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REVIEW

Gene transfer to plants by electroporation: methods and applications

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Abstract
Developing gene transfer technologies enables the genetic manipulation of the living organisms more efficiently. The methods used for gene transfer fall into two main categories; natural and artificial transformation. The natural methods include the conjugation, transposition, bacterial transformation as well as phage and retroviral transductions, contain the physical methods whereas the artificial methods can physically alter and transfer genes from one to another organisms' cell using, for instance, biolistic transformation, micro- and macroinjection, and protoplast fusion etc. The artificial gene transformation can also be conducted through chemical methods which include calcium phosphate-mediated, polyethylene glycol-mediated, DEAE-Dextran, and liposome-mediated transfers. Electrical methods are also artificial ways to transfer genes that can be done by electroporation and electrofusion. Comparatively, among all the above-mentioned methods, electroporation is being widely used owing to its high efficiency and broader applicability. Electroporation is an electrical transformation method by which transient electropores are produced in the cell membranes. Based on the applications, process can be either reversible where electropores in membrane are resealable and cells preserve the vitality or irreversible where membrane is not able to reseal, and cell eventually dies. This problem can be minimized by developing numerical models to iteratively optimize the field homogeneity considering the cell size, shape, number, and electrode positions supplemented by real-time measurements. In modern biotechnology, numerical methods have been used in electrotransformation, electroporation-based inactivation, electroextraction, and electroporative biomass drying. Moreover, current applications of electroporation also point to some other uncovered potentials for various exploitations in future.

Keywords Artificial gene transfer methods · Electrofusion · Electropores · Field strength · Natural gene transfer methods · Plants · Transgenics

Introduction to gene transfer methods

Various natural and artificial methods have been employed to transfer DNA, RNA, and other desired molecules into the cells [1, 2]. Each method possessed its own advantages and disadvantages in terms of transfected cell type and transfection efficiencies, thereby choosing the most convenient method were of higher importance for successful applications [3–5]. Mainly, DNA is the mostly studied transferred molecule because it carries the hereditary information determining the fate of cell and subsequently the whole organism.

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To date, great number of successful applications to introduce the exogenous DNA into cells have been achieved and allowed to acquire transgenic organisms with stable gene expressions [4, 6, 7]. The techniques used for DNA transfer varies depending on type and species of the organism, However, all the available used techniques can be categorized under two main methods of DNA transfer, namely; (i) natural (ii) artificial methods [1].

Natural methods for DNA transfer

Selecting the most suitable method in DNA transfer is an important initial step upon which the success of transfections depends on. Besides, some other factors to be considered for successful applications include the target cell type, transfection endurance (stable or transient), gene selection and isolation, recombinant DNA preparation, identification

of transformed cells, and regeneration of transposed organism [3, 8]. There has been six known methods in natural DNA transfer; (i) conjugation [9], (ii) transposition [10], (iii) *Agrobacterium*-mediated transfer [11, 12], (iv) bacterial transformation [13], (v) phage transduction [14] and (vi) retroviral transduction [15, 16]. Conjugation is a process of transferring genetic material from a donor cell to a recipient cell using a bridge-like connection or by direct cell-to-cell contact [9]. A common way for bacteria to achieve this connection to a recipient cell is by the employment of their pili, which are external appendages with similar helical structure but in different sizes. Bacterial conjugation provides the horizontal gene transfer [9, 17]. Transposition is the movement of transposable elements (TEs). Those elements are basically discrete DNA segments with capabilities of moving, copying, and inserting themselves within genomes [10, 18]. They have been shown to play important roles in genome function and evolution [19]. *Agrobacterium*-mediated transfer is the transfection of desired DNA into the target cells mostly using bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* which are the causal agents of crown gall disease and hairy root formation [11, 12, 20]. *Agrobacterium* DNA transfer capability has been extensively used in biotechnological studies as a means to insert the foreign genes to the plants [12, 21]. Bacterial plasmid T-DNA with inserted desired gene transfects the host DNA to transfer the target gene [22]. Bacterial genetic material transformation is a natural method in which bacteria take up the environmental DNA fragments of other microorganism, which have been left dead or lysed and incorporate them into their genomes by recombination. This process is important for improving the genetic diversity as well as providing chromosomal repair in bacterial cells [9, 13]. Phage transduction involves the DNA transfer from one bacterium to another via bacteriophage, which is basically a viral infection that causes the transferring of genetic materials into the affected bacterial cells [14]. Bacteriophages provide the horizontal gene transfer through transduction mechanism. Both donor and recipient are needed to be susceptible to infection by same phage. Phage is reproduced in donor and subjected to the recipient cells at different multitudes of infection [14, 23, 24]. However, it includes the transfer of DNA by a viral vector retrovirus, which is a single-stranded positive-sense RNA virus. Retrovirus is one of the mainstays in current gene therapy applications [15, 16, 25].

