Chapter 10

Science Behind Cotton Transformation

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Abstract

The introduction of foreign genes into plant has made possible to bring out desired traits into crop of our own interest. With the advancement in cell biology, regeneration of plants from single cell and advent of different procedures for gene transformation to the plants have opened new avenues for the efficient and applicable implementation of biotechnology for the modifications of desired crop characteristics. Identifications and isolation of different genes for various traits from different organisms have made possible to get the crop plants with modified characters. Over time improvement has been made in transformation technology depending upon the crop of interest. The efficiency of plant transformation has been increased with advances in plant transformation vectors and methodologies, which resulted in the improvement of crops. A detailed discussion on advanced techniques for genetic modification of plants with their handy use and limitation has been focused in this chapter.

Keywords: *Agrobacterium*, biolistic gun, infiltration, microinjection, transformation

1. Introduction

Cell theory of Schleiden established the framework of modern plant biotechnology [1], and Schwann's cell theory [2] was based upon phenomenon that cell is the basic unit of living organisms.

The idea of totipotency has been originated from the concept of cell theory, which later on laid the foundation of plant biotechnology. Hernalsteens [3] further provided the evidence and forecasted the production of somatic embryos from vegetative cells.

Among the key discoveries of plant biotechnology, gene transformation in crop plants and regeneration of plants from callus are the most significant achievements. In 1980s, chimeric genes were produced, which resulted in further expansion of genetic modification technology of the plants [1]. It led to the development of new transformation vectors [2], which ultimately changed the ways of DNA delivery systems [3]. Genetic transformation of crop plant can be achieved by a number of ways like *Agrobacterium* Ti plasmid vectors, microinjection, microprojectile bombardment, electroporation of protoplasts, and/or chemical (PEG) treatment of protoplasts. All of these methods have certain merits over each other with some demerits, but transformation using *Agrobacterium* and microprojectile bombardment is currently the most extensively used methods [4]. Due to regeneration ability of tobacco, as done by Skoog and Miller [5] in 1957, tobacco is the first and model plant to be used for genetic transformation. It provided the basis for tissue culture [6]. The ongoing technological expansions in rice, barley, wheat, and cotton may prove to be a hindrance in commercial release of novel GM cereals. Below, a concise narration of all methodologies used in transformation has been presented.

2. Plant regeneration

A number of attempts were made during 1950 to provide clear understanding of the phenomenon of totipotency but it was 1954 when Muir et al. [7] demonstrated the possible aspects of culturing of single plant cell. The cell divisions obtained by him from callus of tobacco placed on a small piece of filter paper provided the basis for totipotency. Davidonis and Hamilton further confirmed the results by obtaining similar results from single cell and group of cells suspended in the agar medium. It was the extension of these experiments when Jones et al. compared with many other crops, reported that it is more difficult to obtain somatic embryogenesis and plant regeneration from cotton except Coker 312. Davidonis and Hamilton [8] first described that plant regeneration from 2-year-old callus of *Gossypium hirsutum* L. [9] was able to grow single isolated cell in microculture chamber providing devised nutrient medium. For the survival and growth of isolated cells, the role of nurse or nutrition medium becomes evident from these studies.

Rao et al. [12] in 2009 were the first who provide the clear evidence of totipotency and its use during regeneration from callus of plants when they were able to regenerate isolated single cells into flowering plants of tobacco, cultured in microchambers, without the aid of nurse cells or conditioned media. Verdiel et al. [9] in 2007 demonstrated that stem cells in meristematic regions are pluripotent and are dependent, whereas those that are present in embryogenic regions are totipotent and hence are independent.

3. Embryo formation

Improvements of many plants, such as cereals, soybean, cotton, canola, cassava, and woody tree species, are dependent on the development of somatic embryos; therefore, the formation of competent embryogenic cultures is imperative for the success of plant biotechnology. Somatic

embryogenesis in cotton species has been reported to be the most difficult to regenerate [10, 11]. Regeneration in cotton has not been achieved rapidly, with the report of regeneration of *G. hirsutum* [12]. Plants obtained from embryogenic cultures are free from any variations phenotypically or genotypically because they are formed from single cells and during the process of embryogenesis normal cells are selected rigorously [13]. Nowadays, embryogenic cultures of extensive range of plant species can be grown on synthetic media due to ever-increasing information about the physiological aspects and genetic regulation of zygotic as well as somatic embryogenesis [14]. In most cases, embryogenic cultures are initiated by using 2,4-dichlorophenoxyacetic acid (2,4-D), and when such plant cell cultures are relocated to media that contains no or very low quantity of 2,4-D they develop into somatic embryos.

4. Binary vectors

In 1984, Braybrook [14] constructed the first binary vector pBIN19, since then efforts are being made to modify binary vectors in order to amplify their efficacy and transformation effectiveness. Japonica rice was successfully transformed using *Agrobacterium* bearing the super binary vector pTOK233 constructed by taking *virB*, *virG,* and *virC* genes from pTiBo542 and cloning them into pGA472 [15]. Afterwards, maize, javanica rice, indica rice [16], Sorghum, and *Allium cepa* [17] transformation was also performed by the same method. The misconception that monocots cannot be transformed by *Agrobacterium*-mediated transformation has been cleared now. Lately, mung bean has been transformed using *Agrobacterium* bearing pTOK233 binary vector [18].

