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Tissue Culture-based *Agrobacterium*-mediated and *in planta* Transformation Methods

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Abstract

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Gene transformation can be done in direct and indirect (*Agrobacterium*-mediated) ways. The most efficient method of gene transformation to date is the *Agrobacterium*-mediated method. The main problem of this method is that some plant species and mutant lines are recalcitrant to regeneration. Requirements for sterile conditions for plant regeneration are another problem of *Agrobacterium*-mediated transformation. The development of a genotype-independent gene transformation method is of great interest in many plants. Some *Agrobacterium*-mediated gene transformation methods independent of tissue culture are reported in individual plants and crops. Generally, these methods are called *in planta* gene transformation. *In planta* transformation methods are free from somaclonal variation and easier, quicker, and simpler than transformation methods based on tissue culture. Vacuum infiltration, injection of *Agrobacterium* culture into plant tissues, pollen-tube pathway, floral dip and floral spray are the main methods of *in planta* transformation. Each of these methods has its own advantages and disadvantages. Simplicity and reliability are the primary reasons for the popularity of the *in planta* methods. These methods are much faster than regular *Agrobacterium*-mediated gene transformation based on tissue culture and success can be achieved by non-experts. In the present review, we highlight all methods of *in planta* transformation comparing them with regular *Agrobacterium*-mediated transformation methods based on tissue culture. Finally, successful recent transformations using these methods are presented.

Keywords: *Agrobacterium*; floral dip; floral spray; pollen-tube pathway; sonication; vacuum infiltration

Gene transformation and genetically modified (GM) plants are the issues that since their appearance have aroused many fears and hopes in the public mind and scientific society. The main public concerns associated with the transgenic plants are potential health and environmental risks (Jouzani & Tohidfar 2013). Since the appearance of *Agrobacterium*-mediated gene transformation in the last three decades great scientific and practical improvements have been achieved in plant breeding and plant biotechnology. Today the genetic engineering of crop plants provides solutions to economic problems of agriculture in arid and tropical areas which rely heavily on agricultural production and crop export (Zalabák *et al*. 2013). Actually new methods of plant breeding such as molecular engineering and plant gene transformation via *Agrobacterium* break down the obstacles that hampered the progress of conventional plant breeding methods. Genetic transformation helps breeders to reach desirable varieties of plants with genes of interest (GOIs) in a shorter time and also remove the obstacles of crossing between different

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plants. In conventional plant breeding, a crossbred is only possible in individuals of the same species (or from closely related species). Traditional breeders cannot create desirable traits or introgress these traits into new varieties if GOIs are not available in these natural gene pools. Therefore, searching for alternative sources of GOIs in unrelated plant species or even in microbial organisms is necessary (Tohidfar & Khosravi 2015).

All of the gene transformation methods for transfer of GOIs to target plants include *Agrobacterium*-mediated transformation, direct gene transfer by imbibition and biolistic transformation (gene gun), osmotic method, liposome method, microinjection and pollen tube pathway, shoot apex method of transformation, infiltration, and silicon carbide mediated transformation (SCMT) (Rao *et al.* 2009). The main problem of direct DNA uptake methods is that the DNA integration patterns are often random and largely unpredictable (Hadi *et al.* 1996). The most effective method for transforming plant nuclear genomes known to date under laboratory conditions is via *Agrobacterium* infection (Meyers *et al.* 2010). Recently some combined methods of gene transformation have been reported such as *Agrolistics* that combines *Agrobacterium*-mediated transformation and biolistics (Mohanty *et al.* 2016). Many methods of plant transformation require the employment of *in vitro* culture, at least during some procedural steps. The generation of transgenic plants is a process that takes months; but the lack of efficient plant tissue culture method in target plant is the factor that can prolong this process significantly. The lack of highly efficient tissue culture regeneration systems is among the main obstacles for generating transgenic plants with modified nuclear or plastid genome in many important crops, such as corn, rice, or tea (Meyers *et al.* 2010). The efficiency of transformation also depends on the ability of selection procedure and the frequency of shoot regeneration and (pollen or somatic) embryogenesis (Sobhanian *et al.* 2012). The difficulties in DNA delivery as well as regeneration of the target plant species are the main challenges for genetic transformation of cereals and monocotyledonous plants using routine methods of gene transformation (Mrízová *et al.* 2014). There are some plant gene transformation methods that are independent of tissue culture procedure and can facilitate gene transformation in plants that do not have developed plant tissue culture protocol. These tissue culture-independent gene transformation methods have their own advantages and disadvantages. Here, we divide *Agrobacterium*-mediated gene transformation into regular tissue culture-based *Agrobacterium*-mediated gene transformation and *in planta* transformation (Figure 1). We then describe the generally used method and recent successful achievements in both tissue culture-based *Agrobacterium*-mediated transformation (TCBAT) and *in planta* transformation.