Artificial methods for DNA transfer

Developing gene transfer technologies have substantially paved the way to genetically manipulate the higher organisms. Many artificial methods for DNA transfer have been developed. Artificial methods are categorized based on their applications. They are physical, chemical, and electrical methods [26–28]. Those methods are classical and powerful tools commonly used in gene transfer applications with their own advantages and disadvantages. The physical methods include the biolistic transformation [29], macroinjection [30], microinjection [31, 32], and protoplast fusion [33]. Biolistic transformation (or more commonly referred to as particle bombardment) employs the accelerated microprojectiles directly to the target DNA or other molecules into the cells [26, 29]. The DNA in which needed to be transferred is coated on microscopic beads, the beads then attached to a plastic bullet and loaded in the gene gun. As the gun is fired, the DNA coated beads penetrate into the cytoplasm. The DNA then disassociate from the beads and join the genome of the transferred cells [26, 34, 35]. Macroinjection is the transfer of DNA using needles with greater diameters than cell's. In plants, the technique was reported to be successful in rye (*Secale cereale* L.) and other economical plants [30]. Contrarily, in microinjection, DNA is injected into the cells using very fine needles or glass micropipettes with 0.5–10 µm diameter [31, 32]. Protoplast fusion (or somatic fusion) is the fusion of two distinct plant species using electric shock or chemical treatment to a plant [33].

The chemical methods of genetic transformation include the calcium phosphate-mediated transfer [36], polyethylene glycol-mediated transfer [27], DEAE-Dextran mediated transfer [37], and liposome-mediated transfer [38]. Calcium phosphate-mediated transfer involves the mixture of desired DNA with calcium chloride and potassium phosphate solutions to form the calcium phosphate precipitate. Then, cells incubated with precipitated DNA are taken inside the tissues by endocytosis [36, 39]. Polyethylene glycol (PEG)-mediated transfer is a method that is particularly used to transfer genes into the protoplasts. Initially, the protoplasts are soaked in PEG-containing solution that facilitate the endocytosis after which DNA uptake occurs [40, 41]. In DEAE-Dextran mediated transfer, DEAE-Dextran is applied as transfection medium, which is commercially available at low cost simple and relevant for transient cells [37]. Liposome-mediated transfer is performed by artificial lipid vesicles known as liposomes that function as delivery agents for exogenous materials [38]. Liposomes surround the delivery molecule and enable its transfer via fusion with cell membrane. The positively charged cationic lipids can more readily interact with negatively charged cell membranes, resulting in fusion and discarding all of its content (*e.g.,* DNA) across the plasma membrane [42]. The electrical transformation methods include the electrofusion and electroporation [28]. Electrofusion provides the electric field-induced cell-to-cell fusion and electroporation allows the electric field-mediated membrane permeabilization [43]. Both methods produce the transient, unstable regions in plasma membranes allowing the passage of molecules to be transported [44, 45].

Currently, target-specific genome editing techniques (Next generation gene transfer methods) were announced as new methods of recombinant DNA technology. These are site-specific editions applying chimeric meganucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas (CRISPR-associated) systems, which are based on RNA-guided DNA endonucleases [46].

Target-specific genome editing technologies first produce DNA double-strand breaks at target site, which activate plant DNA repair mechanism to join the DNA break. The broken DNA is joined by either homologous recombination or non-homologous end joining [47]. The main benefits of these methods are that we can produce transgene-free genetically modified (GM) plants which, are not disturbed to regulatory issues [48, 49].

Electroporation

Electroporation is an electrical transformation method by which transient pores are produced in plasma membranes of prokaryotic and eukaryotic cells [28, 50, 51]. The applied field creates the microscopic pores or electropores in cell membrane, which allow the passage of micro- or macromolecules in and/or outside cells [52] (Fig. 1). Electrically induced formation of aqueous pores in the lipid bilayer, with water molecules first penetrating the bilayer and thus forming unstable hydrophobic pores, and with adjacent lipids then reorienting with their polar headgroups toward these water molecules and thus forming a metastable hydrophilic pore [45, 53]. This reorientation occurs because the energy needed to form an aqueous pore is reduced as the transmembrane voltage is increased and the energy required maintaining the circumference of a large hydrophilic pore is significantly lower than that required to maintain a large hydrophobic pore [51].

Under optimized electric pulse, electropores can be resealable and cells can be recovered (Fig. 2). However, none suitable electric currents (*e.g.,* very high electric field strength) cause the cells to dramatically overheat, resulting in the cell deaths [54]. Since the electropore formations are associated with the amplitude and electric pulse duration applied, this heating effect can be minimized by adopting relatively high amplitude with a short duration pulse or by using two short duration pulses [55]. Thus, depending on the intensity of electric field and duration, the process can be either reversible where electropores in the membrane are resealable and the cells preserve the vitality or irreversible where the membrane is not able to reseal and the cell eventually dies [56]. Depending on the duration of the pulse, resealing is a relatively long process (> 1 s) when compared with the pore formation (ms or µs) [52].

This method has found common grounds and increasing applications in biology, medicine, and biotechnology because of its advantages such as rapid application, low cost, applicability to many cell types, suitability for large number of cells, and providing high stable transformation percentage [51, 57]. From biotechnological perspective, four main application areas have been reported for electroporation [44].