Complete sequencing of pBIN19 has been performed [19]. A modified version pBIN20 has more single restriction sites within the multiple cloning site (MCS) [20]. A newly developed types of pPZP vectors are small and are steady in *Agrobacterium* cells [21]. pCAMBIA series of vectors are extensively used for the transformation of rice. These have kanamycin resistance (neomycin phosphotransferase II; *NPTII)* or bialaphos herbicide resistance (*BAR*) genes as selection markers and beta-glucuronidase *(GUS)* or green florescence protein (*GFP*) as reporter genes and were constructed by using the backbone of pPZP vector.

Plant expression vectors of pRT100 series with a polyA signal under CaMV 35S promoter were developed by Töpfer et al. [22]. Making single-restriction sites available in the expression cassette holding the target gene is usually a very difficult task. Hou and Guo [23] constructed a set of pART7 and pART27 plasmids to deal with this problem. A MCS is present between CaMV35S promoter and OCS polyA signal of the shuttle plasmid pART7. NofI sites (an infrequent 8 bp recognition site) are present on both sides of the expression cassette. In pART27, the coding sequence of a target gene has been cloned in its MCS. NotI is used to cut the expression cassette, which is then cloned in the Not I site of pART27, a binary vector. In pART27 vector, *NPTII* gene is positioned next to the left border to make sure that the selection marker is transferred in the last in the plants. Zapata et al. [24] reported the use of gramineous expression vectors pGU4AGBar and pGBIU4AGBar [23]. Twenty transformants of winter wheat showed protein expression after transforming the snowdrop lectin (*Galanthus nivalis agglutinin*; *GNA)* gene in them through the pollen tube pathway. This method confirms that

whole T-DNA containing the target gene would be present in the transformed plants having kanamycin selection.

5. Methods involved in transformation

Transformation methods can be divided in two main categories: (1) direct and (2) indirect transformations, which are detailed in below sections.

5.1. Indirect transformation

In these methods, plants are transformed using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* to introduce the plasmid construct carrying the target gene into the target cell.

5.2. Direct transformation

In direct methods of transformation, bacterial cells are not used. The most frequently used direct methods include microprojectile bombardment or protoplast transformation. Problems with plant regeneration low transient expression of transgenes arise as a result of protoplast transformation (mostly in monocots).

The chemical substances used to disintegrate the cell walls and electrical field protoplasts lose their viability and ability to divide. In cotton, some attempts have been made to transform cells directly in the shoot apex either through the gene gun or *Agrobacterium* [24], but these methods suffer from extremely low transformation efficiencies. Others have used particle bombardment of suspension cultures of cotton. This method suffers from the fact that additional time is required for establishment of suspension cultures [25]. The most distinguishing limiting factor of using the gene gun is that it causes the presence of multiple copies of the target genes, which may lead to alteration or concealing of the expression. In addition, gene gun is a very expensive method. The usage of in vitro cultures for plant transformation may give rise to few somaclonal changes. Due to this, they cannot be used in further studies, and analysis of the transformants becomes quite complex. As a result of the limitations mentioned, many new transformation methods are being introduced as substitutes. Most commonly mentioned methods out of them are (a) silicon carbide fiber-mediated transformation; (b) microinjection; (c) infiltration; (d) electrophoresis of embryos; (e) transformation through the pollen tube pathway; (f) electroporation of cells and tissues; and (g) liposome-mediated transformation.

6. Overview of transformation techniques

6.1. Protoplasts and somatic hybridization

In 1970 and 1971, two major advances were made, which proved the beneficial role of protoplasts in the enhancement of plants: (i) somatic hybrid cells and novel hybrid plants

are developed by inducing fusion of protoplasts of different species having no taxonomic relationships between them [26], and (ii) use of cultured protoplasts in regeneration of plants [27].

Geerts et al. [28] initiated the micropod culture and Schryer et al. [29] improved it [29]. In grain legumes, protoplast fusion is not well studied [30], but today, a variety of plants can be regenerated from protoplasts. Likewise, vast ranges of somatic hybrids are developed among related as well as unrelated plant species. However, functional hybrids have been developed in case of small number of plants such as Citrus, Solanum, and Brassica. A significant role has been played by protoplasts in the successful genetic transformation of variety of plants [31] including cereal crops [32]. Limitation of protoplast transformation method is given below; Protoplast transformation method consists of variable frequencies of gene transfer in both cases within or between experiments. Therefore, the results of this transformation method are not accurately assessed. This transformation method is recommended only to those plants species which have quality of callus regeneration [33].

6.2. Direct gene transformation through imbibitions

Desiccated plant tissues can uptake foreign DNA through the process of imbibition [34]. Numerous asserts and disproof can be found in literature regarding this method. During the process of desiccation, various physiological and substantial alterations take place such as bursting of the cell wall, leakiness and changes in structure of the cell membrane, quick expansion of the cell, and development of a huge water flow among the peripheral solution and the dehydrated tissue. Uptake and transitory expression, by the cereal and legume seed embryos, of the DNA plasmid bearing the *NPTII* gene through the process of imbibition were confirmed by Töpfer et al. [22].

