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TRANSGENIC TOMATO PLANTS (*Solanum lycopersicum* L.): DIRECT METHODS OF GENE TRANSFER AND FACTORS AFFECTING TRANSFORMATION EFFICIENCY

(review)

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Abstract

Tomato (*Solanum lycopersicum* L.) is the most important food crop which is also widely used as a model plant in molecular genetic investigations of vegetative development and reproductive biology, plant resistance to abiotic and biotic stresses, plant-microbe association and symbiosis, etc., that have both basic and applied value. The production of transgenic tomato plants expressing foreign heterologous genes, as well as with induced silencing or knockout of their own genes, is an important part of modern plant physiology. There are two radically different approaches to introducing foreign DNA into the tomato genome. The first method is based on the natural mechanism of infection with plant-associated bacterial pathogen *Agrobacterium* sp. (*A. tumefaciens*. or *A. rhizogenes*), followed by T-DNA transfer and insertion into the plant genome (*Agrobacterim*-mediated transformation). The second approach is based on the direct introducing of foreign DNA into the plant cells through the plasma membrane by chemical (Ca^{2+}, polyethylene glycol, PEG) or physical exposure (electrical impulse or excessive pressure) (direct methods of tomato genetic transformation). Transgenic tomato plants can be produced both by the classical tissue culture-based transformation procedure and in planta transformation. This review article discusses classical direct methods for introducing foreign DNA into the tomato genome (chemical-mediated transfection, protoplast electroporation, microinjection, biolistic transformation), and in planta transformation methods (pollen-tube pathway, electroporation of mature seed embryo). The review considers features of producing tomato plants both with transient transgene expression and stably inherited insertion into the nuclear or plastid genomes are considered. In addition, the factors affecting the efficiency of transformation are analyzed in detail. A separate section is devoted to the direct tomato genetic transformation methods for delivering various genome editing tools (ZFNs, TALEN, CRISPR/Cas, base editing, prime editing) that have become widespread in the past five years.

Keywords: *Solanum lycopersicum* L., electroporation, PEG-mediated transformation, microinjection, biolistic transformation, transformation in planta, genome editing

Tomato (*Solanum lycopersicum* L.) is important food crop, which ranks second after potatoes among agricultural vegetable plants in terms of the gross harvest of marketable products. Thus, according to the Food and Agriculture Organization of the United Nations (FAO), the global gross harvest of tomato fruits in 2020 amounted to approx. 186.8 million tons when grown on an area of 5.1 million hectares, of which Russia accounted for 2.9 million tons (approx. 1.6%) with an area of 80.7 thousand hectares [1]. In Russia, tomato is grown under various agroecological conditions, both in open and protected ground (about 60 and 40% of the gross harvest, respectively) [2, 3]. The main areas of cultivation of this crop are concentrated mainly in the southern regions of the

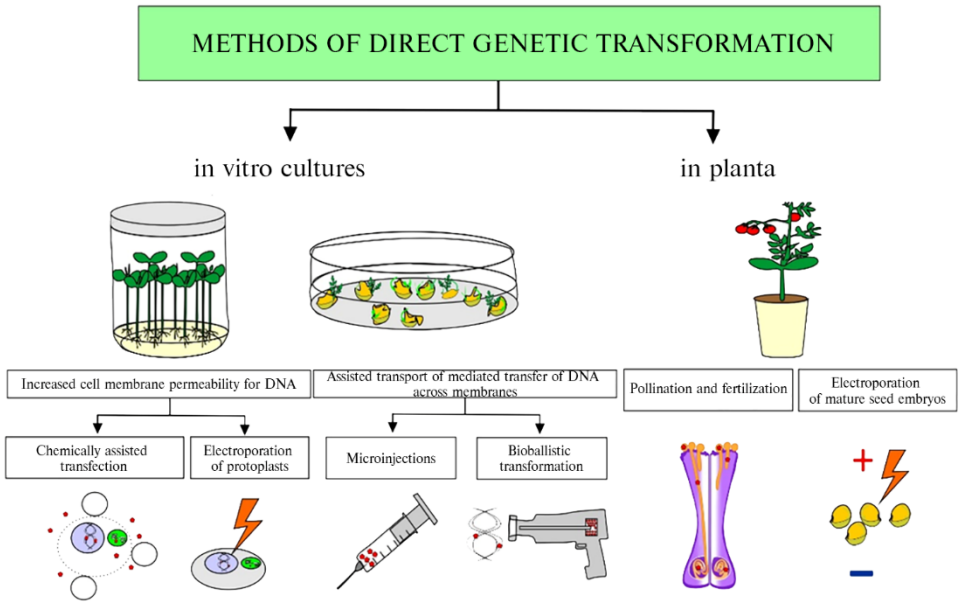
country (Krasnodar and Stavropol Territories, as well as the Volga and Central Chernozem regions), the territories of which are more or less prone to primary and/or secondary salinization, as well as other edaphic stresses (4) . Thus, more than 31% of the soils in the Astrakhan region are characterized by a high degree of salinity (the concentration of Na^+ and SO_4^{2-} ions reaches 7.1 and 12.5 mM per 100 g of soil, respectively), and about 20% of the soils are represented by solonetzic complexes [5]. In addition, the tomato has more than 45 infectious diseases of bacterial, fungal, viral and viroid etiology, which are distributed to varying degrees on the territory of the Russian Federation [6, 7]. As a result, increasing resistance to abiotic and biotic stress factors is one of the priority requirements for modern varieties and hybrids of tomato to realize potential yields. Therefore, it is necessary to constantly expand the range of crops using both traditional breeding approaches and modern methods of biotechnology and genetic engineering [8-10].