I. *In electrotransformation* using reversible electroporation method, desired exogenous DNA can be transfected into the target cells for purposes such as biomolecules production [58], adaptation [59], and basic research [60]. Moreover, electrotransformation of *A. tumefaciens* eliminates the *Escherichia coli* transformation step, thus providing a faster and simpler cloning routine [61].
II. *In electroporation-based inactivation* exposing strong and long enough electric pulse to microorganisms inhibit their cellular activities, which can be exploited for wastewater treatment [62, 63], using irreversible electroporation as a substitute for chlorination of water [64], and non-thermal food pasteurization [65, 66].
III. *In electroextraction* electroporation can be used to extract the potential source of biomolecules for industry from bacteria [67, 68], microalgae [69–71], yeast [72, 73], and multicellular tissues [74, 75].
IV. *Electroporative biomass drying* facilitate the water release from tissue, thereby, significantly contributing to energy savings in drying process [75, 76].

Electroporation process is performed by using an electroporator device, which typically consists of three main parts, (i) a pulse power supply, (ii) electroporation cuvettes, and (iii) electrodes [77] (Fig. 3). The pulse power supply harbors the all control units (*e.g.,* electrical pulse settings). Firstly, the target cell suspension is pipetted into the plastic or glass cuvette. Secondly, the adjusted electric pulse settings are applied to the cells via electrical conductors, electrodes, and this process necessitates a direct contact between cell suspension and electrodes [78]. Every cell type has a distinctive field strength based on the applied pulse parameters (*e.g.,* voltage, resistance and capacitance) and an optimal field strength causes the electropermeabilization by induction of transmembrane voltage [52]. In electrotransformation of nucleic acids, electroporators are often employed with three different electrical wave pulse forms *i.e.* time constant, square wave, and exponential decay [79]. In time constant pulse, a constant pulse is applied to the cells to be electrotransformed at a certain set of voltage and time

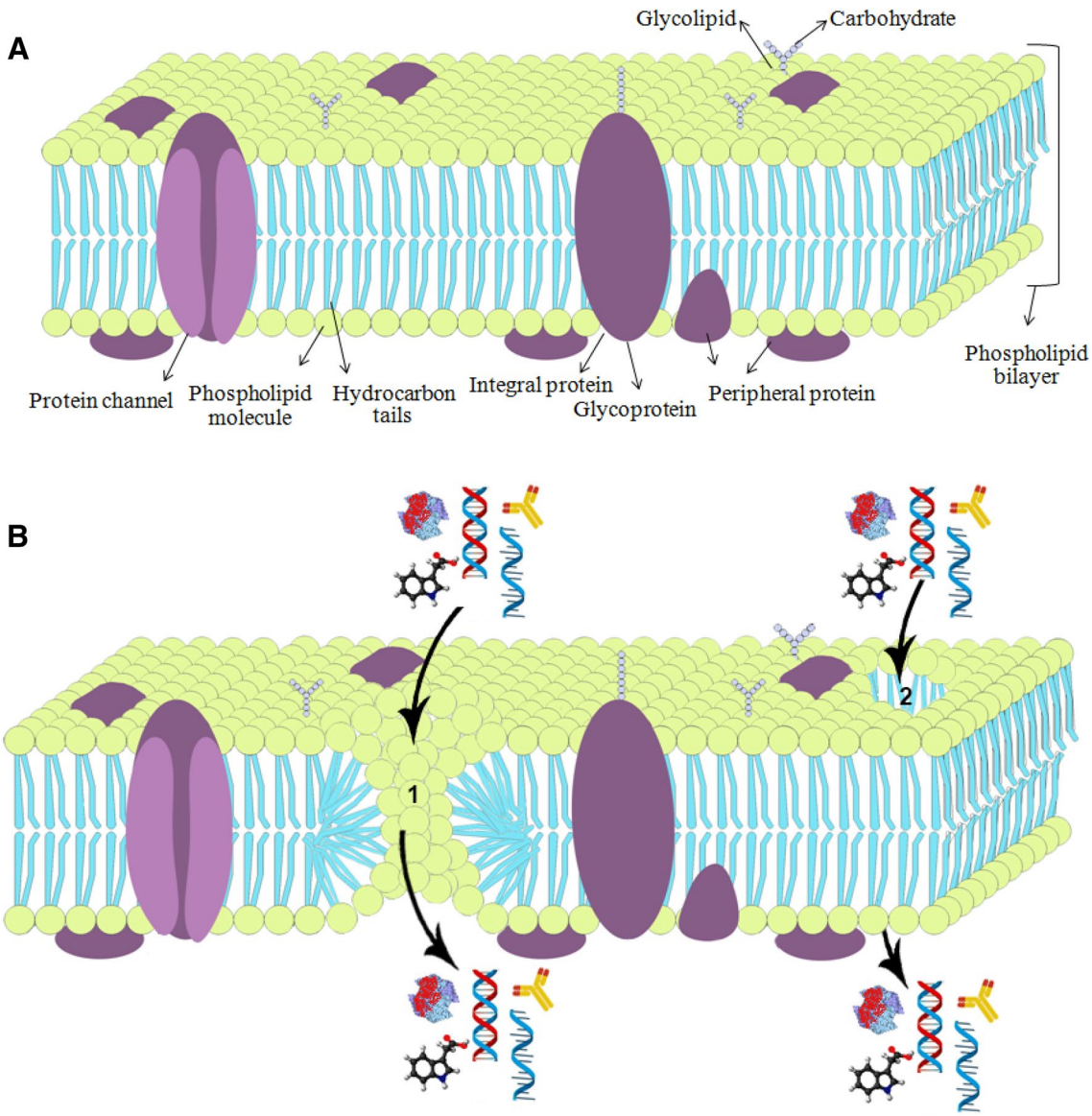

Fig. 1 **a** Basic structure of plasma membrane, **b** Arrangement of plasma membrane lipids in a hydrophilic pore (1) and hydrophobic pore (2) during the pulse and transferring of DNA, RNA, enzyme, antibody, and some chemicals (e.g., hormone) into cell (Copyrighted illustration from Professor Ozyigit)