Permeability of the membrane is a key factor in this process. This was proved by an increase in uptake and expression of the DNA as a result of using 20% DMSO during the process. Successive studies have led to 70% transitory *GUS* expression in dehydrated somatic embryos of alfalfa [35]. Yoo and Jung have also stated a stability of transformation in rice through the process of imbibition. From 30% to 50% incidence of transient expression of *GUSA* and *NPT* genes was observed using vectors, where insertions were driven by CaMV35S promoter. Meristematic regions of the roots and leaf vascular bundles are the major locations for the expression of *GUSA* gene. Different PCR techniques and southern blot analysis of genome showed the uptake, incorporation, and expression of the *HPT* gene in rice. *Hygromycin phosphotransferase* (*HPT*) DNA was not found as a plasmid but rather it was present in an incorporated form in the genome of rice [36]. This method is the easiest of all the direct gene transformation techniques because the formation of target plant tissues is quite uncomplicated and no professional equipment is needed to perform it. Few drawbacks of this transformation method are given below; this method cannot be applied to very specific organs or tissues such as new pollinated flowers or hydrating embryos.

6.3. *Agrobacterium*-mediated transformation

A. tumefaciens causes the crown gal disease in plants. Pett et al. [37] in 1958 performed an experiment and proposed that tumor-inducing property of this soil bacterium is responsible

for transformation in plants. Bacteria-free tumor cells contain large number of opines, which is a new class of metabolites.

Chilton et al. [39] introduced the idea of virulent strain of *Agrobacterium*, which leads to the discovery of mega plasmid. The plasmid were called tumor-inducing plasmid and only a small portion of that plasmid known as T-border is considered responsible for tumor induction [38] and was found to be present in the nuclear DNA of the tumor-inducing cells [39].

Ti plasmid is an integrated part of the plant genome during tumor formation (transformation), suggesting that the plasmid could be used as a vector to transfer other genes. It was reported that various methods were tested to insert genes into the Ti plasmid. Transformed crown-gall tumor tissues, which were grown on hormone-free media, only formed highly aberrant shoots in culture [40]. This was related to the presence of genes controlling the expression of auxin and cytokinin synthesis. Deletion of these genes resulted in the production of transformed tissues that required media supplemented with auxin and cytokinin for continued growth and regeneration of normal shoots and plants. Nadolska-Orczyk et al. [43] showed that efficient *Agrobacterium*-mediated transformation of *Metrosideros polymorpha* provide molecular techniques to facilitate comparative genomics. It was reported that above-discussed facts led to the use of Ti plasmid of *Agrobacterium* as a vector for plant transformation and kanamycin resistance genes as selection marker for transformed cells, and then transformed plants are regenerated [41] (**Figures 1** and **2**). *Agrobacterium*-mediated transformation followed by somatic embryogenesis remains the method of choice for most cotton transformation. However, regeneration aspect of the transformation process remains more difficult and choices are limited in case of cotton, one of the most difficult crop for transformation [42].

As *Agrobacterium* is highly attracted to phenolics, this method of transformation is not preferable for monocots due to production of phenolics, whereas it can be used for dicots. However, recently, *Agrobacterium*-mediated transformation has been optimized and becomes the best method for transformation of monocots [43].

6.4. Biolistic transformation

In early 1980s, direct DNA delivery methods for protoplast were developed [44], especially for the economically important cereal crops, which were not subjected to the umbrella *Agrobacterium*-mediated transformation [45]. In this procedure, osmotic or electric shocks are applied to the protoplasts suspended in solutions containing DNA. After that planting on selection media is performed. Transformation based on embryogenic cell suspension cultures produced the first transgenic cereals [13]. The use of protoplasts for genetic transformation became less striking once it was shown that monocots could be transformed by co-cultivation of embryonic tissues and supervirulent strains of *Agrobacterium* in the presence of acetosyringone [15]. In case of Ricinus (castor oil plant), transformation through gene gun is dependent on many factors such as helium pressure, target distance, osmoticum, microcarrier type and size, DNA quantity, explant type, and number of bombardments had significant influence on transformation efficiency (**Figure 2**). Recommended distance is 6.0 cm, helium pressure of 1100 psi, 0.6-μm gold microcarriers, furthermore single bombardment has shown