In addition to its great practical significance, the tomato is widely used as a model object in various fundamental studies affecting the issues of vegetative development and reproductive biology [11, 12], studying the mechanisms of plant resistance to abiotic and biotic stresses [9, 10, 13], and associative symbiosis with microorganisms [14, 15], meiotic recombination [16] and many others. The choice of such a model object is due to the fact that the tomato is a relatively unpretentious self-pollinating plant in cultivation with a short growing season, high reproductive potential, which is also capable of rapid vegetative reproduction and distant hybridization with some species of the genus *Solanum* [17]. The cultivated tomato and related wild species are diploids, the karyotype of which is represented by 24 chromosomes ($2n = 2\times12 = 24$). In addition, the tomato serves as a convenient model object due to the large number of morphological characters that are clearly identifiable at different stages of ontogeny [18, 19], as well as the availability of detailed genetic and molecular maps [20] (especially after the complete sequencing of the tomato genome in 2012) [21] and developed efficient and reproducible protocols for in vitro cultivation of isolated tissues [10, 22]. These factors ensured the widespread use of transgenic tomato plants as an experimental model for studying the role of expression of heterologous genes of various origins in fundamental biological processes.

Numerous accumulated data indicate that various genetic engineering strategies can significantly accelerate the creation of new tomato genotypes with traits that are difficult or impossible to achieve using traditional breeding. For example, it is possible to increase the resistance of tomato plants to phytopathogens by hybridizing tomato with related wild-growing species, such as *S. pimpinellifolium* L. and *S. habrochaites* S. Knapp & D.M. Spooner, followed by numerous backcrosses [23, 24]. However, only relatively closely related species capable of crossing with cultivated tomato can participate in such breeding programs; the process takes up to 15-20 years [25]. *S. lycopersicum* plants with increased resistance to fungal and bacterial pathogens can be obtained using various genetic engineering strategies: expression of heterologous genes of PR proteins (pathogenesis-related proteins synthesized in a plant cell during a pathogen attack) and antimicrobial peptides of plant, animal and fungal origin; metabolic engineering of phytoalexins and hormones; using resistance genes (R-genes); inhibition of toxic products of the pathogen; lignification of the plant cell wall; activation of plant defense reactions, etc.) [9]. The duration of the selection process is significantly reduced.

Both nuclear and plastid genome can be subjected to genetic transformation. The latter option has a number of undoubted advantages, since foreign genes integrated into the plastid genome are characterized by increased expression

due to the characteristics of plastid DNA [26, 27] — a large number of plastome gene copies per cell (especially the level of transgene expression regardless of the insertion position in plastid DNA due to the lack of compact chromatin packing [30); the absence of epigenetic effects and the resulting silencing of transgenes [29, 30]. All these factors make it possible to increase the yield of transplastomic gene expression product to 40% of total soluble protein (TSP) [28, 29, 31]. Thus, transplastomic plants serve as promising bioreactors for the production of heterologous proteins for medical and veterinary purposes (32-34). Also, polycistronic cassettes are successfully expressed in plastids, which simplifies the co-transformation of one target by many different genes [29]. An undoubted ecological advantage of transgene localization in plastids is maternal inheritance of the plastome, which leads to the absence of the transgene in pollen and the impossibility of genetic contamination of the population [29, 33].



Classification of direct methods of tomato plants genetic transformation.

There are two fundamentally different approaches for introducing foreign DNA into the tomato genome (Fig.). The first one (method of agrobacterial or Agrobacterim-mediated transformation) is based on the natural mechanism of infection of plants with a bacterial pathogen of the genus *Agrobacterim* (*A. tumefaciens* or *A. rhizogenes*) and the transfer of foreign DNA into the plant genome mediated by it [35-37]. The second approach (see Fig.) is based on the direct delivery of foreign DNA into the plant cell through the plasmalemma using chemicals (Ca^{2+}, polyethylene glycol, PEG) or physical effects (electrical impulse or high pressure) (the so-called direct methods of tomato genetic transformation). In this case, transgenic plants can be obtained both by the classical method using the method of culture of isolated organs and tissues in vitro, and without it (transformation in planta) (see Fig.). Regardless of the method used to introduce foreign DNA into the tomato genome, the integration process is random [38].

It should be noted that each of the direct methods for introducing a transgene into the tomato genome has both advantages and disadvantages [39], however, all these methods are used much less frequently than agrobacterial transformation. The main reasons are their low efficiency (especially in the case

of chemically mediated transfection, protoplast electroporation and microinjection), the need for specialized equipment ("gene gun", micromanipulator) and highly qualified personnel, as well as the high copy number of tandemly arranged insertions in plant DNA during bioballistic transformation, leading to low expression or silencing of the transgene [39-41]. However, in the last decade, in connection with the development of technologies for site-specific editing of the plant cell genome (including the tomato model) [42] with the participation of chimeric proteins and nucleoproteins created on the basis of bacterial or yeast endonucleases [43-46], methods of direct genetic transformations are being used more and more.

In addition, bioballistic and PEG-mediated transformation of protoplasts remain an essential tool for creating transplastomic tomato plants [34, 47].

In this review article, we systematized the available experimental data on the genetic transformation of tomato by the direct methods listed above, and analyzed various factors that determine the efficiency of the transformation process.

Chemically mediated transfection. The transformation of cells with chemicals that facilitate the transfer of DNA across the membrane was first carried out in the 1970s. In particular, the fundamental possibility of introducing labeled exogenous DNA into the protoplasts of *Ammi visnaga* (L.) Lam. [48] and *Nicotiana tabacum* (L.) [49] using various chemical compounds.