[50, 80]. Square wave pulse is characterized by the voltage applied, the pulse duration and number, and the interval lengths between pulses [81, 82]. In exponential decay pulse, the adjusted voltage released from capacitor rapidly and exponentially decays over time [83, 84]. Field strength (kV cm^{-1}) and time constant are two important parameters that characterize the pulse delivered. These settings thus can be adjusted to achieve the ideal transfection efficiencies in various cell types [79].

As mentioned above, penetration of water molecules into lipid bilayer initiates the electroporation process, by causing the reorientation of adjacent lipids toward water molecules [45, 85, 86]. The thermodynamics govern the pore formations thereby it is not only attributable to a certain electric threshold [85, 87]. However, electroporation-derived transport is strongly correlated with the electric field-induced transmembrane voltage [88, 89]. The four ranges of electric field strength are reported each with typical characterizations [50]. In no detectable scale, electropores are too small and short-lived to be quantified for transport. In reversible scale, electropores allow a temporary passage for transport and then they are resealed and recovered. In non-thermal irreversible scale, electropores do not reseal or slowly close, causing the cells to break and release their contents but they

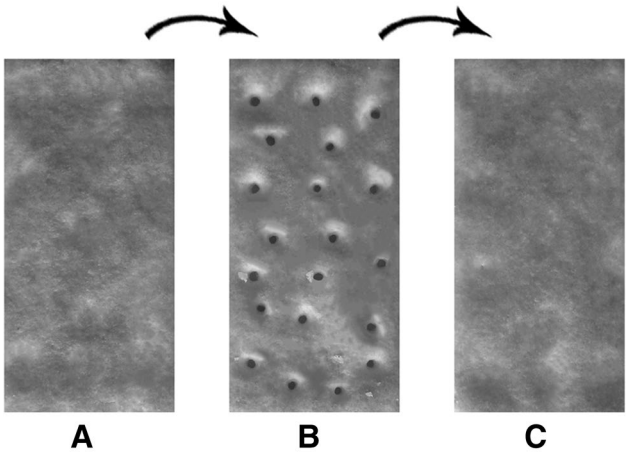

Fig. 2 Membrane before electric pulse (**a**), electropore formations under electric pulse (**b**) and resealing and recovering after the pulse (**c**) (Copyrighted illustration from Professor Ozyigit)

are not thermally damaged. In thermal irreversible scale, applied electric current causes the thermal damage *e.g.,* protein denaturation at > 50 °C and DNA melting at > 70 °C [58, 90–92].

In addition, above-defined ranges also partly overlap with each other because electroporation is a stochastic process, affected by cell type, size, mediated-medium and electrical conductivity, solutes-contained and osmolarity [45, 53]. Moreover, to obtain an ideal uniform electroporation is hard since tissues have various cell types and varying spatial organizations due to gap junctions. Thus, despite the homogeneous application of external electric field, inside cells they are non-homogeneously distributed, leading more electroporation of some cells than others [93–95]. To minimize this, numerical models must be developed to iteratively optimize field homogeneity considering the size, shape, number of cells, and electrode positions as well as complemented by real-time measurements [96–98].

For optimal transformation efficiency, field strengths and pulse durations mainly range of 1–20 kV cm^{-1} and 1–30 ms respectively but optimal values are empirically determined [99]. This efficiency reduces with the thickness or layers of membranes covering the recipient DNA [100], thereby gram-negative bacteria have the highest efficiency with 10^7–10^{10} transformants per mg DNA but it is lower for gram-positive bacteria and archaea due to their thick cell walls (10^5–10^7), and even lower (10^4–10^7) for yeasts

