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Genetic Transformation Methods for Crop Improvement: A Brief Review

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ABSTRACT

Decades of documented successful pieces of evidence in agritech have shown us a clear picture of the importance of biotechnology in crop improvement. The production in agriculture should have steady growth and to achieve these objective conventional methods should go on parallel with biotechnological approaches. Genetic engineering which has revolutionized the path of crop improvement involves the identification and transfer of novel genes into the existing elite cultivars. Different methods of transferring the gene into plant cells have been developed and continuous efforts have been made to increase its efficiency. Both direct and indirect method of gene transfer has its own merits and demerits. Efforts have been made continuously to eliminate drawbacks and to develop an easy, elite and eco-friendly method to transfer genes. The transformation method which is a base of genetic engineering is vital and *Agrobacterium*-mediated gene transfer and gene gun have shown to be doing well in recent years. As a whole the methodology involved, merits and demerits of different methods have been briefly discussed.

Key words: *Agrobacterium,* Gene, Gene gun, Microinjection, Transformation, Ultrasound.

The intensive research works have enhanced the accuracy of the gene to be transferred, integration into the host genome and its post-transcriptional expression. Owing to their importance in plant breeding it is not an alternate option. The continuous existing of humans is possible only by increasing food grain production and productivity. To achieve this conventional plant breeding approaches should be used in combination with biotechnological methods and develop a more reliable efficient method (Altman, 1999).Genetic engineering facilitates the easy transfer of gene which is inter-generic, inter-specific and also even inter-kingdom. Developing a systematic tool to transfer genes is important because it serves as a base for every genetic engineering research works. Both direct and indirect methods developed till date proved its efficiency yet they have its own drawbacks. *Agrobacterium*-mediated gene transfer is an indirect method and is highly efficient compared with other methods that have been discovered unexpectedly during the search for the causal organism for crown gall disease(Que *et al*., 2019). After understanding the molecular mechanism involved in transferring T-DNA into host it has been developed into a successful vehicle to transfer our gene of interest. Gene gun method is a physical method of directly delivering a gene into the target and it is not host specific. Many other methods such as microinjection, electroporation, PEG-mediated gene transfer, liposome-mediated gene transfer, fibre mediated DNA delivery, LASER induced DNA delivery, (Wright, 1999), etc., have been developed. During the last two decades, many improvements have been done in transformation techniques and it leads to rapid growth in the genetic engineering approach in plant breeding programs. Modern biotechnological transfer of gene sequences has overcome the obstacles of incompatibility, sterility, pre-fertilization barriers, post-fertilization barriers, risk in transferring gene between gene pools and also between different unrelated organism, related species wild species (Jeu, 2000), *etc*., This review gives a brief discussion about different gene transfer methods.

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GENE TRANSFERRING METHODS

The gene of interest identified is transferred into the target by different methods which were broadly classified into two types. The methodology that involves the use of a vector to deliver gene is referred to as an indirect method of gene transfer. If the gene delivery is directly by various physical and chemical methods are referred to as a direct method of gene transfer. The genetic diversity exists in available species are analyzed to identify the gene of interest the can satisfy the objective and a suitable method to transfer the identified gene of interest is used to develop an elite cultivar (Table 1).

AN INDIRECT METHOD OF GENE DELIVERY

Agrobacterium-mediated gene transfer

Agrobacterium was first identified as a causal agent of crown gall disease (Stephen G. Rogers, 1986), which is soil borne gram negative rod shaped and independent saprophytes in absence of host or survive as a pathogen in presence of a

Table 1: Different gene delivery methods.