The calcium phosphate method was first used in 1973 for the genetic transformation of human cells with foreign DNA by F.L. Graham and A.J. Van der Eb [50]. The essence of the method is as follows: first, a $CaCl_2$ solution is added to a buffer solution containing DNA, as a result of which the negatively charged phosphate groups of DNA molecules bind to Ca^{2+} ions, and then a phosphate buffer is added to obtain a $Ca_3(PO_4)_2$ precipitate. The resulting DNA-containing solution is added to a suspension culture of isolated protoplasts devoid of a cell wall, which significantly hinders the penetration of macromolecules into the cell. In the first work on the transformation of tomato protoplasts by the described method, its maximum efficiency was 2% [51]. The transgenic nature of six callus aggregates was proved, however, later it was not possible to obtain full-fledged regenerated shoots from them. The authors demonstrated that the choice of plasmid type for genetic transformation has a decisive influence on the efficiency of obtaining transgenic callus tissues.

The use of PEG for DNA transfer across the plasmalemma was first tested for the genetic transformation of protoplasts of two types of tobacco (52). In addition to PEG, the buffer solution for DNA precipitation also contains $MgCl_2$. As a result of PEG-mediated transformation of tomato plastids with the pSSH1 plasmid, transplastomic plants resistant to the selective antibiotic spectinomycin were obtained. The transformation efficiency (TE) value calculated from restriction mapping was 1.5×10^{-6} [53]. S. Ray et al. [54] modified the method of chemically mediated transfection of the tomato plastid genome by a simultaneous presence of PEG and $CaCl_2$ in the buffer solution for DNA precipitation, as well as the addition of an osmotic agent, mannitol, to the solution. As a result, using the pCambia1302 plasmid, the selective and reporter genes (*nptII* and *gfp*, respectively) were integrated into plastid DNA. The authors found that PEG-4000 is more preferable than PEG-6000 due to the greater survival of protoplasts. In addition, a critical factor to maintain the viability of protoplasts was the duration of their incubation in a buffer solution after transformation and before being transferred to a nutrient medium to induce morphogenesis processes. The optimal value was 24 h, while longer incubation negatively affected the viability of protoplasts due to the toxic effect of PEG. The transgenic status of the resulting regenerants was confirmed by polymerase chain reaction

(PCR), as well as fluorescent analysis of the reporter gene expression; The authors did not define TE [54].

Microinjection. The method was developed for the delivery of nucleic acid macromolecules to human and animal cells [55, 56]. In this case, the plasmalemma of the cell is mechanically pierced with a microneedle - a very thin glass pipette with an outer diameter of 1-2 microns, which contains dissolved DNA. Transformation is performed by a specially trained operator under a microscope equipped with a micromanipulator.

The application of this method is significantly complicated by the presence of a strong cell wall and a large vacuole in a plant cell. The cell wall makes it difficult to visualize the nucleus, and its fragments clog the microneedle, so the microinjection method was developed for the transformation of naked protoplasts. Accuracy of microneedle targeting of specific cell compartments is improved by immobilizing protoplasts during microinjection, for example, if cells are placed on agar medium [57] or attached to a glass slide with poly-L-lysine (58). H. Morikawa and Y. Yamada [59] developed a mechanical method for holding protoplasts using additional pipettes; they also used fluorescent labels to visualize the DNA introduced into the cell [59]. The accuracy of micromanipulation is critical, since when the microneedle enters the vacuole, aliet DNA is destroyed by hydrolytic enzymes; in addition, disruption of the integrity of the vacuole can cause cell rupture and death due to the entry of toxic metabolites into the cytoplasm [60].

In the first works on the model of protoplasts isolated from hypocotyls of *Brassica napus* L., the survival of protoplasts after the introduction of alien DNA into them, as well as their subsequent ability to divide and form microcallus with a frequency of up to 70, 65 and 50%, respectively, was estimated. The authors found that the efficiency of these processes is affected by the following factors: genotype, age of the intact explant, the composition of the nutrient medium for cultivating protoplasts, and the pH of the buffer solution [61]. However, transgenic plants were subsequently obtained only for a limited number of crops, including plants of the *Solanaceae* family — petunias [62], tobacco [63], and barley [64]. The experimental work of Japanese researchers on the genetic transformation of intact tomato callus cells by microinjection was one of the first published in 1988. Using the pE2KX plasmid containing the *nptII* gene, which causes resistance to aminoglycoside antibiotics, the authors optimized the parameters that determine TE, the size of the microneedle (the use of a microneedle with an outer diameter of more than 0.3 μm leads to irreversible damage to cell structures and the death of 90-95% of the transformed cells) and the duration of the microinjection procedure. If it exceeds 20 s, irreversible structural and functional disorganization of the nucleus and other membrane organelles occurs). As a result of the selective selection of surviving injected cells on a nutrient medium with the addition of kanamycin, the frequency of callus aggregate formation after 1 month of cultivation was 22%; their transgenic nature was confirmed by PCR analysis for the presence of the *npt II* gene [65]. The TE values, which varied within 15-20% depending on the studied factors, were comparable with those obtained in alfalfa protoplast culture (15-26%) with the introduction of various plasmids (pTiC58, pMON8015, pMON120, pAL4404/pMON120) [66].