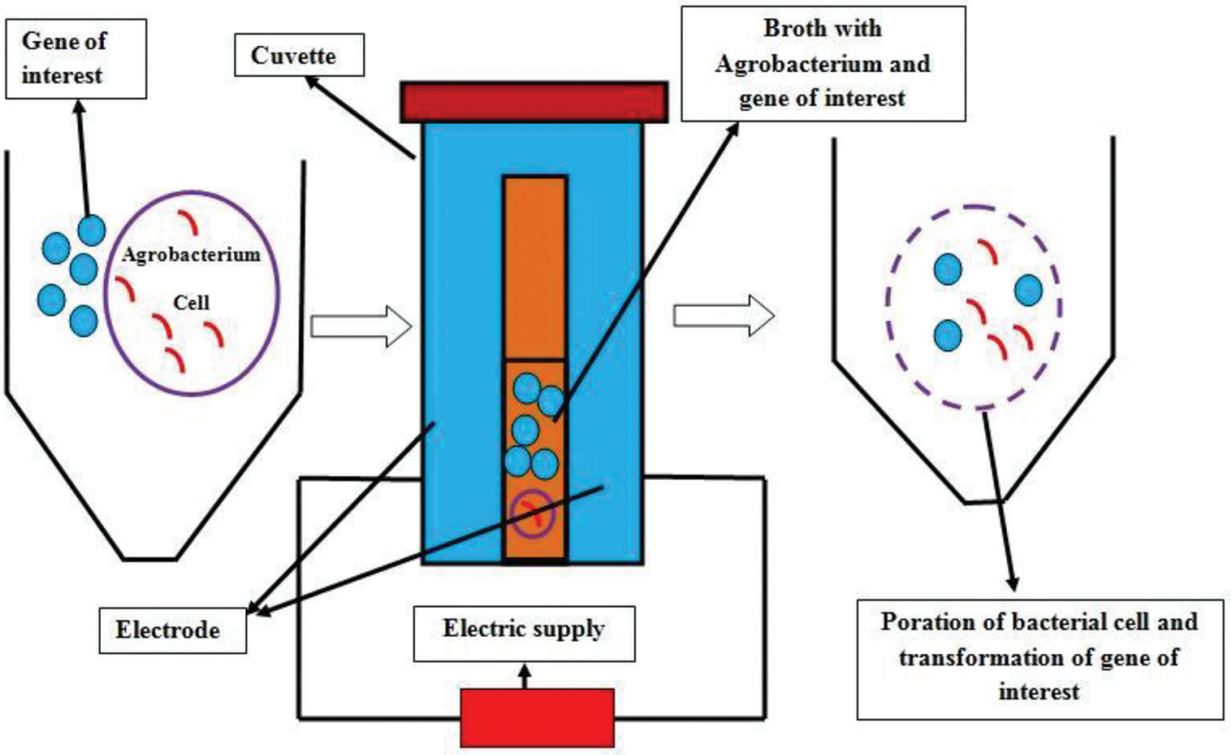

Figure 1. Basic concept of gene transformation through *Agrobacterium*.

positive results [46]. Finer and McMullen [47] demonstrated the successful recovery of transgenic cotton plants by biolistic bombardment of embryogenic suspension cultures and reported a transient to stable conversion frequency of about 0.7%. Biolistic method is not recommended for the crops, which cannot be tissue cultured.

6.5. Biolistic with *Agrobacterium*

Limitations of *Agrobacterium*-mediated transformation and difficulties involved in isolation and maintaining of embryogenic cultures have led to the discovery of universal transformation method redundant [48]. However, not a single transformation technique had proved satisfactory to be universal; therefore, a novel transformation technique for transformation was developed by Sanford et al. [49]. DNA-coated gold or tungsten particles are bombarded with high velocity to the intact cells or tissues. Combination of *Agrobacterium* and biolistics methods of transformation is most widely used method. However, choice depends on the individual researcher or plant tissue/sample [50]. **Figure 2** explains the schematic overview of biolistic gene transformation in plants.

6.6. Chemical method

To enhance the uptake of DNA, a combination of polybrene-spermidine treatment is used to obtain non-chimeric transgenic cotton plants. Polybrene-spermidine combination treatment for plant genetic transformation has the advantage because it is less toxic than the other chemicals; furthermore, it also protects condensation effect, DNA shearing, and integration of the plasmid with host genome [35]. To deliver plasmid DNA into cotton suspension culture obtained from