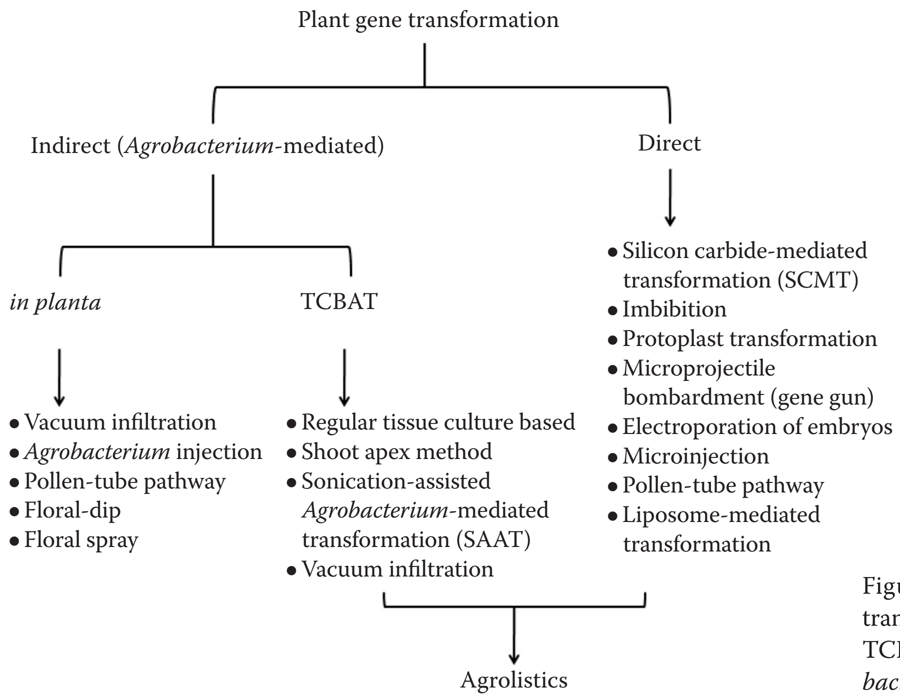

Figure 1. Direct and indirect gene transformation methods in plants TCBAT – tissue culture-based *Agrobacterium*-mediated transformation

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Principles of *Agrobacterium*-mediated gene transformation

Agrobacterium tumefaciens that causes crown gall disease in plants is a gram-negative soil phytopathogenic bacterium. Indeed, crown gall disease is a result of the integration of transferred DNA (T-DNA), derived from the bacterial tumour-inducing (Ti) plasmid, into the plant nuclear genome (Gelvin 2000; Tzfira & Citovsky 2002). The T-DNA contains a series of genes that are responsible for uncontrolled cell division, as well as genes promoting the production of opines (Hooykaas & Beijersbergen 1994). *Agrobacterium* is the most famous natural way of genetic transformation of plants (Zupan *et al*. 2000). T-DNA is one of the best vehicles to integrate GOIs into the plant genome (Meyers *et al*. 2010). Bacterial proteins encoded by the virulence (vir) region of the Ti plasmid, and numerous hijacked host-encoded factors are the primary elements that are involved in the integration of T-DNA into plant genome (Gelvin 2000; Tzfira & Citovsky 2002; Meyers *et al*. 2010). *Agrobacterium* T-DNA can be used in two ways for the integration of GOIs into plants: (*i*) direct cloning of GOIs into the T-DNA region of the Ti plasmid, and (*ii*) binary vector systems. The binary vector systems consist of helper plasmid and binary vector that are working together to gene transformation. Helper plasmid is the *Agrobacterium* Ti carrying the *vir* genes, and the binary vector is a DNA backbone derived from commonly used *E. coli* cloning vectors and carrying the GOI flanked by 25 bp-long right and left T-DNA border sequences (RB and LB) (Meyers *et al*. 2010).

Tissue culture-based *Agrobacterium*-mediated transformation (TCBAT)

Regular tissue culture-based Agrobacterium-mediated gene transformation

In regular tissue culture-based *Agrobacterium*-mediated gene transformation different parameters of tissue culture should be studied and developed for target plant. One of the most important parameters usually considered in this type of studies is a presence of PGRs. Also, besides tissue culture parameters, there are many other parameters related to gene transformation that should be optimized. Parameters such as bacterial strains and concentrations, addition of phenolic compounds to plant culture medium, plant genotypes, type and concentration of plant growth regulators, explant type, light and temperature during co-cultivation, antibiotics, wounding the target tissue and suitable method for the selection of transgenic cells can affect transformation efficiency (Tohidfar & Mohsenpour 2010). The main additive in this method is acetosyringone, as an inducer of T-DNA transfer, although other treatments such as vacuum infiltration, sonication and additives such as surfactant Silwet L-77 can be used for higher efficiency (Table 1). Many other additives have been used, e.g. sucrose, cysteine, 2-(N-morpholino)ethanesulfonic acid (MES), $MgCl_2$, and $AgNO_3$.

Shoot apex method

One of the gene transformation methods that can be used in both direct and indirect transformation is the meristem-based regeneration method (shoot apex method). The advantage of using the shoot apex for *Agrobacterium*-mediated gene transformation is a low incidence of tissue culture induced genetic changes, and a simple and direct development of transformed plants (Gould *et al*. 1991). Recently the shoot apex method has been used successfully in *Agrobacterium*-mediated transformation of rice (Clement *et al*. 2016), cucumber (Baskaran *et al*. 2016), and broccoli (Ravanfar & Abdul Aziz 2015) (Table 1).