Electroporation. The essence of the method lies in the fact that under the influence of an electric pulse, pores with a diameter of about 30 nm are formed in the plasma membrane of the cell, which exist for several minutes, followed by the restoration of the normal state of the membrane. The short-term formation of pores is sufficient for the penetration of large water-soluble

macromolecules, in particular DNA, into the cytoplasm of the cell [67-70]. Transformation is carried out with the help of an electroporator device, which consists of a cuvette, a pair of electrodes, and a pulse generator of a given shape [71]. A suspension of transformable cells and vector constructs with target genes are added to a cuvette with a buffer solution, after which an electrical pulse (usually rectangular or exponential) is passed through the solution [72]. The highest ET is achieved at a field strength of 1-20 kV/cm and a pulse duration of 1-30 ms, however, for each type of cell, the optimal values are selected empirically. The critical values of these physical parameters for the formation of pores and successful transport of DNA through the plasma membrane of a cell are determined by its own membrane potential, as well as by the potential arising under the action of an external electric field [73]. TE in most cases has a random component, since cells in solution are under different conditions and, therefore, acquire different potentials [74]. TE can be increased by adding various components to the electroporation buffer, such as Ca^{2+} or Mg^{2+} ions, PEG to increase membrane permeabilization [75], or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to maintain for the optimal pH value [76].

A significant limitation of the wide application of the electroporation method for plant objects is the presence of a cell wall [77, 78]. The use of a culture of protoplasts devoid of a cell wall significantly reduces ET, since the subsequent production of full-fledged regenerated plants from them is extremely difficult for many species. Obtaining stable transgenic lines of a number of cultures turned out to be possible in the case of adapting the electroporation technique developed for isolated protoplasts for use on intact cells. This approach proved to be effective in obtaining a stably inherited transgene insertion in the maize genome; however, to achieve a positive result, the authors demonstrated the need for partial disruption of the cell wall and increased membrane permeabilization in transformed cells [79].

The production of transgenic tomato plants by electroporation was first reported back in 1989 by two independent groups of researchers [80, 81]. So, C. Bellini et al. [80] introduced alien DNA into the protoplasts of two species of the genus *Solanum* — *S. lycopersicum* L. and wild-growing tomato species *S. peruvianum* L. TE calculated as the ratio of the number of transformed colonies resistant to a selective antibiotic to their total number, varied within 0.3-2.5%. The maximum TE value was achieved when protoplasts were treated with a three-time pulse of 100 μs duration, creating a voltage of 250 V or 300 V. The regenerated plants were successfully obtained from transformed protoplasts [80]. M. Tsukada et al. [81] used single exponential pulses resulting from the rapid discharge of a pre-charged capacitor for the genetic transformation of protoplasts of cultivated tomato cv. Petit Tomato. An electric field strength of 1 kV/cm with a capacitor discharge of 47 μF provided the best results [81].

On the model of protoplasts of the wild species *S. peruvianum* L., we optimized a number of physical and physiological factors affecting the process of introducing foreign DNA, such as the composition of the buffer solution for electroporation (Ca^{2+}- or Mg^{2+}-containing buffer solution with or without PEG), cell concentration in suspension of protoplasts, their pretreatment (heat shock), as well as pulse parameters [75]. The maximum efficiency of the electroporation process was achieved under the following conditions: the use of a Ca^{2+} buffer solution and a heat shock for 5 min at a temperature of 45 °C, followed by the addition of 8% PEG, as well as a rectangular pulse with a duration of 30 μs and an electric field strength of 1.5 kV/cm.

1. Bioballistic method of tomato genetic transformation and factors affecting its efficacy

Transformation method[1] and transgene localization[2]	Explant, genotype	Device (manufacturer)	Parameters of bioballistic transformation			Selective and/or reporter gene[4]	TE, %	Note	References
			distance between the macrocarrier and stop screen, cm	helium pressure, psi	type[3] and microparticle size, μm				
T	Suspension cell culture of tomato variety VFNT Cherry and *Solanum pennellii* Correll.	PDS-1000/He™ (Bio-Rad)	8.5	nd	W, 1.0	*nptII*, *uidA* (*gus*)	1.2 (VFNT Cherry) and 45.0	TE was the ratio of the number of kanamycin-resistant calli to the number of cells in which transient expression of the reporter gene occurred after 48-h shell transformation	[83]
T	Leaves, 5-8 mm unripe fruits of tomato variety VFNT Cherry LA 1221	PDS-1000/He™ (Bio-Rad)	8.5	1500, 1800 (optimum 1800)	Au, 0.4-1.2	*luc ιιu uidA* (*gus)*	nd	Optimyzed bioballistic transformation parameters for transient expression	[89]
SP	Leaves of tomato variety IAC-Santa Clara	PDS-1000/He™ (DuPont)	nd	1100	Au, 0.6	*aadA*	2.5* 1.15**	* The ratio of the number of calli resistant to spectinomycin to the total number of transformed explants ** The ratio of the number of regenerants with PCR-confirmed transgene insertion to the initial number of transformed explants	[28]
SP	Leaves of tomato line IPA-6	PDS-1000/He™ (Bio-Rad) c Hepta Adaptor (Mologen)	nd	nd	Au, 0.6	*aadA*	nd	Production of stably inherited transplastomic plants expressing bacterial lycopene-β-cyclase with increased herbicide resistance and a 4-fold increase in provitamin A content in fruits	[90]
SN	Parts of hypocotyl of tomato variety CastleRock	PDS-1000/He™ (Bio-Rad)	6.0 and 9.0	1350	Au, nd	*bar*, *uidA* (*gus)*	26.5	TE was the ratio of the number of primary transformants with PCR-confirmed status to the total number of regenerants	[91]