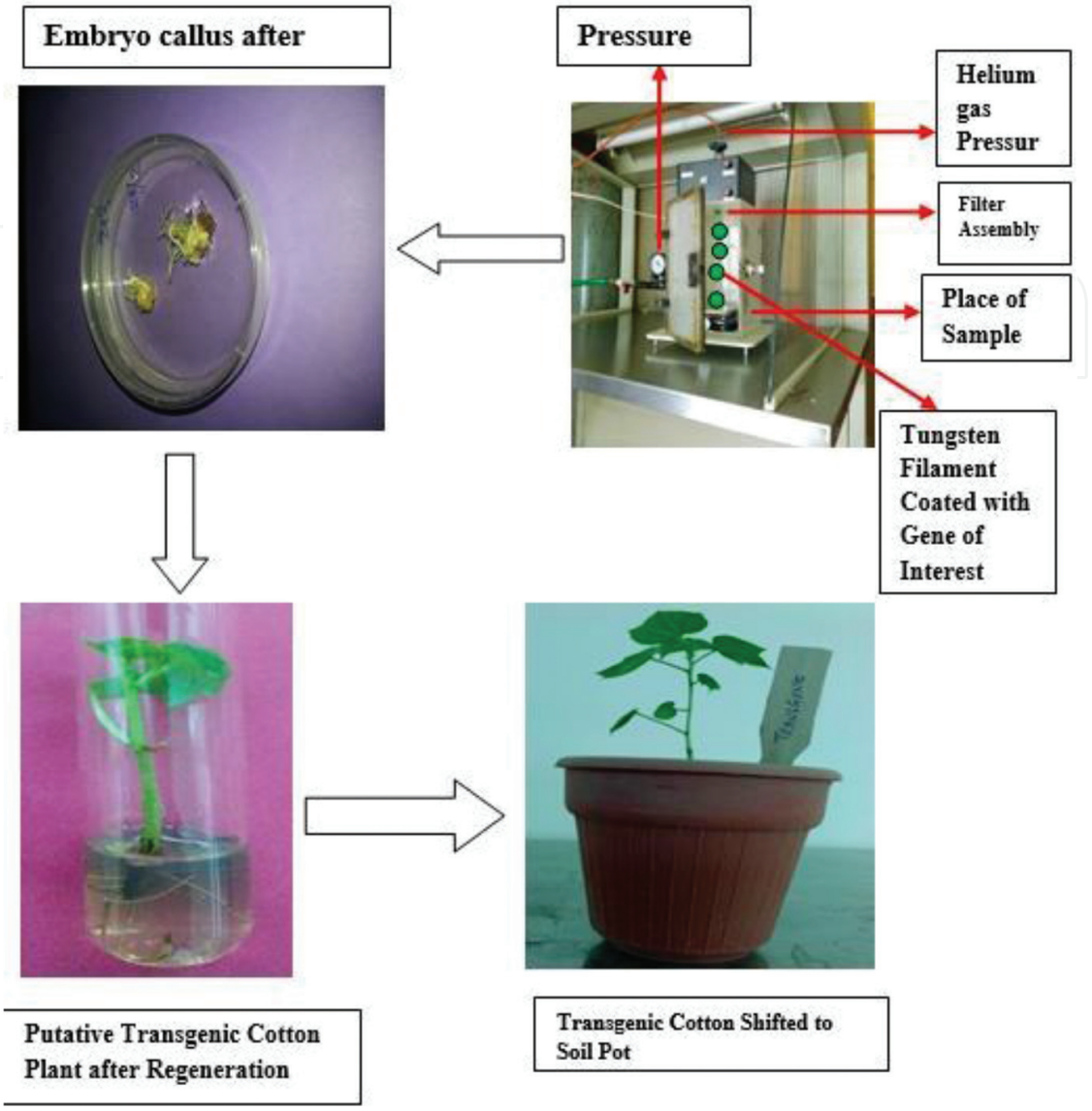

Figure 2. Schematic overview of gene transformation through gene gun.

cotyledon-induced callus, polybrene and/or spermidine treatments were used. Researchers have also regenerated and analyzed the cotton plants containing *HPT* genes as a selectable marker [51]. Major limitation in chemical transformation technique is its low efficiency when compared to other transformation techniques.

6.7. Microinjection

Microinjection technique is based on introducing DNA in the cells using injection pipette of microcapillary glass [51]. This operation requires a micromanipulator. In this case, cells are immobilized by holding glass and gentle suction. Both pipettes are filled with mineral oil.

Microinjection is mostly used for animal cells, while with plants, a cell wall causes hindrance for transformation by microinjection as it works as barrier for microglass tools. Using

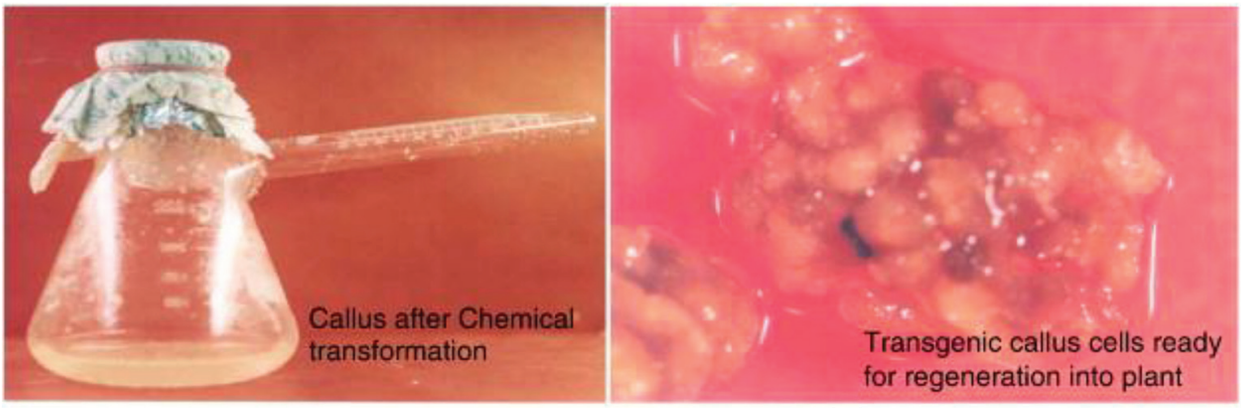

Figure 3. Schematic overview of gene transformation through chemical [12].

microinjection technique for protoplast, there is a risk of toxic compounds to be released, which may cause sudden death of the protoplast. It is also possible to remove vacuoles before microinjection but regeneration and division may be decreased [52].

Protoplast microinjection involves different methods for immobilization, in which instead of using sucking poly-L-lysine is coated to the protoplasts. One of the major advantages of the microinjection is that it not only allows the transformation of the DNA plasmid but also the whole chromosome [53]. This technique is being used for the cellular mechanism and functions of the plant cells and to study the physiology of the plastids especially for tobacco [54] (**Figure 3**). Major limitation of microinjection method involves the use of expensive micromanipulator and it is a time-consuming procedure. Furthermore, frequency of transformation is very low and dependent on the species, i.e., proved to be successful in tobacco [55], Petunia [53], Rape [56], and Barley [57].