Sonication-assisted Agrobacterium-mediated transformation (SAAT)

Manipulation of the bacterium and the target tissue can enhance transformation of monocots and certain dicot tissues that are not very receptive to *Agrobacterium* infection. Some manipulations include the addition of antioxidants to the co-culture medium and wounding the target plant tissue to enhance transformation rates in *Agrobacterium*-based transformation (Trick & Finer 1997). One of the integral steps in *Agrobacterium*-based transformation is wounding that allows the bacterium to easily penetrate into the target tissue and also stimulate the production of T-DNA transfer inducers (Stachel *et al*. 1985). The advantage of wounding, compared to acetosyringone, is that besides increasing the accessibility to target tissue for *Agrobacterium*, it can also enhance other inducers of T-DNA transfer. Different types of wounding include simple wounds made during the normal course of explant prepara-

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tion (Horsch *et al.* 1985), particle gun-mediated micro-wounding (Bidney *et al.* 1992), using syringes filled with *Agrobacterium* (Chee *et al.* 1989), and the use of sonication (Trick & Finer 1997). Sonication can overcome barriers of the host specificity and the inability of *Agrobacterium* to reach the proper cells in the target tissue and thus enhances DNA transfer (Trick & Finer 1997). In this method, plant tissues are subjected to brief periods of ultrasound in the presence of *Agrobacterium*. However, sonication is also used with naked DNA for the transfer of exogenous DNA (Joersbo & Brunstedt 1990, 1992; Zhang *et al.* 1991). Recently successful *Agrobacterium*–mediated transformation reports using SAAT are presented in Table 1.

In planta transformation

Various *in vitro* difficulties limit the development of gene transformation technique, also the mode of regeneration affects a successful rate of the genetic transformation (Mariashibu *et al.* 2013). *In planta* gene transformation is referring to an alternative method in which *Agrobacterium* is used to infect the explants but it does not involve in *in-vitro* culture and regeneration of plant cells or tissues (Kalbande & Patil 2016), thereby reducing time, labour cost and most importantly avoiding somaclonal variation encountered during *in vitro* culture-mediated genetic transformation and regeneration (Mayavan *et al.* 2013). In this kind of gene transformation, infected explant can be the whole plant (plantlet), flower or plant tissue. The main methods of *in planta* gene transformation are as follows: vacuum infiltration, *Agrobacterium* injection, pollen tube-mediated gene transfer (PTT), floral dip and floral spray methods (Figure 1).

Vacuum infiltration-assisted Agrobacterium-mediated genetic transformation (VIAAT)

In VIAAT, plant tissues are submerged in a liquid suspension of *A. tumefaciens* and subjected to decreased pressure followed by rapid repressurization (Bechtold *et al.* 1993; Bechtold & Pelletier 1998). Actually, vacuum treatment exposes *Agro-*

Table 1. Examples of tissue culture-based *Agrobacterium*-mediated gene transformation in different plants

Plant species	GOI/SMG/RG	Additive/treatment	Efficiency (%)	Reference
Cucumber (*Cucumis sativus* L.)	*act/nptII/gfp*	acetosyringone/ vacuum infiltration	11.9 ± 3.5	Nanasato *et al.* (2013)
Chickpea (*Cicer arietinum* L.)	–/*nptII*//*uidA*	SAAT/ acetosyringone	–	Tripathi *et al.* (2013)
Soybean (*Glycine max* L.)	–/*hptII/gus*; –/*hptII/gfp*;*gat/nptII/gus*	vacuum infiltration/ SAAT/Silwet L-77	5.7	Mariashibu *et al.* (2013); Arun *et al.* (2015); Guo *et al.* (2015)
Blackgram (*Vigna mungo* L. Hepper)	–/*nptII/uidA*	acetosyringone	–	Sainger *et al.* (2015)
Broccoli (*Brassica oleracea* var. *italica*)	*athsp101/nptII/luc*	acetosyringone	5	Ravanfar and Abdul Aziz (2015)
White ash (*Fraxinus americana* L.)	–/*nptII/gusA&egfp*	vacuum infiltration/ SAAT/acetosyringone	–	Palla and Pijut (2015)
Indian ginseng (*Withania somnifera* L.)	–/*nptII/gusA*	SAAT/vacuum infiltration/ acetosyringone	–	Sivanandhan *et al.* (2015)
Cucumber (*Cucumis sativus* L.)	–/*nptII*/–	acetosyringone/ microinjection	–	Baskaran *et al.* (2016)
Chrysanthemum (*Chrysanthemum indicum* L.)	*rsmyb1/bar*/–	acetosyringone	8	Naing *et al.* (2016)
Jerusalem artichoke (*Helianthus tuberosus* L.)	–/*hptII/gus*	–	–	Kim *et al.* (2016)
Indica rice (*Oryza sativa* L.)	–/*hptII/gus&gfp*	heat/hydrolytic enzymes/ acetosyringone	–	Clement *et al.* (2016)

GOI – genes of interest; SMG – selection marker genes; SAAT – sonication-assisted *Agrobacterium*-mediated transformation; RG – reporter genes

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bacterium to plant cells that are more susceptible to transformation than those present on the explant epidermis and this phenomenon occurs when vacuum is broken and pressure rapidly increases and the cell suspension may be driven into the explant to replace the discharged gases (Mariashibu *et al*. 2013). Using VIAAT solves the problems of regeneration in recalcitrant plants and sterile conditions that are required to regenerate plants (Tague & Mantis 2006). Besides the low efficiency, this method is easier to transform recalcitrant plants and the transformation process is significantly shortened (Lin *et al*. 2009). Currently successful *in planta* transformations using VIAAT are presented in Table 2.