Continued Table 1

T	Apical meristem, cotyledons and hypocotyl parts of tomato line IPA-3	PDS-1000/He™ (Bio-Rad)	2.5-10.0 (optimum 7.5)	1100	W, 0.6	*uidA* (*gus)*	from 22.69 to 36.56	TE was the ratio of the number of explants with GUS activity to the total number of transformed explants; the maximum TE value (36.56%) when using parts of hypocotyls	]84]
SN	Parts of hypocotyl of tomato variety CastleRock	PDS-1000/He™ (Bio-Rad)	6.0 and 9.0	1350	Au, 1.0	*hpt*	42.5	ET was the ratio of the number of transgenic regenerants confirmed by PCR analysis to the total number of hygromycin-resistant regenerants	]92]
SP	Parts of hypocotyl with cotyledons (hypocotyledonary) of tomato variety CastleRock	PDS-1000/He™ (Bio-Rad)	6.0 and 9.0	1350	Au, 1.0	*hpt*	52.3	The same as in [92]	]93]
T, SP	Parts of leaves and fruits of tomato variety Ferum	Gene Gun Helios System (Bio-Rad)	nd	210	W, 1.1	*luc* или *uidA* (*gus)*	nd	Study of the activity of the fruit-specific promoter of the *SlPPC2* gene encoding tomato phosphoenolpyruvate carboxylase (EC 4.1.1.31)	]85]
T	Fragments of various parts of fruits of tomato line F-144	Scientz GJ-1000 (Ningbo Scientz Biotechnology Co., Ltd.)	1.0. 3.0 and 6.0 (optimum 6.0)	500, 650 and 1100 optimum 1100)[5]	W, 1.0	*uidA* (*gus)*	nd	The largest number of blue dots in GUS reaction (2456.91/cm^2) at the points where the tomato fruit is attached to peduncle	]86]
SP	Callus tissue derived from leaves of tomato variety Pusa Ruby	PDS-1000/He™ (Bio-Rad)	6.0. 9.0 and 12.0 (optimum 9.0)	1100	nd	*nptII*, *uidA* (*gus*)	75.0	The 9.0 cm distance between the macrocarrier and the stop screen provided the maximum TE value (75%)	]102]
T	Callus tissue derived from mature embryos of tomato variety Ventura	Salyaev's pneumatic gene gun [102]	nd	nd	W, 1.1-1.2	*uidA* (*gus*)	80.0	Transient expression of the *gus* gene was confirmed by histochemical method with X-Gluc[5]	]87]
SP	Callus tissue derived from mature embryos of tomato variety Ventura	Salyaev's pneumatic gene gun [102]	nd	nd	W, 1.1-1.2	nd	nd	Production of transplastomic tomato plants containing the *hpv16 L1* gene encoding the synthesis of the main antigenic protein of the highly oncogenic human papillomavirus type HPV16 L1 which is up to 5300 ng/mg of total soluble protein	]88]

Continued Table 1

SN	Parts of hypocotyl of tomato-variety Rutgers	PDS-1000/He™ (DuPont)	6.0 and 9.0 (optimum 9.0)	1200	Au, 0.73	*nptII*, *uidA* (*gus*)	nd	PCR analysis and the reporter gene expression, confirmed by histochemical staining for β-glucuronidase activity, indicated the production of transgenic plants	[94]
SP	Leveas of tomato variety Micro-tom	PDS-1000/He™ (Bio-Rad)	12.0	1100	Au, 0.6	*aadA*	nd	Production of stable transplastomic plants with induced RNA interference to control insect pests	[95]

N o t e. [1] — stable transformation (S) or transient expression (T); [2] — nuclear (N) or plastid (P) transformation; [3] — microparticles of gold (Au) or tungsten (W); [4] — *nptII* and *hpt*, selective genes for neomycin phosphotransferase II and hygromycin phosphotransferase of *Escherichia coli*, respectively; *aadA*, a selective gene encoding the enzyme aminoglycoside-3'-adenyltransferase; *uidA* (*gus*) and *luc*, reporter genes for β-glucuronidase and luciferase, respectively; [5] — X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide); excess pressure may be created not by helium but by nitrogen in the "gene gun" model; nd — no data.

Bioballistic transformation. This direct method for delivering nucleic acid macromolecules to plant cells was developed by John Sanford at Cornell University (USA) in 1984 [82]. The essence of the method is that DNA molecules are deposited on the surface of carriers — gold or tungsten microparticles [83-95] (Table 1) or nanoparticles [96], which are accelerated to a 300-600 m/s due to the helium pressure. They carry out the bombardment of various explants placed on the target. Since particles with such a high velocity successfully penetrate the cell wall, the bioballistic method is suitable for the transformation of intact cells, which has an important advantage in avoiding the steps of protoplast isolation and subsequent low-efficiency morphogenesis [83].

The development of a device for shelling microparticles (the so-called gene gun) began in 1984, and the idea was subsequently patented by the developers [97]. Subsequently, the original model was developed in modifications: He Biolistics Particle Delivery System (PDS-1000/Hetm; licensed by DuPont, USA); non-commercial variant of Accel™ Particle Gun (98); Particle Inflow Gun [99]; microtargeting device designed for transformation of apical meristems [100] and Helios Gene Gun (Bio-Rad, USA). The latter option does not require a vacuum chamber to hold the target tissue and can be used as a portable device [101].