6.8. The pollen tube pathway method

Transformation by pollen tube pathway has got great intention in molecular breeding [58]. After pollination, a foreign DNA/plasmid is applied to the styles. To reach ovule, DNA uses the pollen tube pathway. This method of transformation was first used by Luo et al. [60] in rice [59]. In case of rice, a high frequency of transgenic plants was obtained, and this method was then applied to the other commercially important crops, such as wheat [60], soybean [61], *Petunia hybrid* [62], and watermelon [63]. At the premeiotic stage, bacterial inoculum can be applied to the inflorescence without removing the stigma. Pena et al. [65] used this protocol for transformation in rye. Limitation of pollen tube pathway method reported by Shou et al. [66] collected pollens from the genetically engineered plants and reported 10-fold less efficiency in soybean. It was concluded that pollen tube pathway is not reproducible in case of cotton and soybean (**Figure 4**).

6.9. Liposomes

Direct transformation of the foreign DNA into the plant cells using liposomes was Employed in the 1980s. Liposomes are phospholipids with spherical shape, carry nucleic acid, and

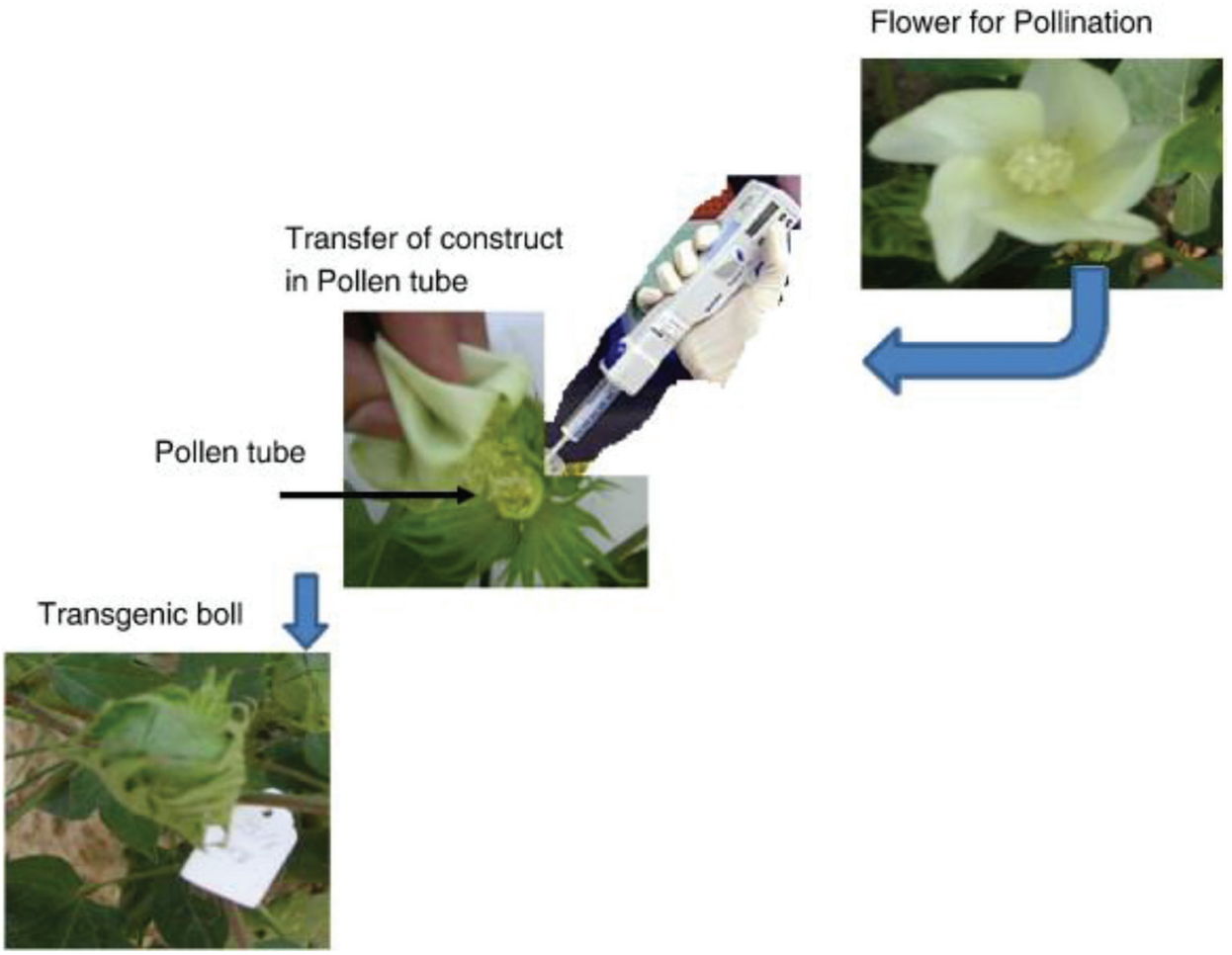

Figure 4. Schematic overview of gene transformation through pollen tube [12].

internally aqueous. Liposomes were put in a nutshell with the DNA fragments to get attached to the cell membranes. Thus, in this way DNA enters the cell and then to the nucleus. For the transfer of the bacterial, plant, and animal genes, lipofection has been a very competent technique. Lipofection takes place by fusion through membrane, and it has improved transformation efficiency because the genetic material used for lipofection is not naked as used in conventional techniques [64, 65]. In spite of cheap and less equipment demanding technique, liposome transformation is not so common. Major limitations in this technique are its low efficiency and being so hectic. Therefore, success story for liposome-mediated transformation have been published so far only for tobacco [66] and wheat [67].