Injection of Agrobacterium culture into plant tissues

Another type of *in planta* transformation is the injection of bacterial culture into meristematic and other target tissues of plants. Kalbande and Patil (2016) reported an *in planta* gene transformation in upland cotton. They bisected the shoot tip to expose the cells at apical meristem and then infected this site with *Agrobacterium*. Finally they reported successful transformation of *At-NPR1*gene (Kalbande & Patil 2016). In another *in planta* transformation, Xu *et al*. (2014) used a modified agroinfiltration method by the injection of *Agrobacterium* suspension to epidermal cells of onion. Successful transformation of *DREB1A* gene for resistance to cold stress was reported using piercing and injection of *Agrobacterium* to the 3-days-old shoot apical meristem of developing seedling of tomato (Hussain Shah *et al*. 2015). One disadvantage of using this method of *in planta* transformation is the production of chimeric transgenic plants especially when the shoot tip regeneration is applied for injection or inoculation with *Agrobacterium* (Kalbande & Patil 2016).

Pollen tube-mediated gene transfer (PTT)

Another alternative for regular *Agrobacterium*-mediated gene transformation is PTT. This method was first reported in cotton (*Gossypium hirsutum* L.) by Zhou *et al.* (1983). Pollen tube pathway-mediated genetic transformation is much simpler than the tissue culture-based transformation method and consists of three major steps that include injection of foreign genes into pollen tube, integration of foreign

Table 2. Examples of *in planta* gene transformation methods applied in plants

Plant species	GOI/SMG/RG	Method of gene transformation	Efficiency (%)	Reference
Indica rice (*Oryza sativa* L.)	*at2g47750/bar/gfp*	vacuum infiltration	6	Lin *et al*. (2009)
Soybean (*Glycine max* L.)	–/–/*gus*	pollen tube	–	Liu *et al*. (2009b)
Wheat (*Triticum aestivum* L.)	–/*nptII*/–	floral dip	–	Zale *et al*. (2009)
Maize (*Zea mays* L.)	–/*hptII/gfp*	floral dip	–	Mu *et al*. (2012)
False flax (*Camelina sativa*)	*rev/als/*–	floral dip	*0.8*	Liu *et al*. (2012)
Cotton (*Gossypium hirsutum* L.)	–/*nptII*/–	pollen tube	–	Wang *et al*. (2013)
Onion (*Allium cepa* L.)	–/–/*gfp*	injection of *Agrobacterium* culture	–	Xu *et al*. (2014)
Okra (*Abelmoschus esculentus* L.)	–/*bar& hptII/gus*	vacuum infiltration/ SAAT	–	Manickavasagam *et al*. (2015)
Sugarcane (*Saccharum officinarum* L.)	–/bar *& hptII/gus*	vacuum infiltration/ SAAT	–	Mayavan *et al*. (2015)
Tomato (*Solanum lycopersicum* L.)	*dreb1a/hptII*/–	injection of *Agrobacterium* culture	5.49–8.28	Hussain Shah *et al*. (2015)
Black gram (*Vigna mungo* L. Hepper)	–/*bar & hptII/gfp-gus*	vacuum infiltration/ SAAT	–	Kapildev *et al*. (2016)
Cotton (*Gossypium hirsutum* L.)	*at-npr1/nptII*/–	injection of *Agrobacterium* culture	6.89	Kalbande and Patil (2016)

GOI – genes of interest; SMG – selection marker genes; SAAT – Sonication-assisted *Agrobacterium*-mediated transformation; RG – reporter genes

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genes into plant genome, and selection of transgenic plants (Wang *et al.* 2013). Pollen tube-mediated gene transformation can be done by three major methods including microinjection, direct drop of DNA to the stigma, and culture of foreign genes with pollen and pollination with the pollens (Wang *et al.* 2013). Transport of exogenous DNA to the ovary of the recipient plant through growing pollen tube leads to integration of exogenous DNA with the undivided but fertilized recipient zygote(s) (Ali *et al.* 2015). In this method, the incorporation of exogenous DNA occurs at the stage of embryo formation and final produced seeds are transformed, so neither cell culture nor plant regeneration procedures are required (Zhou *et al.* 1983; Luo & Wu 1988). PTT avoids the traditional regeneration process that is essential in regular *Agrobacterium*-mediated gene transformation (Ali *et al.* 2015) but the transformation rate of this method is low, so it is better to do a field bioassay before the molecular techniques to test those potential transgenic plants (Wang *et al.* 2013). Usually the development of somatic embryogenesis and plant regeneration protocol is a genotype-dependent event, and thus PTT is a genotype-independent gene transformation method that is of great interest. Recently PTT was successfully used in some important crops such as soybean (Liu *et al.* 2009a; b) and cotton (Bibi *et al.* 2013) (Table 2).