Bioballistic transformation is the most widely used direct method for introducing foreign DNA into tomato cells, which is used both to study the transient expression of heterologous genes [83-87, 99] and to obtain transgenic plants with a stably inherited nuclear [85, 91-94, 102] or plastid [28, 88, 90, 95] transgene insertion (see Table 1). Most often, the shelling of tomato plant tissues with microparticles is carried out using the PDS-1000/He™ device from DuPont or Bio-Rad [28, 83, 84, 89-93, 94, 95] (see Table 1), significantly less commonly, serial devices from other manufacturers [86] or devices developed independently [103]. The target with transformable isolated cells or explants is installed in a vacuum chamber with a pressure of about 0.1 atm. At the moment of pressure release, the particles are ejected from the gun towards the target. Typically, cells in the center of the target die due to physical damage [104]. The applied model of the installation determines the following set of physical parameters of bioballistic transformation that affect the efficiency of the process: DNA concentration, type of microparticles and their size, varying from 0.4 to 1.2 μm, distance between the particle macrocarrier and the stopping screen (from 6.0 to 9 .0 mm), helium pressure (from 200 to 1800 psi), as well as the multiplicity of shots (single or multiple). These parameters are optimized taking into account the age and physiological characteristics of specific explants, the cells of which are subjected to shelling. Tomato transformation protocols have been developed to obtain stable and transient expression of heterologous genes on the model of suspension [83] and callus [87, 88, 102] cell cultures, apical meristem [84], hypocotyl fragments [84, 91, 92, 94], cotyledons [84], leaves [85, 89, 90, 95] and fruits of various degrees of maturity [85, 86, 89]. ET varies widely (from 1.2 to 80.0%) depending on the type of transformation (stable or transient), genotype characteristics, explant type, and many other physiological and physical factors (see Table 1). It is also important to note the lack of a universal methodology for determining ET, as a result of which the authors propose radically different methods for calculating this indicator (see note in Table 1), the values of which can differ even by an order of magnitude.

In 1995, stable transgenic plants of the tomato and its wild relative *S. pennellii* Correll were obtained for the first time by bioballistic transformation [83]. Suspension culture cells were subjected to shelling, which were subsequently cultivated on a nutrient medium to induce morphogenesis processes with the addition of a selective agent, kanamycin. The study used a yeast chromosome (YAC) vector and three types of plasmids carrying the *uidA* (*gus*) and *nptII* genes. The authors

demonstrated transient expression of GUS in all cases, however, the number of selected kanamycin-resistant calli in the wild species was significantly greater than in the cultivar at a comparable level of transient expression. This fact indicates that stable transformation is genotype-specific, and the reason for this is the genetic determination of various genotypes and somatic tissues to in vitro morphogenesis [10, 22, 105, 106]. In general, the genotypes of representatives of the genus Solanum can be arranged in the following order according to their morphogenetic potential: closely related wild tomato species *S. pimpinellifolium* L., *S. peruvianum* L., and *S. glandulosum* (L.) Morong > *S. lycopersicum* L. [107); model genotypes with no practical value > commercially important F_1 varieties and hybrids [10]; cultivars *S. lycopersicum* L. > hybrids F_1 *S. lycopersicum* L. [108].

D. Ruma et al. [84] performed optimization of the physical parameters of bioballistic transformation during transient expression of the reporter gene uidA (gus) in various tomato explants of the IPA-3 line. The maximum ET (34.12, 36.56 and 22.69%, respectively, for the apical meristem, fragments of hypocotyls and cotyledons) was achieved by double bombardment of the explant with microparticles from a distance of 7.5 cm and a helium pressure of 1100 psi [84]. In addition, the authors studied the biological factors that affect the frequency of transient expression of the reporter gene. For one shot, the optimal amount of plasmid DNA was 1.89 μg per 1125 μg of microparticles. The use of DNA in excessive concentrations reduces ET due to the adhesion of microparticles [109]. Explant preculture reduced ET due to the loss of mechanical strength of cell walls in competent cells. Similar results were observed in the case of pretreatment of tomato explants with 0.3 M mannitol solution [84]. In another study, on the contrary, pretreatment of tomato leaves and fruits with osmotic (12% mannitol) before shelling led to a 30-fold increase in the level of expression of the heterologous luciferase gene [89]. L. Sun et al. [86) performed shelling with tungsten particles of tomato fruits of different maturity with the "gene gun" (Scientz GJ-1000, Scientz, China). The excess pressure was generated not with helium, but with nitrogen. The maximum TE was achieved with a single shelling of explants with microparticles (0.83 μg DNA per shot) from a distance of 6 cm at a nitrogen pressure of 1100 psi [86].

In order to obtain transgenic tomato plants with a stably inherited gene with a high frequency (26.5%), G.A. Abu-El-Heba et al. [91] proposed other parameters of bioballistic transformation, i.e., 1 μg aliqute of DNA, double shelling with gold microparticles at a 6 and 9 cm distance between the macrocarrier and the stop screen for the first and second shots, respectively; helium pressure of 1350 psi [91]. These values have been successfully applied in a number of research works for shelling fragments of hypocotyls, as well as other types of tomato plant tissue [92, 93, 102], taking into account the individual characteristics of the composition of the nutrient medium for the induction of morphogenesis.

Bioballistic transformation is the main method for creating transplastomic tomato plants, which were first obtained at the beginning of the 21st century based on the commercial variety IAC-Santa Clara [28]. The peculiarity of such plants is the inheritance of the transgene in generations along the maternal line. The selection of homoplastomic cells and plants is carried out on a selective nutrient medium with the addition of spectinomycin at concentrations of 300-500 mg/l [28, 90, 95] with a photoperiod of 16/8 h (day/night) and reduced illumination (15 or 25 μE) [28, 95]. Expression of the target gene was observed in different types of plastids: leaf chloroplasts, fruit and flower chromoplasts [28].

Transformation in planta. Transformation in planta makes it possible to obtain transgenic plants, bypassing the long and laborious stage of cultivating isolated organs and tissues in vitro. The main advantages of in planta transformation

are the relative simplicity and speed, since these methods do not involve equipping the laboratory with specialized biotechnological equipment due to the absence of an in vitro culture stage, thereby eliminating somaclonal variability [110, 111]. Pollen can be considered the best material for in planta transformation: it can be obtained in large quantities, it contains haploid cells, and practically any of the methods previously tested for the transformation of protoplasts and intact cells can be used for transformation - electroporation, microinjection, DNA packaging into liposomes, bioballistic method, as well as *Agrobacterium*-mediated transformation [112].