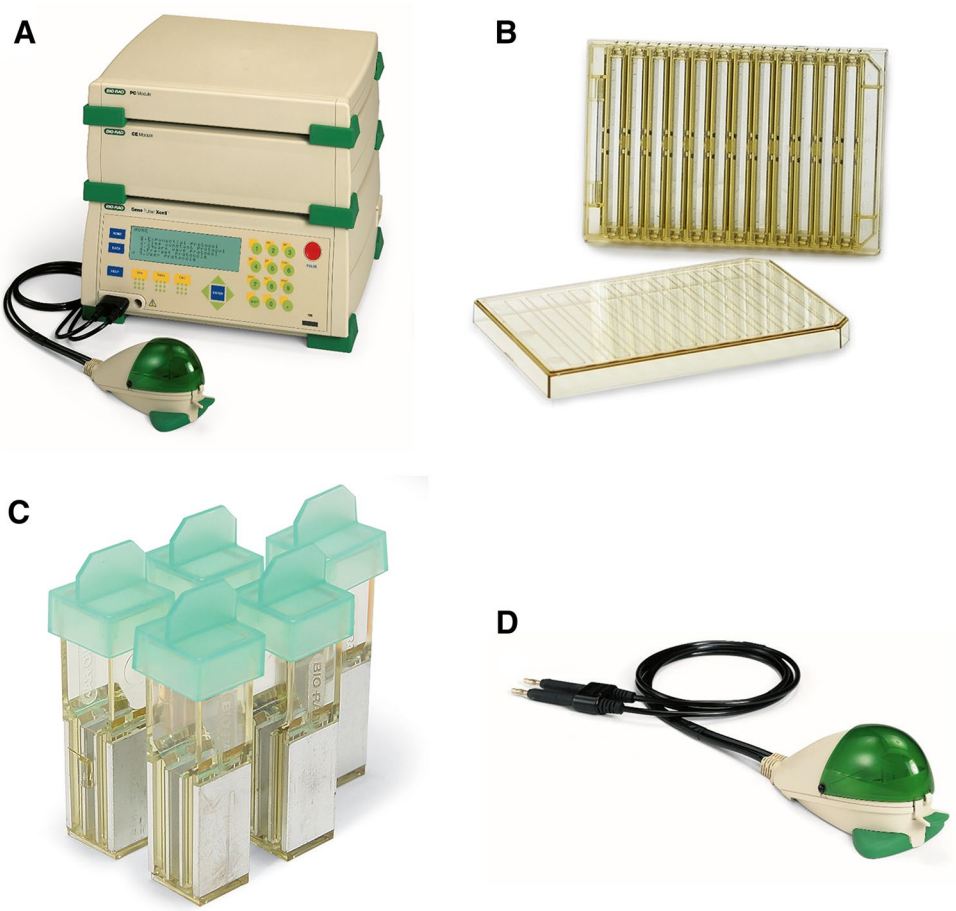

Fig. 3 Gene Pulser Xcell™ Electroporation Systems (**a**) and its accessories. Electroporation plate (**b**), Cuvettes (**c**) and Cuvette chamber (**d**) (With the permission of Bio-Rad Laboratories, Inc.)

and microalgae due to their nuclear membranes [101, 102]. The use of electroporation is higher for smaller organisms such as bacteria and archaea with 5–20 kV cm^{-1}, and microalgae and yeasts with 1–12 kV cm^{-1}. The supercoiled circular dsDNA has the highest transformation efficiency but it is lower for circular ssDNA, relaxed circular dsDNA, and linear dsDNA with homologous/non-homologous ends [103, 104]. The divalent cations such as Mg^{2+} and Ca^{2+} can be avoided since they interact with DNA [105]. To some limited extend, transformation efficiency can be increased by hyperosmolarity [106, 107] and chemical pretreatments [84, 108]. All these considerations require a better and deeper understanding of electroporation and its effects on cell membranes or cell walls.

In vascular plant species, different pulses were varied in a range of field strengths and pulse durations related to cell types (stomata guard cell, anther, microspore, zygote, mature and/or immature embryo, mesophyll, nodal meristems) their derivates (callus, protoplast) and applied organelle (chloroplast and/or mitocondria). Some applications in selected literature are given below.

- In *Solanum dulcamara* L. (Bittersweet nightshade) and *Prunus avium* x *pseudocerasus* (Colt cherry), the duration of the pulse decay constant was 10–50 µs by discharging 10–50 nF capacitors at voltages from 250 to 2000 V cm^{-1}, respectively [109, 110].
- In *Beta vulgaris* L. (Sugar beet) and *Nicotiana tabacum* L. (Tobacco), rectangular pulses were varied in a range of field strengths between 70–300 V mm^{-1} and pulse durations between 25–1000 µs while exponentially decaying pulses were produced using a capacitor of 800 µF and the pulse length was 22.4 ms [111].
- In *Eucalyptus citriodora* (Hook.) K.D. Hill & L.A.S. Johnson (Lemon-scented eucalyptus), rectangular pulses were applied at a range of voltages between 400–1600 V cm^{-1} with pulse durations of 100–2000 µs [112].
- In interspecific hybrid of Poaceae family member *Saccharum* spp. (Sugarcane) protoplasts, 5–10 ms pulses were used at voltages from 385 to 540 V cm^{-1}[113].
- In *Zea mays* L. (Maize), one successive pulse at 375 V cm^{-1} from a 900 µF capacitor was applied [114].
- In *Triticum aestivum* cv. Hartog (Wheat), the pulse length was 3 ms by discharging 120 µF capacitor at 667 V cm^{-1} voltage [115].
- In *S. officinarum* L., the pulses were at a range of voltages between 600–850 V cm^{-1} and capacitances of 440, 660, and 880 µF were evaluated [116].
- In *Hordeum vulgare* L. (Barley), the pulses were applied at the voltage of 670 V cm^{-1} by discharge of a 200 µF capacitor [117].
- In the leaf protoplasts of *Vitis* sp. (Grapevine), one pulse was at 150, 174, and 200 V cm^{-1} and 150 or 175 µF, and in embryogenic protoplasts, one pulse was at 200 V cm^{-1} and 100 or 150 µF [118].
- In *N. tabacum* L., the pulses were applied at a voltage of 900 V cm^{-1} by discharge of a 21 µF and a pulse time of 13 ms [119].
- In *H. vulgare* L., the pulses were used at a range of voltages 500, 750, and 1000 V cm^{-1} with two capacitance values of 500 and 960 µF [120].
- In *H. chilense* Roem. x *T. turgidum* L. Conv. durum (Tritordeum), a single electric pulse of field strength at 550 V cm^{-1} was discharged from a 960 µF [121].
- In *Asparagus officinalis* L. (Sparrow grass), the pulses were applied at a range of voltages 250, 500, 750, 1000, 1500, or 2000 V cm^{-1} by discharging of 25, 50, 75, 100, or 125 µF capacitors [122].
- In *Citrus sinensis* L. (Sweet orange), a single exponential pulse with a 500 V cm^{-1} field strength and three capacitors (250, 500, and 960 µF) were tested [123].
- In *Pelargonium* × *hortorum* 'Panaché Sud', the pulses were tested at a range of voltages between 250–300 V cm^{-1} by discharging 10, 33, and 50 µF capacitors [124].
- In *Pinus armandii* Franch. (Armand pine), the pulses were practiced at the voltage of 375 V cm^{-1} by discharging of 900 µF capacitor [125].
- In *Gentiana kurroo* Royle (Himalayan gentian), the duration of the pulse decay constant was between 20–40 µs and 1–5 ms at voltages from 0 to 1.75 kV cm^{-1}, respectively [126].
- In *Arabidopsis thaliana* Heynh., the standard electroporation program was consisting of 375 V cm^{-1} (150 V setting), 10 ms and 50 ms for poring pulses, and 50 V cm^{-1} (20 V setting), and 50 ms for transferring a square wave pulse [127].