Transformation method	Properties	References
Agrobacterium-mediated gene transfer	Ti-plasmid containing T-DNA is used in the transfer of the DNA segment	Slater *et al*., 2009)
Non-*Agrobacterium* mediated gene transfer	Microbes containing plasmidof order *Rhizobiales* are used	Mullins, 2018
Viral method of gene transfer	Integration of gene into the viral genome	Fiandaca and Federoff, 2014; Patel and Misra, 2011
Gene gun	Microcarrier coated with DNA is directly delivered into target under high pressure	Rajasekaran, 2013
Electroporation	Electrical charges are used to make pores in the membrane that helps in the entry of DNA	Young and Dean, 2015
Microinjection	Micropipette loaded with aqueous DNA deliver the target genes to nucleus or cytoplasm	Saito, 2005
Sonoporation	Ultrasound waves play a role in gene delivery	Akowuah *et al*., 2005; Cochran and Wheatley, 2013
Hydro-dynamic gene transfer	Gene delivery is by the hydrostatic pressure	Jinturkar, Rathi and Misra, 2011; Weiwei Wang, 2013
Polyethylene glycol (PEG) – induced DNA uptake	Polyether compound PEG adhere to DNA and transport it into a target	Sahab, Hayden, Mason and Spangenberg, 2019
Liposome-mediated gene delivery	Cationic liposome deliver DNA into the host cell	Kulkarni, Greiser, O'Brien and Pandit, 2010
Fibre mediated gene delivery	Silicon carbide fiber facilitated the transfer	Simon and Foroughi-Wehr, 2000

suitable host. A unique virulence mechanism of *Agrobacterium* was studied over hundreds of years and now it was developed as a potential technique to transfer gene. This mode of transformation is host specific which means genotype dependency (BoucHEZ, 1995).

The pathogenic *Agrobacterium* consists of three biovars (Slater *et al*., 2009),

- *Agrobacterium tumefaciens* species complex (biovar I)
- *Agrobacterium rhizogenes* (biovar II)
- *Agrobacterium vitis* (biovar III)

The biovar I strain *Agrobacterium tumefaciens* C58 genome was first sequenced and was renamed as *Rhizobium radiobacteria*. They produce tumour at the root and shoot intersection in many monocots of various families (Frame *et al*., 2006). The *Agrobacterium tumefaciens* with Ti plasmid are virulent due to the presence of T-DNA in Ti plasmid and this DNA has the capacity to cause neoplastic growth in host with the help of vir genes (virulence genes).

Ti plasmid structure and molecular mechanism of T-DNA transfer

Ti plasmid consists of two region T- DNA region which is transferred into the host cell and Vir region that helps in transferring T-DNA into the target host (Zhang, *et al.* 2006).

T-DNA is flanked by direct repeats of nearly 25bpthat is imperfect and is said to be left border (LB) and right border (RB). The genes that codes forauxin, cytokinin and opine are present in T-DNA. Auxin and cytokinin produce uncontrolled cell division that results in tumour formation and opines act as a nutrient to bacterial growth. The vir region consists of vir genes (virulence genes) with nearly 11 operons and can code for 35 proteins called vir proteins among them 20 proteins are involved in tumour formation. The vir protein recognizes the LB and RB sequence and creates nick to produce a single strand of linear T-DNA that gets transferred to the host cell to produce pathogenicity. This natural mechanism was exploited to transfer a gene of interest to the target cell by inserting the sequence to be transferred to the place of T-DNA (Christine Desfeux, 2000).

Molecular mechanism

There are three two-component systems were as follows (Nester, 2014).

1) The chemotaxis system that acts as a sensorfor chemical released from the wounded site of the plant and makes the bacterium to move to the target site.
2) The chromosomal virulence gene system chvG/chvI ascertain acidity and activate VirG.
3) VirA/virG system which is a key player that promote the coding of vir protein as a retort to plant phenols.

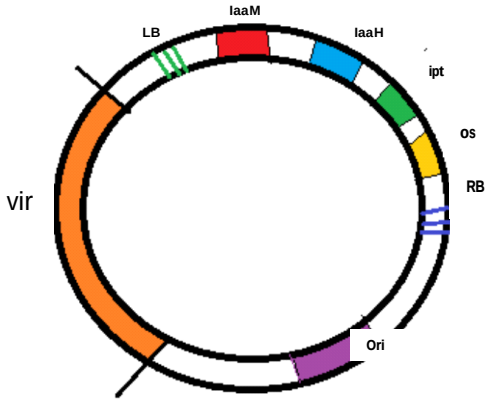

Fig 1: Ti plasmid in which LB-left border, RB- right border, vir- vir region,iaaM and iaaH codes for auxin, ipt codes for cytokinin and os – codes for opine respectively.