During the germination of transformed pollen in planta, exogenous DNA enters the embryo sac, followed by the possible formation of a transgenic diploid embryo [113, 114]. This method, called the pollen tube pathway, was developed and first applied in 1974 on plants of the *Solanaceae* family *Petunia hybrida* Vilm. [115, 116] and *Nicotiana glauca* Graham [115], and also on barley plants [117]. The pollen tube method was effective to produce transgenic plants of cotton [118], barley [119], rye [120] and other crops, including various tomato genotypes [121-126].

The first work showing the possibility of genetic transformation of tomato using the pollen tube method was published in 1989 [121]. This study was continued by other works, in particular, transgenic hybrids of *S. lycopersicum* L. cv. Fakel and *Solanum penelii* Cor. [125]. In this case, the genetic transformation of pollen and the process of pollination were carried out simultaneously. Freshly dried pollen was placed in a nutrient medium supplemented with 15% sucrose, 0.018% boric acid, and 0.04% $Ca(NO_3)_2$, to which a solution of plasmid DNA was then added. Immediately after the addition of DNA, pollination was carried out with the transformed pollen of previously castrated and isolated flowers. ET calculated as the ratio of the number of kanamycin-resistant seedlings derived from immature embryos to the total number of transformed embryos was 2.2%. The ratio of the number of kanamycin-resistant and kanamycin-sensitive seedlings obtained from seeds was 3:1, which proves their transgenic status and indicates a single-locus model of transgene inheritance. The relatively low efficiency of transformation by the pollen tube pathway method is largely associated with the nuclease activity of germinating pollen, which degrades most of the exogenous DNA [112].

Another modification of the pollen tube method, based on an earlier work by N.V. Turbine et al. [117], involves the introduction of a DNA-containing solution into the ovule of an already fertilized flower. After pollination, the stigma is removed and a solution containing the target DNA sequence is injected with a fine needle [114]. This method has also been used to obtain transgenic tomato plants [122-124]. R. Wang et al. [124] studied the effect of genotype, plasmid DNA concentration, and components of the buffer solution for injection on the ET of tomato varieties Zhongshu 6, Liaoyuanduoli, and Jinguan 9 using a vector construct containing the selective *bar* gene, which causes resistance to phosphinothricin, as well as the yellow fluorescent reporter protein gene *yfp*. The authors established the absence of significant differences in ET between the studied varieties. At the same time, the highest yield of transgenic plants was provided by the addition of 600 ng/μl of plasmid DNA, as well as 5% sucrose, and 0.05% Silvet-L-77 surfactant to the solution for injection [124]. For the described modification of the pollen tube pathway method, the time interval from pollination to transformation and the preservation of the ovule upon removal of the pistil are critically important [123, 127]. It has been established that the introduction of foreign DNA should be carried out 24 hours after pollination; shortening this period reduces ET

[123]. As regards the safety of the ovule, various methods are used to break the integrity of the pistil before the introduction of alien DNA, for example, the complete removal of the pistil before injection [123] or the removal of only part of it (stigma with part of the style) [122]. The contribution of the latter factor is difficult to assess due to the low efficiency of the method as a whole. Depending on the above factors, ET ranges from 0.2% [124] to 1.4% [128].

A variation of the pollen tube pathway method is the agroinfiltration of foreign DNA into various plant generative organs [129, 130]. The effectiveness of various modifications of this method reaches an average of 3-4%. The procedure is actively used to obtain transgenic *Arabidopsis* plants and other cultures of the *Brassicaceae* family [131]. This method can be considered as a hybrid between pollen tube pathway and agrobacterial transformation. In tomato, the discussed method was successfully applied to introduce the *LFY* and *GUS* genes [132].

A highly efficient protocol for electroporation of mature tomato seeds was proposed by Z. Hilioti et al. [133] for the delivery of genome editing systems. The sterilized seeds were incubated for 12 h in the dark at a low temperature (10 °C) in a solution supplemented with 5% sucrose, 3% H_3BO_3, and 1.3 mM $Ca(NO_3)_2$. The swollen seeds were subjected to vacuumnation in a buffer solution (80 mM KCl, 5 mM $CaCl_2$, 10 mM HEPES, and 0.5 M mannitol) and kept on ice for 1 h. Electroporation of pretreated seeds was carried out in a buffer solution with the addition of plasmid DNA (50 μg per 200 μl of buffer) in the mode of three pulses of 4 ms each at a field strength of 6.25 kV/cm. The authors demonstrated that 65% of tomato plants grown from transfected seeds contained various mutations of the target gene, which indicates successful transformation of the embryos with the vector encoding ZFN nucleases and their subsequent expression in electroporated embryos [133].

Use of direct methods of tomato genetic transformation for the delivery of genome editing systems. Over the past 5 years, there has been a boom in research work devoted to the targeted introduction of changes in the tomato genome using genomic editing systems ZFNs (zinc-finger nucleases), TALEN (transcription activator-like effector nucleases) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats) [43, 134]. Depending on the system used, various variants of genomic editing can be carried out - through knockout mutations resulting from insertions or deletions of a part of nucleotides in the editing site due to non-homologous combination of repaired ends or through homologous recombination; knock-in gene editing by introducing exogenous oligonucleotides or longer DNA fragments after the introduction of breaks and subsequent homologous recombination; the introduction of single nucleotide substitutions due to the deamination of nitrogenous bases (CBE, cytosine base editors, ABE, adenine base editors). The mechanisms of action of the genomic editing systems ZFNs, TALEN, CRISPR/Cas, CBE, and ABE are considered in detail in a number of review articles [135-139].