Floral dip and floral spray

The removal and repotting of plants limit the utility of VIAAT, but subsequent research revealed that the uprooting and replanting of plants are unnecessary. By elimination of the uprooting and replanting of plants from the VIAAT method, a flower dip method of *Agrobacterium*-mediated gene transformation was created (Clough & Bent 1998; Tague & Mantis 2006). In comparison with PTT, in floral dip, the removal of the recipient plant's stigma is not required, neither is floral dip limited to flower structure (Ali *et al.* 2015). In this method, in the absence of vacuum infiltration, the inflorescence of plants is submerged at the early stages of flowering in an *Agrobacterium* suspension. The mode of application of the *Agrobacterium* inocula is variable from the application of drops of inocula to closed flower buds (Trujillo *et al.* 2004) through the immersion of shoots in *Agrobacterium* suspension (Tague & Mantis 2006), to the spray of *Agrobacterium* suspension cells to the inflorescence shoots (Chung *et al.* 2000). One of the critical points in floral dip transformation is the use of Silwet L-77 surfactant that dramatically increases the transformation efficiency (Richardson *et al.* 1998). In addition to Silwet L-77 surfactant, another key ingredient in floral dip transformation is sucrose. Compared to VIAAT, floral dip may result in lower overall transformation rates and higher seed set, but repeated application of *Agrobacterium* can improve the transformation rate (Clough & Bent 1998). The most important feature in floral dip transformation is the number of seeds produced on an individual plant. Since the efficiency of the floral dip method is about 0.1 to 5%, this method is more efficient in plant species that form more than 100 seeds per reproductive cycle (Tague & Mantis 2006). Although *in planta* transformation methods were developed with a floral dip method for *Arabidopsis* in the past decade (Clough & Bent 1998; Kojima *et al.* 2006), it seems that this method could be used easily in plants of *Apiaceae* species whose umbrella-like inflorescence can easily be exposed to *Agrobacterium* suspension (Figure 2). Theoretically, the umbel inflorescences of *Apiaceae* species host a large number of exposed flowers and produce many seeds (Baranski 2008), but unfortunately there has been no successful report on floral dip in *Apiaceae* so far. Successful reports on *Agrobacterium*-mediated gene transformation using floral dip are listed in Table 2.

Advantages and disadvantages

Disadvantages of plant tissue culture-based gene transformation include: (*i*) requirement of highly

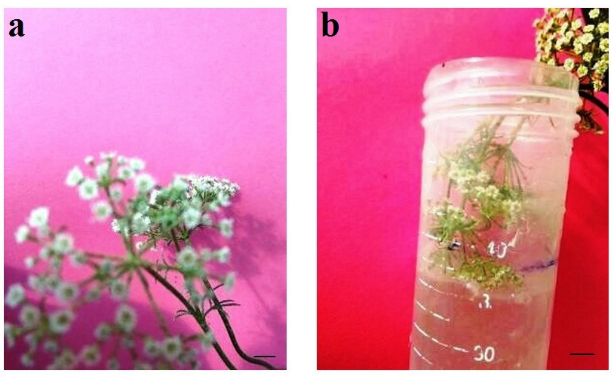

Figure 2. Floral dip of ajowan medicinal plant: umbrella-like inflorescence of ajowan ready for inoculation with *Agrobacterium* (bar = 0.5 cm) (a), submerging of ajowan inflorescence in *Agrobacterium* cell culture (bar = 0.5 cm) (b); (unpublished data of the authors)

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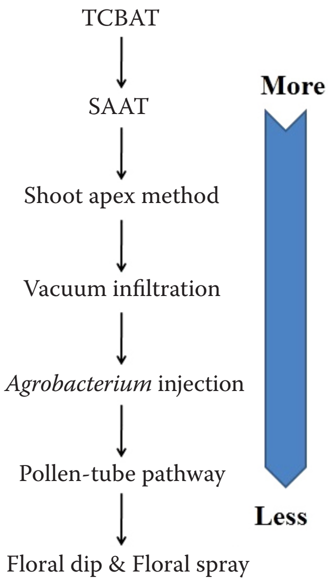

Figure 3. Degree of complexity, costs and technical skill requirement in different methods of TCBAT and *in planta* transformation
TCBAT – tissue culture-based *Agrobacterium*-mediated transformation; SAAT – sonication-assisted *Agrobacterium*-mediated transformation

sterile conditions, (*ii*) time consumption, (*iii*) occurrence of somaclonal variations, (*iv*) genotype specificity, (*v*) recalcitrance, (*vi*) failure in the acclimatization of valuable transgenic plantlets during the hardening of regenerated plants (Mayavan *et al*. 2013), (*vii*) harmful effects of selectable antibiotics such as kanamycin on the rooting of GM plants (Tohidfar *et al*. 2008), (*viii*) as different explants can be used in different varieties of plants, therefore detection of appropriate varieties and protocols is needed (Niapour *et al*. 2013). So, because of these disadvantages in regenerated GM plant through tissue culture, commercial application of transgenic plants needs considerable refinements of current transformation systems. Degree of complexity, cost effectiveness, time consumption and requirement of labour skills and expertise of different methods of tissue culture-based and *in planta* transformation are presented in Figure 3. List of other advantages and disadvantages of regular tissue culture-based *Agrobacterium*-mediated gene transformation and *in planta* gene transformation is presented in Table 3.