Although principles of electroporation extend to the second half of the twentieth century, its real applications in biotechnology and other areas have been recently emerging. The spontaneous transform of foreign genes in microorganisms provided motivation to develop more controlled transformation methods [44]. Various physical and chemical approaches have been proposed but by mid-1980s transformation with electric field or electrotransformation has come forward because of its efficiency and applicability [99]. Despite some limitations, electrotransformation has been effectively used in bacteria; *Brevibacterium lactofermentum* [128], *Corynebacterium glutamicum* [129], *Mycobacterium aurum* [130] (Actinobacteria), *Bacteroides fragilis* [131], *B. ruminicola and B. uniformis* [132], *Prevotella ruminicola* [133] (Bacteroidetes), *Chlamydia psittaci* [134], *C. trachomatis* [135] (Chlamydiae), *Chlorobium vibrioforme* [136] (Chlorobi), *Spirulina platensis* [137], *Fremyella diplosiphon* [138], *Synechococcus elongatus* [139]

(Cyanobacteria), *Deinococcus radiodurans* [140], *Thermus thermophilus* [141] (Deinococcus-Thermus), *Bacillus cereus* [142], *Clostridium perfringens* [143], *Enterococcus faecalis* [144], *Lactobacillus acidophilus* [145], *L. brevis*, *L. bulgaricus*, *L. casei*, *L. plantarum* [146, 147], *Listeria monocytogenes* [148], *Streptococcus pyogenes* [149], *S. thermophilus* [150], (Firmicutes), *Fusobacterium nucleatum* [151] (Fusobacteria), *Planctomyces limnophilus* [152, 153] (Planctomycetes), *Campylobacter jejuni* [154], *Escherichia coli* [155], *Klebsiella pneumoniae* [101], *Pseudomonas fluorescens* [156], *Salmonella typhimurium and S. typhi* [157], *Sinorhizobium meliloti* [158], *Thiobacillus neapolitanus* [159], *Yersinia pestis* [160] (Proteobacteria), *Borrelia burgdorferi* [161] (Spirochaetes), *Mycoplasma pneumoniae* [162] (Tenericutes), *Thermotoga neapolitana* [163] (Thermotogae), archaea; *Metallosphaera sedula* [164], *Sulfolobus islandicus* [165], *S. solfataricus* [166] (Crenarchaeota), *Methanococcus voltae* [167], (Euryarchaeota), microalgae; *Ankistrodesmus falcatus* [168], *Auxenochlorella protothecoides* [169], *Chlamydomonas reinhardtii* [170], *Chlorella ellipsoidea* [171], *C. vulgaris* [172], *Dunaliella salina* [173], *Scenedesmus obliquus* [174] (Chlorophyta), *Nannochloropsis* sp.[175], *Phaeodactylum tricornutum* [176] (Heterokontophyta), *Cyanidioschyzon merolae* [177] (Rhodophyta), and yeasts; *Candida albicans* [178], *C. maltosa* [179], *Hansenula polymorpha* [180], *Pichia pastoris* [181], *Saccharomyces cerevisiae* [182], *Schizosaccharomyces pombe* [183] (Ascomycota), *Cryptococcus neoformans* [184], *Pseudozyma antarctica* [185], *P. flocculosa* [186] (Basidiomycota) as mostly reported by Kotnik et al. [44] and other researchers given above. Known of these limitations was mainly derived from species sensitivity for usual electroporation process or its resistance for pore formation [187–189].