4) The peak expression of vir genes takes place when chvE attach to monosaccharide released by plants.

The signal cascade of acidity, phenols and sugar activate vir gene that has developed by evolution.

Signal receiving

VirA is a membrane receptor (histidine kinases) with four domains(periplasmic domain- percept monosaccharide and slight acidity, linker domain–recognize phenolic compounds,kinase domain–act as a catalyst for phosphorylation, receiver domain- singing up factor for virG) and virG act as intercellular response regulator. The virA autophosphorylates a specific histidine residue and transfers phosphate to aspartic acid residues of virG that ultimately initiate transcription of vir operons.

T-DNA production

The T-DNA is produced by a complex of proteins called the relaxosome protein complex. The protein complex consists of virD2, virD1, virC1 and virC2 attach to the border sequence of T-DNA. The linear single strand T-DNA is produced by VirD2 and it remains covalently attach to 5' end of T- strand (single strand of T-DNA) to produce virD2-T-DNA nucleoprotein complex. The action of virD1 is to cleave vir D2 from superhelical T- border (type –I DNA topoisomerase) and virC1 And virC2 act as T-DNA production enhancer (Meyer *et al*., 2018).

Adhesion of bacterial cell and host cell

The binding system is of two type's unipolar polysaccharide (UPP) dependent polar attachment and T pilus-mediated attachment.

Transport of T-strand

The pore channel across cell envelope is produced by Type – IV secretion system (T4SS) (Lacroix and Citovsky, 2018) that consists of 11 vir B protein and vir D4 which will be represented as virB/virD4 T4SS. Therefore, the virD2T-DNA nucleoprotein complex is transferred via virB/virDA T4SS along with four vir proteins (virD5,virE2,virE3 and virF) (Nester, 2014). The virE2 act as a single-strand binding protein and protect the complex from host nucleases. The virD2-T-DNA nucleoprotein complex along with VirE2 coat is called "T –complex" and together with other translocated vir protein called "super T- complex" (M. Guo, Ye, Gao, Xu and Yang, 2019).

Integration of T- strand

The virE2 is trafficked by the endoplasmic reticulum and degrade by virF. The vir F interacts with vip1 protein to degrade virE2 and protected from proteolysis and stabilized by VirD5.The integration of T-DNA into the host genome is by using the host DNA repair mechanism but it is not proved completely. The T-DNA that contains the only exon get integrated into the actively transcribed region of the host genome. The 3' end of T-DNA has promoter of eukaryotes called microhomologies that helps in the expression of T-DNA(M. Guo *et al*., 2019).

Expression of T-DNA

They show two types of expression,one is a transient expression in which the gene is transcribed without integration into the genome. This happens within 2-4 days of transfer but it is not transferred to progeny. The stable transformation that is the transferred DNA can be passed to progeny and this is by the integration of transferred DNA into the host genome within 10-14 days. Therefore the host cell with integrated transferred DNA should be selected and the transgenic plant should be regenerated (Toth *et al*., 2006).

The disarmed Ti plasmid that is the oncogenes (genes that code for auxin and cytokinin) are excised. The gene of interest is placed between the borders. Since wild strain Ti plasmid is 100-2000 kb have demerit of breakage, few sites of restriction by enzymes and to avoid this binary vector one with only T-DNA (mini Ti) and other with only vir region (helperTi) have been developed (Anami, 2013) (Fig 2).

Now, this method is exploited more efficiently by pharmaceutical industries to produce bio-pharmaceutical and enzymes in plants. The DNA has been transferred to yeast, fungi and cultured human cell in *in-vitro* condition. In plant breeding, DNA has been transferred to nearly 23 crops (Anna *et al*. 2000; Wilkins, *et al*. 2010) (host and non-host plant) for about 50 characters. The potential risk of accidental release of modified *Agrobacterium*into the environment should be avoided by adopting standard and improved protocols.