6.10. Shoot apex method of transformation

Transformation by shoot apex method is a rapid method of transformation in cotton, and it is a genotype independent method. In this method, shoots are isolated from the plant and subjected to a virulent strain of *A. tumefaciens*, an antibiotic selection is also applied, and shoots are regenerated *in vitro*. In shoots, dedifferentiation does not take place and is less prone to mutations. Rooting occurs in 6–10 days depending on the cotton cultivar [68]. The shoot apex

gives hairy transformed roots because it is most reactive to develop tumor. Shoot apex method is a substitute method for *Agrobacterium*-mediated transformation of dicotyledonous cultivars in which regeneration by protoplasts, leaf disks, or epidermal strips does not take place. This technique offers a rapid method for transformation with less risk of mutations [27]. Katageri et al. [59,71] transformed an Indian variety with shoot apex method and showed a great potential for transformation. Shoot apex transformation method is a time consuming and laborious method for transformation, and there are higher rates of somaclonal variations.

6.11. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT)

SAAT method is based on principle of causing thousands of wounds by ultrasound. These wounds allow the *Agrobacterium* to infect and cause gall formation. The chance of transformation of gene of interest is increased in sonication-assisted method due to infecting deeper plant cells. For the *GUS*A, transient expression protocol was optimized. This method has shown competitive advantage for being used as a routine method [10]. A sonication-assisted method of transformation was used to improve the transformation efficiency of the flax. These wounds created by the ultrasounds allow the uptake of the plasmid DNA into the flax hypocotyls and cotyledons, and its efficiency is dependent on duration and frequency of the sonication applied. SAAT could be a potential tool for increasing transformation efficiency in flax [11]. Due to low efficiency of *Agrobacterium*-mediated method in cotton, an alternative method was required to overcome the barrier of efficiency. SAAT should be a valuable and alternative method for demonstrating the stable cotton transformation [69].

6.12. Infiltration

In vitro culture is required for some transformation procedures. Some transformation procedures do not require *in vitro* culture. In the case of infiltration, plant parts at meiotic or mitotic stages are applied with bacterial inoculums. This method is mainly applied for *Arabidopsis* species for the past many years.

This simple procedure in which plant at the early stages is placed upside down in the beaker containing 5% sucrose solution with *A. tumefaciens* in such a way that only the inflorescences are submerged in the inoculum. Beakers are placed in vacuum chamber with usually 0.05 bar pressure. Seeds are collected and sown on selected media under sterile condition in order to protect from microbial contamination. Up to 95% plants with transgenic seeds can be obtained by keeping optimal conditions.

Vacuum infiltration was applied for the first time in 1993 for transformation of *Arabidopsis* [11]. Over the next 5 years, the optimal conditions were improved and efficiency increased about 2%. Another version of this protocol is to use optimum concentration of sucrose and bacterial inoculum by the surfactant, Silwet L-19 [70]. This modified protocol, which was optimized by Clough and Bent, gives the best result in transformation of *Arabidopsis* [71]. Modified infiltration method was replaced the vacuum infiltration, and the protocol was modified a bit by immersing flowering plant in bacterial culture or spraying bacterial culture on plant. In another study, which was performed by Chung et al. [72], there is a comparison of the classical vacuum infiltration and new version which include modification. The results indicated that

plants with bacterial suspension produced much better results, i.e., 2.41% vs. 1.76% for vacuum infiltration and 2.09% for immersion.

Infiltration method is most suitable to plants which have smaller genome. Vacuum infiltration is excluded for plants having genome greater than *Arabidopsis*. Attempts were failed with Chinese cabbage so far. Hence this technique is species specific [12].

6.13. Silicon carbide-mediated transformation (SCMT)

SCMT is less complicated method of plant transformation. Silicon carbide fibers are added to a suspension containing plant tissue (cell clusters, immature embryos, and/or callus) and plasmid DNA; it is mixed and then vortexed. Kaeppler et al. [73] demonstrated that DNA-coated fibers penetrate the cell wall in the presence of small holes created by collisions between the plant cells and fibers. The fibers mostly used in this procedure are single crystals of silica organic minerals like silicon carbide, elongated in shape having a length of 10–80 mm, and a diameter of 0.6 mm and show a high resistance to expandability.

The factors controlling the efficiency of SCMT are fiber size, vortexing parameters, shape of the vessels used, the plant material, and the characteristics of the plant cells, especially the thickness of the cell wall. The main advantages of this procedure are the low expenses and its usefulness for various plant materials. Disadvantages of this method are low transformation efficiency, damage to cells negatively influencing their further regeneration capability, and the necessity of obeying extraordinarily rigorous precaution protocols during laboratory work, as breathing the fibers in, especially asbestos ones, can lead to serious sicknesses [74].