In human tissues, electroporation is efficient, feasible, and tolerable, and mostly used clinical application is electrochemotherapy (ECT). Electrochemotherapy has the capability to develop the efficacy of drugs that have impeded transport through the plasma membrane to treat tumors [92, 190]. Additionally, reversible electroporation is gaining momentum as an effective technique for gene electrotransfer and DNA vaccination [92]. Some drugs tested and found in vitro potentiation in combination with electroporation are; actinomycin D [191, 192], bleomycin [193], carboplatin [194], cisplatin [195], cytarabine [196], mitomycin C [197], netropsin [196], 2-N-methyl-9-hydroxy-ellipticinium (NMHE) [191], oxaliplatin [198], vinblastine [199], and vincristine [200].

In recent years, in vitro electroporation researches focused on calcium electroporation. Dose-dependent reduction in cell viability was showed for all experienced cell lines [201, 202]. Also, electroporation has been used for gene editing with CRISPR/Cas technology [203, 204]. After additional preclinical developments, electroporation based therapy with different types such as reversible/irreversible electroporation, calcium electroporation, electrochemotherapy, DNA vaccination, combined with CRISPR/Cas9 was tested in different cancer types such as colorectal [202], brain [205], breast [206], liver [207], pancreatic [208], and prostate [209] cancers and some promising results have been obtained.

Electrotransformation applications in plants

The usage of electric pulse techniques in biology, medicine, biotechnology, and many other biological sciences have found huge support because of their efficiency and wider applicability. However, optimal electrotransformation applications have required a number of parameters to be considered and numerical models to be constructed for efficient applications [96–98]. So, this is an empirical process, at each application providing a better and deeper understanding for further steps. Below has been chronologically compiled some successful and promising electroporation-mediated applications from plants.

- Transgenic rice (*Oryza sativa* L.) plants regenerated via somatic embryogenesis were electroporated with plasmid carrying *nptII* gene and production of kanamycin-resistant plants demonstrated the usability of protoplast in genetic engineering studies [210].
- Protoplasts of woody medicinal plant bittersweet nightshade (*Solanum dulcamara* L.) were electroporated, and protoplast-derived tissues exhibited increased morphogenesis compared to untreated protoplasts. Regenerated shoots also rooted more readily and developed more prolific root systems than shoots from untreated protoplasts [109].
- High frequency regeneration and the number of shoots per callus were observed in electropulsed colt cherry (*Prunus avium* × *pseudocerasus*) protoplasts. Also, these electropulsed tissues generated more prolific root systems when compared to non-electropulsed ones [110].
- Uptake and expression of cucumber mosaic viral (CMV) RNA by tobacco (*Nicotiana tabacum* L.) protoplasts were examined using both square and exponential wave electroporation pulses [211].
- Transgenic rice (*O. sativa* L.) plants were electrotransformed with multiple copies of introduced genes in a complex manner in plant genome. As a result of the research, *hpt* were detected and expressed in the progeny of transformants [212].
- The electroporation efficiency in sugar beet (*Beta vulgaris* L.) and tobacco (*N. tabacum* L.) protoplasts by alternating, rectangular and exponentially decaying

pulses were studied by assaying transient expression of an introduced *cat* gene activity [111].