NON- *AGROBACTERIUM*-BASED METHODS

Four decades before it was identified that some members (*Rhizobia* spp) of *Rhizobiaceae* family also have the capacity to transfer the gene to the host. *Ensifer adhaerens, Ochrobactrum haywardense and Rhizobium etli* are some of the species related to *Agrobacterium* have used in gene transfer yet they have the disadvantage of limited host range (Mullins, 2018) (Fig 3).

Viral-mediated gene transfer

Plant-infecting RNA and DNA viruses can be used as a vector to transfer genes to the target. The gene to be transferred is made integrated into the viral genome and now the virus acts as a vector to transfer the gene. The virus with the transferred gene is made infect the target cell and its results in successful transformation. The main disadvantage is the high copy number per cell and viral-

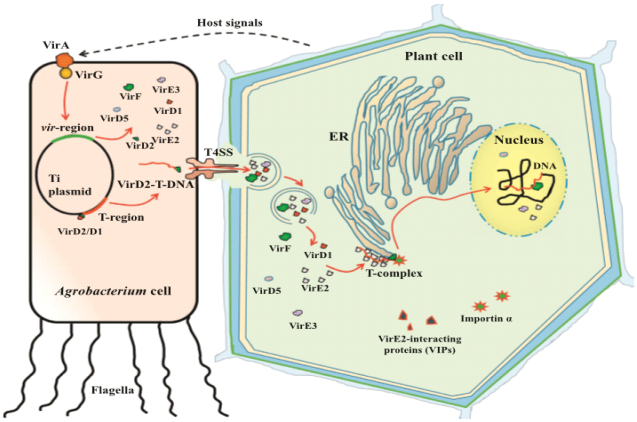

Fig 2: The detailed process involved in the transfer of gene segment mediated by *Agrobacterium* has been diagrammatically explained (Guo *et al*., 2019).

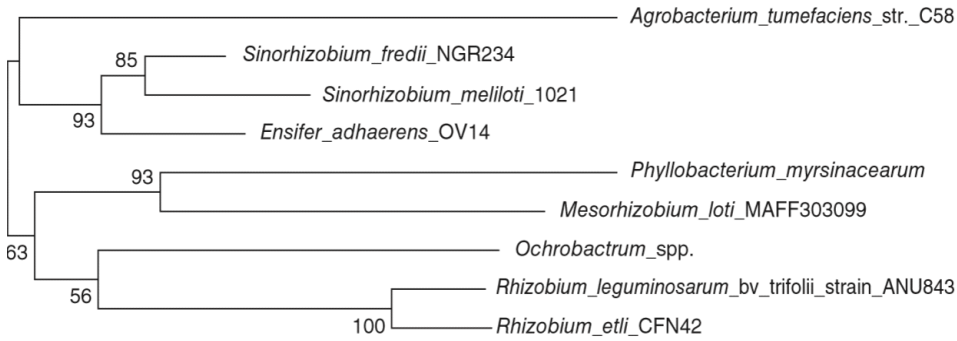

Fig 3: Tree diagram shows the phylogenetic relationship between *Agrobacterium* and other non- *Agrobacterium* species belong to the common order Rhizobiales (Slater *et al*., 2009).

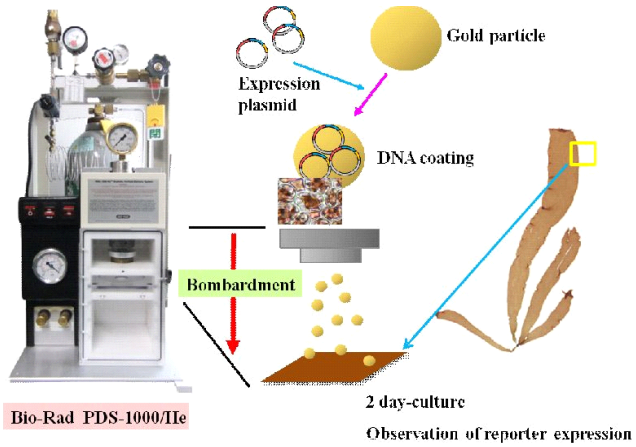

Fig 4: Biolistic gun (researchgate.net).