CONCLUSIONS

In a standard gene transformation project two sets of optimization are needed: (*i*) optimization of tissue culture protocol parameters, such as concentration of plant growth regulators (PGRs), type of media, type of explant, etc., and (*ii*) optimization of gene transformation protocol parameters such as *Agrobacterium* strain, time of inoculation, type and concentration of antibiotics to kill *Agrobacterium*, type and concentration of selectable antibiotics, concentration of acetosyringone, etc. These two sets of optimization make the *Agrobacterium*-mediated gene transformation a complex procedure, especially when we consider the interaction of these factors with plant genotype. Somaclonal variation is also another problem of standard *Agrobacterium*-mediated gene transformation. Although in some tissue

Table 3. Advantages and disadvantages of tissue culture-based *Agrobacterium*-mediated transformation and *in planta* transformation

Method	Advantages	Disadvantages
TCBAT	• More reliable for transformation, especially in plant with the limited number of seeds produced per reproductive cycle	• Complex and time consuming • Dependent to plant genotype • Require development of efficient *in vitro* regeneration protocol • Requirement of sterile conditions for regeneration • Optimal cell densities of *Agrobacterium* is required
in planta	• Easier and faster • Independent from plant genotype • *Agrobacterium* can be applied to plants at a range of cell densities	• Water-logged plants in vacuum treatment • Susceptibility of plant to damage from vacuum treatment • Low efficiency of floral dip in plants with limited seed production per reproductive cycle • Identification of developmental stage of the plant at the time of inoculation is required

TCBAT – tissue culture-based *Agrobacterium*-mediated transformation

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culture-based *Agrobacterium*-mediated transformation methods such as sonication, at least the risk of somaclonal variation is decreased when seedlings are used as target tissues, the requirement of *in vitro* regeneration remains in this method. In plants recalcitrant to tissue culture, by using *in planta* gene transformation methods, in addition to the removal of tissue culture optimization, some parts of gene transformation optimization such as antibiotics usage to kill *Agrobacterium* and to select transgenic tissue are also removed. The main methods of *in planta* transformation include vacuum infiltration, *Agrobacterium* injection, pollen tube pathway, floral dip and floral spray. Each of these *in planta* methods needs specific equipment and additives. In the vacuum method, a vacuum unit and its accessories are needed. In the floral dip method Silwet L-77 surfactant and sucrose are crucial additives. Germ-line transformation methods (pollen tube pathway and floral dip) are very simple and success can be achieved by non-experts. One of the most important variables in these methods that need experience is the identification of developmental stage of the plant at the time of inoculation with *Agrobacterium* (Clough & Bent 1998). The simplest methods of *in planta* transformation are floral dip and floral spray. In comparison with vacuum infiltration and pollen tube pathway, floral dip does not need the uprooting and replanting of plant and the removal of the recipient plant's stigma. The floral dip transformation method is more efficient in species in which each plant produces more than 100 seeds per reproductive cycle and theoretically this method can be used successfully in *Apiaceae* species. Sonication and vacuum infiltration are the treatments that can be used in both TCBAT and *in planta* transformation.

Although all the above-mentioned *in planta* transformation methods can help to enhance transformation efficiency in plants, other alternatives such as transcription factors (Lowe *et al*. 2016) can help to achieve the genotype-independent high efficiency protocol for plant transformation and enhance the regeneration and transformation efficiency in tissue culture and *Agrobacterium*-recalcitrant plants.

In addition, there are contemporary trends to genome editing methods such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR/Cas) system, but this promising method also needs a suitable delivery method (Kumar & Jain 2015) and it is worth noting that our ability to transform plants efficiently is the limiting factor in reaping the benefits of the novel tools for genome engineering methods such as CRISPR/Cas9 for co-delivering of multiple genes.

References

Ali A., Bang S.W., Chung S.M., Staub J.E. (2015): Plant transformation via pollen tube-mediated gene transfer. Plant Molecular Biology Reporter, 33: 742–747.

Arun M., Subramanyam K., Mariashibu T.S., Theboral J., Shivanandhan G., Manickavasagam M., Ganapathi A. (2015): Application of sonication in combination with vacuum infiltration enhances the *Agrobacterium*-mediated genetic transformation in Indian soybean cultivars. Applied Biochemistry and Biotechnology, 175: 2266–2287.

Baranski R. (2008): Genetic transformation of carrot (*Daucus carota*) and other Apiaceae species. Transgenic Plant Journal, 2: 18–38.

Baskaran P., Soós V., Balázs E., Van Staden J. (2016): Shoot apical meristem injection: A novel and efficient method to obtain transformed cucumber plants. South African Journal of Botany, 103: 210–215.

Bechtold N., Pelletier G. (1998): *In-planta Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods in Molecular Biology, 82: 259–326.

Bechtold N., Ellis J., Pelletier G. (1993): In-planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. Comptes rendus de l'Académie des sciences. Série 3, Sciences de la vie, 316: 1194–1199.

Bibi N., Kai F., Shuna Y., Mi N., Mosaddek I.M.,Waqas M., Xuede W. (2013): An efficient and highly reproducible approach for the selection of upland transgenic cotton produced by pollen tube pathway method. Australian Journal of Crop Science, 7: 1714–1722.

Bidney D., Scelonge C., Martich J., Burrus M., Sims L., Huffman G. (1992): Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Molecular Biology, 18: 301–313.

Chee P.P., Fober K.A., Slightom J.L. (1989): Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. Plant Physiology, 91: 1212–1218.

Chung M.H., Chen M.K., Pan S.E. (2000): Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. Transgenic Research, 9: 471–476.

Clement W.K.F., Lai K.S., Wong M.Y., Maziah M. (2016): Heat and hydrolytic enzymes treatment improved the *Agrobacterium*-mediated transformation of recalcitrant indica rice (*Oryza sativa* L.). Plant Cell, Tissue and Organ Culture, 125: 183–190.

doi: 10.17221/177/2016-CJGPB

Clough S.J., Bent A. (1998): Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant Journal, 16: 735–743.