To date, various methods of editing the tomato genome are widely used in both fundamental [140] and pronounced applied studies, for example, in order to increase plant resistance to abiotic [141, 142] and biotic [143-146] stressors of various nature, increase keeping quality of fruits and improving their quality [147-152], as well as to accelerate the process of domestication of wild *Solanum* species [153, 154]. Delivery of genome editing systems is carried out mainly through agrobacterial transformation with vector constructs expressing these systems. Direct methods for introducing alien DNA to obtain genome-edited tomato plants also occupy a significant place in this list (Table 2).

2. Direct methods for delivering genome editing systems to tomato cells

Editing system	Delivery method	Genotype	Gene	Trait	Editing efficacy, %	References
TALEN	PEG-Ca^{2+}-mediated transformation	Micro-Tom	*ANT1*	Anthocyanin biosynthesis; purple color	7.28	[155]
CRISPR/Cas9	PEG-Ca^{2+}-mediated transformation	Micro-Tom	*ANT1*	Anthocyanin biosynthesis; фиолетовой окраски	2.75-8.8	[155]
	PEG-mediated transformation	Micro-Tom	*CCD7* *CCD8*	Strigolactone biosynthesis	30 90	[156]
	PEG-Ca^{2+}-mediated transformation	*Solanum peruvianum* L.	*SpRDR6* *SpSGS3* *SpPR-1* *SpMlo1* *SpProSys*	Resistance to plant pfthogenes	13.2 8.3 13.9 63.6 45.8	[157]
ZFNs	Electroporation of mature seed embryos	Heinz 1706	*LIL4*	Transcription factor to control the development of cotyledon, true leaves, flowering and fruit ripening	65	[133]
Prime editing	Биобаллистика	Micro-Tom	*NanoLucM*	Back mutation that restores NanoLuc luciferase activity	0.26[a]	[158]

N o t e. [a] — transient expression efficacy.

Thus, using the TALEN and CRISPR/Cas9 systems and PEG-Ca^{2+}-mediated transformation of tomato protoplasts of the Micro-Tom model variety, the CaMV35S promoter was accurately inserted between the promoter and transcribed regions of the *ANT1* gene, which controls anthocyanin biosynthesis. The constitutive promoter-mediated overexpression of the *ANT1* gene contributed to the ectopic accumulation of the pigment in plant tissues. Depending on the type of genetic construct and genomic editing system, ET varied from 2.75 to 8.8%. More than two thirds of the transgene insertions were accurate and stably inherited in the T_1 seed generation according to Mendelian segregation [155].

The PEG-mediated delivery of the CRISPR/Cas9 system into tomato protoplasts made it possible to edit two genes for carotenoid-cleaving dioxygenases (*CCD7* and *CCD8*) involved in strigolactone biosynthesis. In this case, multiplexing was used, that is, the simultaneous targeting of several guide RNAs to both genes. For transfection of protoplasts, the authors used a multicomponent buffer solution containing 12.5% PEG-4000. As a result, out of 50 randomly selected callus aggregates formed in the protoplast culture, one (2%) and five (10%) had monoallelic mutations (in the *CCD7* and *CCD8* genes, respectively), while 13 (26%) and 36 (72%)) calluses contained biallelic mutations of the same genes (homozygous or heterozygous state) [156].

Y.C. Lin et al. [157] obtained independent regenerants with point mutations introduced by CRISPR/Cas9 into the following genes, the expression products of which confer resistance to phytopathogens, from the protoplast culture of *S. peruvianum*: *SpRDR6* (RNA-dependent RNA polymerase 6), *SpSGS3* (suppressor gene silencing 3) (two key RNA silencing genes mediating protection against tomato yellow leaf curl virus), *SpPR-1* (pathogenesis-related protein-1), *SpProSys* (prosystemin), and *SpMlo1* (one of the gene family of the O locus causing resistance to snow mold). As a result, the frequency of editing, depending on the gene, varied between 8.3 to 63.6% [157].

The use of mature embryos of tomato cv. Heinz 1706 seeds as explants for electroporation with plasmids with the ZFN sequence made it possible to obtain independent lines with the edited *LIL4* gene encoding the transcription factor LEAFY COTYLEDON1-LIKE4, which controls the development of cotyledon and true leaves, as well as flowering and fruiting. It was found that 65% of tomato plants grown from transfected seeds contained various mutations of the target gene

[133].

The delivery of genomic editing systems is also carried out using the bioballistic method. An example is the editing of the Micro-Tom tomato genome using the prime editing system based on the Cas9 nuclease, cross-linked with mouse leukemia virus (MuLV) reverse transcriptase and containing a new guide RNA variant, prime editing guide RNA (pegRNA), which not only directs the nuclease to the desired site of DNA, but also serves as a template encoding changes [158]. The efficiency of genome editing using this technology was 0.26% (the indicator was estimated by means of restored transient expression of luciferase 7 days after microparticle bombardment).

Thus, this review article discusses various direct methods (chemically mediated transformation, microinjection, electroporation, bioballistic transformation) of introducing foreign DNA to obtain transgenic tomato plants, as well as factors of various nature (physical, genetic, and physiological) that affect the efficiency of this process. The first three methods are characterized by low efficiency, and therefore have not been widely used. The most common direct method of tomato genetic transformation is the bioballistic method, which produces plants with a stably inherited transgene insertion in both the nuclear and plastid genomes with varying efficiency. It also should be noted that a direct comparison of transformation efficiency (TE) is not always possible, since different authors use different methods for its determination. In addition, in a number of research papers, no TE occurred, since, apparently, the resulting transgenic tomato plants were single or TE determination was not a goal. It should be noted that with the wide development of various genome editing systems, direct methods of tomato genetic transformation are used more and more often, especially with protoplast culture.

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