mediated gene transfer can only produce transient transfer and not the stable transformation that is they are unable to transfer to the progeny. Some of the viral vectors used are a Retrovirus, Adenovirus (Chailertvanitkul and Pouton, 2010), Adeno-associated virus, Herpes virus, Pox virus, Human Foamy Virus (HFV) and Lentivirus (Fiandaca and Federoff, 2014; Patel and Misra, 2011).

DIRECT GENE DELIVERY METHODS

PHYSICAL METHOD

Particle gun/biolistic/ballistic method of DNA delivery

Gene gun/ballistic DNA injection/DNA-coated particle bombardment is an efficient physical method of gene delivery into cell/tissue/organ (Fig 4). The particles of gold, tungsten, silver or any other heavy metal which is usually of 0.5-5µm in size and 25mg in weight are used as a DNA carrier and they were referred as microcarriers (John O'Brien 2002). As like firearm, microcarriers coated with DNA cassette are accelerated under high pressure of 900psi (helium gas) and shot over the plant material (cell/tissue/organ). The DNA cassette carried by microcarrier is delivered directly into tissues to few millimeters deep. The DNA delivered to the germline layer (L2) is only useful to have stable transformation. Unlike indirect method of gene delivery, it is not host specific and so used successfully in many crops like tobacco,soybean, rice, maize (Liu *et al*., 2019), wheat (Lazzeri, 1999), cotton (Rajasekaran, 2013),mango (Andres Cruz-Hernandez)etc., the DNA coating over the carrier is very important and many protocols have been developed while the generally used methods were Ca2+ protocol and PEG /MgCl2 protocol. the DNA and adhesive chemical mixture in micro-vial are constantly vortexed to prepare microcarrier (Rajasekaran, 2013).

The transformation frequency is decided by gene size, particle size, explants used in transformation, speed of the particle, etc. and the average transformation frequency is 9.9% if the nano quantity of DNA is used along with PEG as coating material (Ismagul *et al*., 2018). The main drawback noted was high copies of transgene in a single target and tissue damage. This will leads to the silencing of the transgene, mislay of transgene expression and transgene may be lost. The method to have a transgenic material with a single copy of transgene is under research. Using the nanogram quantity of DNA cassette (40 nm projectiles coated with 1µg YFP-DNA) instead of full plasmid has increased the frequency of transgenic material with single-copy transgene and less tissue damage (O'Brien and Lummis, 2011). The gene gun is fixed inside the aseptic condition generally laminar air flow chamber to facilitate transformation without contamination. The material to be transferred is bombarded to a maximum of one time because more times of bombardment will causetissue damage, chromosome truncations, large deletions, partial trisomy and breakage-fusion bridge cycling, etc., (Liu *et al*., 2019) which ultimately may cause mortality. The plants that show low response to tissue culture find difficult to use gene gun for the transformation of a gene. Although the method has disadvantages like many laboratories are devoid of gene gun and standardizing the protocol is quite difficult.

Electroporation

The non-viral method of DNA delivery needs two basic things nucleic acid along with cell suspension and electric field (Young and Dean, 2015). The protoplast along with nucleic acid is subjected to an electric field for a brief period of time. The cell membrane which is charged by hydrophobic lipid bilayer gets aligned by the applied electric field and that leads to a pore in the cell membrane called hydrophobic pore (Fig 5).

A) once the electric field is applied the charge molecules

in cell membrane get aligned and forms a dipole that results in the formation of hydrophilic and hydrophobic pores (Henshaw, *et al.* 2006). B) a large number of pores are formed at both the poles but the entry of DNA is only at anode pole because DNA is negatively charged get aligned at anode pole(Young and Dean, 2015).