Gelvin S.B. (2000): *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annual Review of Plant Biology, 51: 223–256.

Gould J., Banister S., Hasegawa O., Fahima M., Smith R.H. (1991): Regeneration of *Gossypium hirsutum* and *G. barbadense* from shoot apex tissues for transformation. Plant Cell Reports, 10: 12–16.

Guo B., Guo Y., Wang J., Zhang L.J., Jin L.G., Hong H.L. Chang R.Z., Qiu L.J. (2015): Co-treatment with surfactant and sonication significantly improves *Agrobacterium*-mediated resistant bud formation and transient expression efficiency in soybean. Journal of Integrative Agriculture, 14: 1242–1250.

Hadi M.Z., McMullen M.D., Finer J.J. (1996): Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Reports, 15: 500–505.

Hooykaas P.J.J., Beijersbergen A.G.M. (1994): The virulence system of *Agrobacterium tumefaciens*. Annual Review of Phytopathology, 32: 157–181.

Horsch R.B., Fry J.E., Hoffman N.L., Eicholtz D., Rogers S.G., Fraley R.T. (1985): A simple and general method for transferring genes into plants. Science, 227: 1229–1232.

Hussain Shah S., Ali S., Jan S.A., Din J.U., Ali G.M. (2015): Piercing and incubation method of *in planta* transformation producing stable transgenic plants by overexpressing *DREB1A* gene in tomato (*Solanum lycopersicum* Mill.). Plant Cell, Tissue and Organ Culture, 120: 1139–1157.

Joersbo M., Brunstedt J. (1990): Direct gene transfer to plant protoplasts by mild sonication. Plant Cell Reports, 9: 207–210.

Joersbo M., Brunstedt J. (1992): Sonication: A new method for gene transfer to plants. Physiologia Plantarum, 85: 230–234.

Jouzani G.S., Tohidfar M. (2013): Plant molecular farming: Future prospects and biosafety challenges. BioSafety, 2: 1000e136.

Kalbande B.B., Patil A.S. (2016): Plant tissue culture independent *Agrobacterium tumefaciens* mediated *in-planta* transformation strategy for upland cotton (*Gossypium hirsutum*). Journal of Genetic Engineering and Biotechnology, 14: 9–18.

Kapildev G., Chinnathambi A., Sivanandhan G., Rajesh M., Vasudevan V., Mayavan S., Arun M., Jeyaraj M., Alharbi S.A., Selvaraj N., Ganapathi A. (2016): High-efficient *Agrobacterium*-mediated *in planta* transformation in black gram (*Vigna mungo* (L.) Hepper). Acta Physiologiae Plantarum, 38: 205.

Kim M.J., An D.J., Moon K.B., Cho H.S., Min S.R., Sohn J.H., Jeon J.H., Kim H.S. (2016): Highly efficient plant regeneration and *Agrobacterium*-mediated transformation of *Helianthus tuberosus* L. Industrial Crops and Products, 83: 670–679.

Kojima M., Sparthana P., Teixeira da Silva JA., Nogawa M. (2006): Development of *in planta* transformation methods using *Agrobacterium tumefaciens*. In: Teixeira da Silva J.A. (ed.): Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues. Isleworth, Global Science Books: 41–48.

Kumar V., Jain M. (2015): The CRISPR-Cas system for plant genome editing: advances and opportunities. Journal of Experimental Botany, 66: 47–57.

Lin J., Zhou B., Yang Y., Mei J., Zhao X., Guo X., Huang X., Tang D., Liu X. (2009): Piercing and vacuum infiltration of the mature embryo: a simplified method for *Agrobacterium*-mediated transformation of *indica* rice. Plant Cell Reports, 28: 1065–1074.

Liu J., Su Q., An L., Yang A. (2009a): Transfer of a minimal linear marker-free and vector-free smGFP cassette into soybean via ovary-drip transformation. Biotechnology Letters, 31: 295–303.

Liu M., Yang J., Cheng Y.Q., An L.J. (2009b): Optimization of soybean (*Glycine max* (L.) Merrill) *in planta* ovary transformation using a linear minimal *gus* gene cassette. Journal of Zhejiang University Science B, 10: 870–876.

Liu X., Brost J., Hutcheon C., Guilfoil R., Wilson A.K., Leung S., Shewmaker C.K., Rooke S., Nguyen T., Kiser J., Rocher J.D. (2012): Transformation of the oilseed crop *Camelina sativa* by *Agrobacterium*-mediated floral dip and simple large-scale screening of transformants. In Vitro Cellular & Developmental Biology – Plant, 48: 462–468.

Lowe K., Wu E., Wang N., Hoerster G., Hastings C., Cho M.J., Scelonge C., Lenderts B., Chamberlin M., Cushatt J., Wang L., Ryan L., Khan T., Chow-Yiu J., Hua W., Yu M., Banh J., Bao Z., Brink K., Igo E., Rudrappa B., Shamseer P., Bruce W., Newman L., Shen B., Zheng P., Bidney D., Falco C., Register J., Zhao Z.Y., Xu D., Jones T., Gordon-Kamm W. (2016): Morphogenic regulators *Baby boom* and *Wuschel* improve monocot transformation. The Plant Cell, 28: 1998–2015.

Luo Z.X., Wu R.A. (1988): Simple method for the transformation of rice via the pollen-tube pathway. Plant Molecular Biology Reporter, 6: 165–174.