- Among selectable markers delivering herbicide tolerance in maize (*Zea mays* L.) plant, *bar* gene from *Streptomyces hygroscopicus* was first obtained as an herbicide marker [114].
- Stably transformed calli were regenerated under selection for kanamycin resistance following introduction of *nptII* gene into protoplasts of sugarcane (interspecific hybrids of Poaceae family member *Saccharum* spp.) [113].
- Protoplasts isolated from cotyledons of lemon eucalyptus (*Eucalyptus citriodora* (Hook.) K.D. Hill & L.A.S. Johnson) were electroporated using a rectangular pulse, with plasmid carrying *cat* gene [112].
- Fertile transgenic rice (*O. sativa* var. IR36) plants were obtained by electrotransformation of *nptII* gene into the seed embryo cells [213].
- Protoplasts were isolated from embryogenic suspension cultures of wheat *(Triticum aestivum* cv. Hartog) were electroporated in the presence of plasmid pEmuGN and/or pEmuPAT, which contained *gus* and *bar* genes respectively, and up to 0.9% of viable protoplasts displayed the *gus* activity two days after electroporation [115].
- First successful electrotransformation into protoplasts of an important temperate grass species, (*Agrostis alba* L.) was achieved. The *nptII* gene was used as a selectable marker [214].
- Commercial sugarcane (*S. officinarum* L. var. Ja 60–5) was electrotransformed with a plasmid conferring *gus* activity into cell clusters from embryogenic calli and it was reported to be an effective and reproducible method for sugarcane [116].
- Barley (*Hordeum vulgare* L.) protoplasts were transformed with *nptII* gene by electroporation. Analysis of 2nd and 3nd generation plants demonstrated the successfully integration of transgene by inheriting new trait to progenies [117].
- Analysis with discriminative molecular markers demonstrated that two artificial gene transfer methods such as particle bombardment and intact cell electroporation are better in production of transgenic rice (*O. sativa* L.) plants (cv. Lido, Carnaroli and Thaibonnet) with insignificant genomic changes [215].
- Somaclonal variation in t*cryIA(b)* gene transformed sugarcane (*Saccharum* hybrid cv. Ja60-5) clones by intact cell electroporation was analyzed with molecular marker (RAPD, AFLP, and RAMP) techniques [216].
- Optimum conditions were detected for DNA transfer to mature embryos of barley (*H. vulgare* L.) via electroporation [120].
- Direct inoculation of *grapevine fanleaf virus* (GFLV) into grapevine (*Vitis* sp.) protoplast, which were isolated from mesophyll cells of in vitro grown plants and from embryogenic cell suspensions via electroporation [118].
- Transformed *gfp* constructs demonstrated a transient expression in ~ 2.6% of electroporated tobacco (*N. tabacum* L.) zygotes [119].
- Fertile transgenic plants were generated from tritordeum (*H. chilense* Roem. × *T. turgidum* L. Conv. *durum*) inflorescences using tissue electroporation. The expression of both inserted marker genes (*uid*A and *bar*) was confirmed using standard assays, while transgene integration was confirmed using PCR and Southern hybridization analyses [121].
- Mechanically isolated microspores of three different sparrow grass (*Asparagus officinalis* L.) genotypes were submitted to electric fields in order to modulate their competence for embryogenesis [122].
- Genetic transformation though protoplast electroporation was established in a tropical forage legume, stylosanthes (*Stylosanthes guianensis* (Aublet) Sw.) and *gus* expression was assayed [217].
- Embryogenic protoplasts of sweet orange (*Citrus sinensis* (L.) Osbeck) were effectively electrotransformed and transformed plants were regenerated. The plasmid vector pBI221 containing the *gus* coding sequence under the control of the CaMV 35S promoter was used and *gus* activity was measured 24 h after electroporation [123].
- Anther culture-derived embryos of wheat (*T. aestivum* L.) were electrotransformed by a plasmid pAM2100 carrying *bar* and *gus* genes [218].
- An exogenous substance, fructan was electrotransformed into perennial ryegrass (*Lolium perenne* L.) protoplasts and substance concentrations in protoplasts were reported to increase by electroporation [219].
- Maize (*Z. mays* L.) inbred line Pa91 was electrotransformed by a plasmid pGREEN0229 carrying an *Arabidopsis* trehalose phosphate synthase (*AtTPS1*) gene and a selective gene *bar*. As a result of the research, successfully transformed plants were obtained [220].
- Plantain (*Musa* sp. cv. harton) shoot apices were electrotransformed by a plasmid pCAMBIA 3201 carrying a *Basta* (herbicide) resistant gene. Introduction of *bar* genes into plantain has been successful by electroporation [221].
- Nodal buds of cowpea (*Vigna unguiculata* (L.) Walp.) were electrotransformed by a plasmid carrying an insecticidal *Cry1Ab* and *nptII* genes. T1-3 plant progenies were reported to significantly reduce the larval survival [222].
- Mesophyll protoplasts of (*Pelargonium* × *hortorum*) 'Panaché Sud' were transformed by electroporation, which was reported to be more efficient for protoplast survival, membrane permeation and cell division. Calcein and *gfp* genes were used to set up the process. PCR

analysis of in vitro micropropagated plants showed that 18 clones out of 20 contained the *nptII* gene [124].

- Mature embryos of Armand pine (*Pinus armandii* Franch.) were electrotransformed by a plasmid pBSbtCryIII(A) carrying a selectable *nptII* gene and an insecticidal *SbtCryIII(A)* gene, with a successful genomic integration [125].
- Isolated cucumber (*Cucumis sativus* L.) mitochondria were successfully transformed by electroporation. Comparison of mitochondrial RNA before and after applications demonstrated no RNA degradation by electroporation [223].
- Pollens of common wheat (*T. aestivum* L.) were transformed by electroporation and transformants demonstrated stable expression in transgenic progeny [224].
- Protoplasts of Himalyan gentian (*Gentiana kurroo* Royle) embryogenic cells were electrotransformed by a plasmid carrying *nptII* and *bar* genes, and the highest electroporation efficiency in respect to protoplast survival rate was evaluated under specific physical conditions [126].
- Pre-treatment by irreversible electroporation in Genovese basil (*Ocimum basilicum* L.) leaves was reported to shorten the drying times but quality characteristics of dried leaves were lowered [225].
- Protein directly delivered into *A. thaliana* cells in the presence of a cell wall with 83% efficiency rate, which also proved to be less toxic. This is a step towards nucleic-acid free genome engineering in plants [127].

Taken collectively, historical development of electroporation and its present applications point that this method also includes some other uncovered potentials for various exploitations in future.

Conclusion and future perspective

Various natural and artificial methods have been developed to transfer DNA, RNA, and other molecules into the cells, each with its own advantages and disadvantages. To select the most convenient method is a crucial factor in successful transformations. Transformed cell type, transfection endurance (stable or transient), recombinant preparation, regeneration, gene selection, and isolation are also some other important parameters to be considered. Among gene transfer methods, electroporation has found grounds because of its both, higher efficiency and broader applicability. It has been effectively used in many disciplines such as biology, medicine, and biotechnology. In biotechnology it was employed in electrotransformation, electroporation-based inactivation, electroextraction, and electroporative biomass drying. In electrotransformation studies, uniform electroporation has been a challenging issue due to the varying cell types, and spatial and temporal organizations of cellular components. However, this problem was minimized by developing numerical models considering the all involved parameters. Thus, electroporation is an empirical process, at each application a better and deeper understanding is acquired for further steps. Moreover, current applications of electroporation also point to some other uncovered potentials of this method for various exploitations.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the author.

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