The DNA gets aligned to the anode pole and gain entry into the cell by the pore. This method causes both transient and stable transformation in the cell (Yoshito *et al*, 1991). The protoplast prepared for transformation along with DNA to be transferred are subjected to an electric field created in specialized equipment for a brief period usually microseconds (Wang and Lu, 2006). The pore formed facilitates the entry of DNA and result in protoplast with transgene from which transgenic plants can be regenerated.

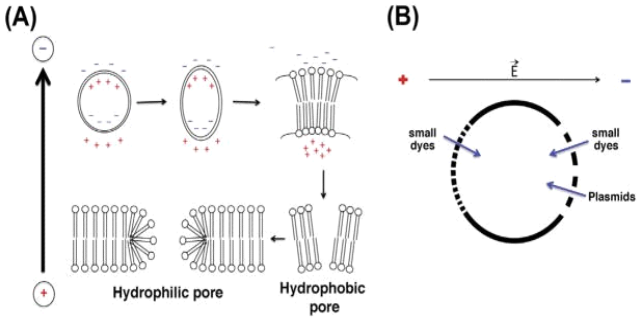

Fig 5: Formation of the pore in the cell membrane during electroporation (researchgate.net).

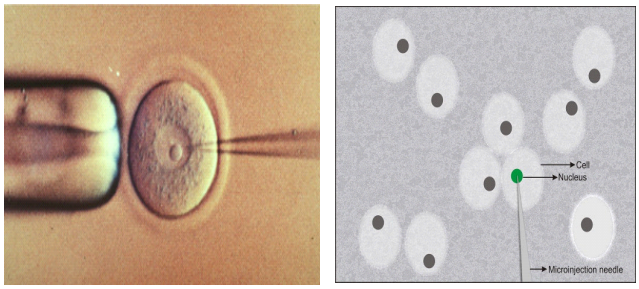

Fig 6 and 7: DNA delivery by microinjection (Jinturkar *et al*., 2011).

The efficiency of this method is controlled by many factors such as the strength of the electric field, duration of treatment, nature of cell membrane, properties of nucleic acid, etc., (Kim, Lee and Jung, 2010). The linear DNA fragment is easily transformed when compared with circular DNA. The very high voltage leads to stable transformation while low voltage leads to transient transformation (Guo *et al*., 2012; Markelc *et al*., 2012). This method is achieved in rice, wheat, maize tobacco *etc*.

Microinjection

Microinjection is a surgical technique that uses the micropipettes to directly deliver the DNA into the nucleus or cytoplasm. The microinjector connected to a micromanipulator deliver the DNA into the target (Baskaran and Dasgupta, 2011). The holding pipette holds the cell and the injector pipette injects the DNA and all these processes happen under a microscope (Hosokawa *et al*., 2009). It is an easy direct method of DNA delivery and now in order to increase its efficiency capillary microinjection has been developed (Fig 6 and 7). The size of the micropipette is usually 0.5-5µm (Saito, 2005).

Sonoporation

Sonoporation, as the name suggests, it involves making the pore by using the properties of ultrasound waves. The membrane is destabilized by using sound waves. This method is under research work and not fully established but it facilitates a safe delivery of DNA (Akowuah *et al*., 2005; Cochran and Wheatley, 2013; Du *et al*., 2018; Saliba *et al*., 2012; Weiwei Wang, 2013; Yu, Chen and Yan, 2019).

Hydrodynamic gene transfer

The non viral based and physical method of DNA delivery is done by destabilizing the cell membrane by applying hydrostatic pressure. The DNA can easily enter through the destabilized membrane and its result in either transient expression of the transgene or stable expression of the transgene. This method is more readily utilized in many pharmaceutical industries (Jinturkar *et al*., 2011; Weiwei Wang, 2013).

Table 2: Merits and demerits of different gene transformation methods.