Transgenic forms, cell colonies, or plants were derived from maize [75], and rice [76], from wheat [77], from tobacco [78], and from *Lolium multiflorum, Lolium perenne, Festuca arundinacea*, and *Agrostis stolonifera* [79]. Maize variety Black Mexican Sweet (BMS) cell suspension was transformed with the plasmid carrying the *BAR* gene [73]. They found that 3.4% transgenic cell lines were expressing both transgenes from a 300 ml of packed cell volume, which shows that the integration of transgenes occurred in one per one million cells. The efficiency was significantly lower as described earlier by the same team or other authors employing micro bombardment for transformation. Vortexing with silicon carbide fibers caused the damage to cells decreasing their viability about 29% and causing the decrease in the efficiency of transformation. However, SCMT is speedy and cost-effective with easy to perform. Therefore, this method may prove an alternative method for plant transformation where gene gun method is restricted (i.e., most of monocots). It was suggested that SCMT system of using commercial shakers, which has been reported for maize, seemed to be very good for commercial large-scale transformation [80]. Frame et al. [81] obtained first fertile transgenics for maize in 1994. Out of 22 independent transgenic cell lines, 311 transgenic plants were derived, and 8 of those turned out to be stable transformants. The efficiency of SCMT was 5- to 10-fold lower than gene gun-mediated transformation. Petolino et al. [79] reported that efficiency is much lower in comparison with micro bombardment. The authors concluded that silicon carbide fibers cause damage to transformed cells that is why the results obtained were unsatisfactory [75].

6.14. Electroporation of intact plant cells and tissues

The principles of electroporation are the same for plant cells and protoplasts. However, difference may exist in other plant tissues such as pollen, microspores, leaf fragments, embryos, callus, seeds, or buds. During electroporation, the material used can be in the form of plasmid DNA and *Agrobacterium* inoculums. To check the transient expression of transgenes, different efforts were performed in early 1990s by applying electroporation technology.

Protocols were established for successful electroporation in cell suspensions, e.g., in tobacco [82], rice [24], and in wheat [83]. In early 1990s, experiments were performed to obtain transgenic plants. It was reported that the best results were obtained for maize. Researchers transformed immature embryos and embryogenic callus type I, which were treated by a solution of pectolytic enzymes, and then transferred into electroporating cuvettes [66]. The electroporation efficiency was relatively high when compared with micro bombardment conducted for same species, and about 90 transgenic plants were regenerated from 1440 embryos (6.25%) and 31 plants from 55 callus clusters (54.6%).

Laursen et al. [86] obtained similar results for this species. The authors estimated that the integration of transgenes took place approximately in one per 10,000 cells. Sorokin et al. [87] reported that much lower efficiency, i.e., about three transgenic plants from 1080 immature embryos (0.28%), was observed in the case of wheat electroporation. The transformation efficiency could be increased by the post-pulse addition of ascorbic acid or another ascorbate without any negative influence on cell viability [69]. Tissues were electroporated in liquid media containing 8 mg/L benzyl adenine that showed maximal regeneration through secondary somatic embryogenesis. DaSilva et al. [88] reported that the secondary somatic embryos regenerated from electroporation were positive for *GUS* expression. PCR analysis was positive for the *GUS* and *BAR* genes at torpedo shape somatic embryos. For some species, electroporation is an effective method but it is not widely used for plant transformation.

6.15. Electrophoresis

At the end of the 1980s, Songstad et al. [78] developed a method employing electrophoresis for the transformation of immature embryos, especially in monocotyledonous plants. This method was adopted as an alternative for transformation, but it is very expensive and yield poor results when compared with micro-projectile bombardment [53]. Transfected embryos were placed between the tip of two pipettes and connected to electrodes. The pipette connected to the anode was filled with agar / agarose followed by an EDTA containing electrophoresis buffer.

The pipette connected to the cathode contained agar that was mixed with DNA and an electrophoresis buffer. This pipette was in contact with the apical meristem of the embryo, whereas the second one was located near basal apical part of embryo. Electrophoresis-mediated transformation efficiency depends on various factors, such as electrical field parameters, duration of electrophoresis, contents of electrophoresis buffer, and physicochemical properties of the embryonic tissues. Voltage of 25 mV and an amperage of 0.5 mA for 15 minutes are mostly used for electrophoresis program [74]. In spite of its simplicity, electrophoresis is not

considerable method in plant transformation, and the reason behind is less viability of treated embryos. Ahokas et al. [84] showed that none of the plants showed expression of transgene inserted. Griesbach et al. [85] obtained successfully transformed plants of *Calnthe orchid* L.

7. Conclusion

Plant transformation is an essential tool for incorporating new characteristics in crop plant like cotton. Cotton is recalcitrant crop hence a reproducible regeneration is not available in local cotton varieties. Among all strategies developed by different researchers, a little success in cotton (*G. hirsutum*) genetic modification has been reported using *Agrobacterium*-mediated shoot apex cut method of cotton or embryo and sonication-assisted *Agrobacterium*-mediated transformation, except Coker genotypes, where regeneration potential leads to the use of particle bombardment or even *Agrobacterium*-mediated gene transformation as the best procedure. Limitations of gene transformation in cotton are observed in almost all of other procedures. One the most listed limitations of their application is the decreased viability of cells. Infiltration is the main transformation method for *Arabidopsis*, whereas SCMT is for maize, which does not have much success in cotton. With optimizing conditions, these transformation procedures might soon be available for a broad spectrum of plant transformation. The investigations like RNAseq of cotton regeneration in comparison with Coker genotypes or 2D-gel electrophoresis of embryogenic callus of cotton in comparison with control can give better insight to further improve the regeneration potential of cotton cultivars for improvement of cotton regeneration and for better cotton genetic modification in future.

Author details

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