with spatial and temporal specificity [71,72]. However, it has been reported that ultrasound can damage the cell, completely breaking its membrane [73]. Crucial parameters are the intensity,

exposure time, central frequency, the type of application (continuous or pulsed), the pulse repetition frequency, and the duty cycle [70].

Shock-waves

Shock waves are pressure 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 with duration of 2 to 20 μs. They are produced by electrohydraulic, electromagnetic or piezoelectric shock wave generators [74]. The exact mechanism responsible for shock wave-assisted cell permeabilization is still not clear, but there is evidence that it is due to shock wave-induced cavitation [2].

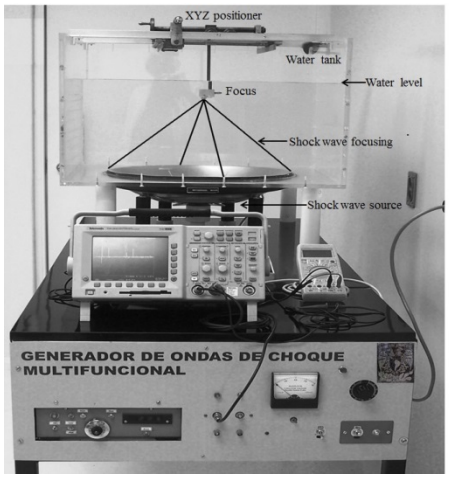

Figure 1: Experimental shock wave generator

Shock-wave-mediated insertion of DNA has been reported in Escherichia coli, Pseudomonas aeruginosa, Salmonella [75-77], Aspergillus niger, Trichoderma reesei, Phanerochaete chrysosporium, and Fusarium oxysporum [78]. Several authors have published articles on shock-wave-mediated DNA delivery in eukaryotic cells and prokaryotes [79-85].

Certainly, the application of shock waves to transform cells has several advantages. Expensive enzymatic cocktails are not required, the transformation frequency is higher in comparison with other available methods, and the method is fast, easy to perform and reproducible. Additionally, the same frequency, energy, voltage and number of shock waves can be used to transform diverse species of fungi [78]. At present, the main drawback for the use of shock waves is the need for relatively expensive equipment. Figure 1 is a photograph of the experimental piezoelectric underwater shock wave generator, used to transform filamentous fungi placed at its focus [2,78]. The system uses a Piezolith 2501 shock wave source manufactured by Richard Wolf GmbH.

Conclusions

Different physical methods have been developed to manipulate and incorporate specific genetic sequences into the genomes of bacteria, plants, fungi and animals (Table 1). There is no single method applicable to all types of cells and the different variables described in Table 1 should be considered to choose a suitable method for a particular organism. Random integration of the transgenes, transgene silencing and low stability and efficiency continue to be major issues that need addressing. The increasing availability of novel DNA sequences and a deeper knowledge of the mechanisms underlying gene expression will provide tools to target genes, to improve expression and stability and to prevent silencing. In addition, a better understanding of the physics involved will help make more efficient protocols and may open new strategies for genetic transformation of cells. Novel methods such as shock-wave-mediated transformation, have contributed to increase the efficiency of transformation but they still need refining.

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