Transformation method	Merits	Demerits
Agrobacterium-mediated gene transfer	Stable transformation and high transformation frequency	Host-specific
Non-*agrobacterium* mediated gene transfer	An alternate approach to *Agrobacterium*	Limited host range and low frequency of transformation
Viral method of gene transfer	Wide host range	Only transient transformation
Gene gun	Easier and highly adaptable	High copy number
Electroporation	Applicable to many species and equipment is simple	Standardization of dose is difficult
Microinjection	Easy method	Time-consuming
Sonoporation	Eco-friendly approach	Under research and need more development
Hydro-dynamic gene transfer	Easy and reliable	Need more development
Polyethylene glycol (PEG) – induced DNA uptake	No need for specialized equipment. Simple procedure	Preparation of transgenic plant from protoplast is difficult.
Liposome-mediated gene delivery	Easy to formulate, less toxicity	Possible only in a limited number of species
Fibre mediated gene delivery	Technically simple	Less Transformation frequency

CHEMICAL METHOD

Polyethylene glycol (PEG) – induced DNA uptake

The polyethylene glycol is a polyether compound that has the capacity to attach with DNA and along with Ca2+ transfer the DNA into the target by penetrating the membrane(Robert *et al*, 1983). The method is simple and does not require specialized equipment (Liu and Vidali, 2011). In this method the target is protoplast and so the method starts with the preparation of protoplast. Usually, leaf mesophyll is used to prepare protoplast. The leaf disc is treated with cellulose and protease enzymes to degrade cell wall (Sahab *et al*., 2019). The enzyme mixture contains osmoticum because maintaining tonicity is very important while preparing protoplast and the protoplast is purified with a mannitol solution. The protoplast prepared are made suspend in the solution of PEG/Ca2+ and the DNA solution of concentration 60µm. The gentle shake to the solution of DNA and protoplast and incubated to a brief period of time. Finally, the protoplast treated with DNA is spread over the suitable medium and the protoplast containing the transferred DNA is isolated by using marker and regeneration of the whole plant from the transferred protoplast that results in the transgenic plant (Sahab *et al*., 2019). Though the methodology is simple and devoid of costly equipment, it suffers difficulty in the regeneration of plant from a protoplast. The concentration of various chemicals and DNA are standardized based on the target. The multiple copies of target DNA are produced either by *E.coli* or PCR.

Liposome-mediated gene delivery

The vesicle with a small aqueous solution surrounded by lipid bilayer is called liposome that has the property of transferring substances into the cell by fusing with the cell membrane. These are artificial vesicles that can act as delivery agents for exogenous materials including transgene. They are considered as a sphere of lipid bilayers surrounding the molecule to be transported and promote transport after fusing with the cell membrane (Q-R Chen, 2000). Cationic lipids are those having a positive charge are used for the transfer of nucleic acid. The liposome is able to interact with the negatively charged cell membrane more readily than uncharged liposome (Munye *et al*., 2015). Due to the fusion between cationic liposome and cell surface result in the delivery of DNA directly across the cell membrane (Kulkarni *et al*., 2010).

Fibre mediated gene delivery

The silicon carbide fiber facilitates the delivery of DNA into the cell. This method involves the vortexing of cell suspension along with silicon carbide and plasmid DNA(Simon and Foroughi-Wehr, 2000). The plasmid DNA gets attached to silicon carbide fiber that has the unique capacity to penetrate the cell membrane and produce transferred cells. The treated cell is examined under the microscope to identify the cell with transgene and the transgenic plant is regenerated from the identified cells (Skokut, 1999).

Merits and demerits of different gene transformation methods

Each and every method has its own advantage and disadvantage in relation to stability, host specificity and copy number *etc*., are presented in Table 2 as suggested by Jinturkar *et al*., 2011.

CONCLUSION

The last 30 years of data show that there was a continuous improvement in the gene transformation approaches. The transformation methods adopted in genetic engineering have changed the path of crop improvement and lead to much significant improvement in agricultural production, crop protection and crop improvement. Upcoming years will show much improvement regarding the development of an easy, cost-effective and more significant method of gene transformation.

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