Manickavasagam M., Subramanyam K., Ishwarya R., Elayaraja D., Ganapathi A. (2015): Assessment of factors influencing the tissue culture-independent *Agrobacterium*-mediated *in planta* genetic transformation of okra [*Abelmoschus esculentus* (L.) Moench]. Plant Cell, Tissue and Organ Culture, 123: 309–320.

Mariashibu T.S., Subramanyam K., Arun M., Mayavan S., Rajesh M., Theboral J., Manickavasagam M., Ganapathi A.

doi: 10.17221/177/2016-CJGPB

(2013): Vacuum infiltration enhances the *Agrobacterium*-mediated genetic transformation in Indian soybean cultivars. Acta Physiologiae Plantarum, 35: 41–54.

Mayavan S., Subramanyam K., Arun M., Rajesh M., Kapil Dev G., Sivanandhan G., Jaganath B., Manickavasagam M., Selvaraj N., Ganapathi A. (2013): *Agrobacterium tumefaciens*-mediated *in planta* seed transformation strategy in sugarcane. Plant Cell Reports, 32: 1557–1574.

Mayavan S., Subramanyam K., Jaganath B., Sathish D., Manickavasagam M., Ganapathi A. (2015): *Agrobacterium*-mediated *in planta* genetic transformation of sugarcane setts. Plant Cell Reports, 34: 1835–1848.

Meyers B., Zaltsman A., Lacroix B., Kozlovsky S.V., Krichevsky A. (2010): Nuclear and plastid genetic engineering of plants: Comparison of opportunities and challenges. Biotechnology Advances, 28: 747–756.

Mohanty D., Chandra A., Tandon R. (2016): Germline transformation for crop improvement. In: Rajpal R.V., Rao R.S., Raina S.N. (eds): Molecular Breeding for Sustainable Crop Improvement. Cham, Springer International Publishing: 343–395.

Mrízová K., Holasková E., Tufan Öz M., Jiskrová E., Frébort I., Galuszka P. (2014): Transgenic barley: A prospective tool for biotechnology and agriculture. Biotechnology Advances, 32: 137–157.

Mu G., Chang N., Xiang K., Sheng Y., Zhang Z., Pan G. (2012): Genetic transformation of maize female inflorescence flowering floral dip method mediated by *Agrobacterium*. Biotechnology, 11: 178–183.

Naing A.H., Ai T.N., Jeon S.M., Lim S.H., Kim C.K. (2016): An efficient protocol for *Agrobacterium*-mediated genetic transformation of recalcitrant *Chrysanthemum* cultivar Shinma. Acta Physiologiae Plantarum, 38: 1–9.

Nanasato Y., Konagaya K., Okuzaki A., Tsuda M., Tabei Y. (2013): Improvement of *Agrobacterium*-mediated transformation of cucumber (*Cucumis sativus* L.) by combination of vacuum infiltration and co-cultivation on filter paper wicks. Plant Biotechnology Reports, 7: 267–276.

Niapour N., Baghizadeh A., Tohidfar M., Pourseyedi Sh. (2013): *Agrobacterium* mediated transformation of *fld* and *gus* genes into canola for salinity stress. Journal of Stress Physiology & Biochemistry, 9: 74–85.

Palla K.J., Pijut P.M. (2015): *Agrobacterium*-mediated genetic transformation of *Fraxinus americana* hypocotyls. Plant Cell, Tissue and Organ Culture, 120: 631–641.

Rao A.Q., Bakhsh A., Kiani S., Shahzad K., Shahid A.A., Husnain T., Riazuddin S. (2009): The myth of plant transformation. Biotechnology Advances, 27: 753–763.

Ravanfar S.A., Abdul Aziz M. (2015): Shoot tip regeneration and optimization of *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var. *italica*) cv. Green Marvel. Plant Biotechnology Reports, 9: 27–36.

Richardson K., Fowler S., Pullen C., Skelton C., Morris B., Putterill J. (1998): T-DNA tag-ging of a flowering-time gene and improved gene transfer by *in planta* transformation of *Arabidopsis*. Functional Plant Biology, 25: 125–130.

Sainger M., Chaudhary D., Dahiya S., Jaiwal R., Jaiwal P.K. (2015): Development of an efficient in vitro plant regeneration system amenable to *Agrobacterium*-mediated transformation of a recalcitrant grain legume blackgram (*Vigna mungo* L. Hepper). Physiology and Molecular Biology of Plants, 21: 505–517.

Sivanandhan G., Kapil Dev G., Theboral J., Selvaraj N., Ganapathi A., Manickavasagam M. (2015): Sonication, vacuum infiltration and thiol compounds enhance the *Agrobacterium*-mediated transformation frequency of *Withania somnifera* (L.) Dunal. PLoS ONE, 10: e0124693.

Sobhanian N., Habashy A.A., Farshad Far E., Tohidfar M. (2012): Optimizing regeneration and reporter gene (*gus*) transformation of alfalfa (*Medicago sativa*). Annals of Biological Research, 3: 2419–2427.

Stachel S.E., Messens E., Van Montagu M., Zambryski P. (1985): Identification of the signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. Nature, 318: 624–629.

Tague B., Mantis J. (2006): *In planta Agrobacterium*-mediated transformation by vacuum infiltration. In: Salinas J., Sanchez-Serrano J.J. (eds): Arabidopsis Protocols. New York, Humana Press: 215–223.

Tohidfar M., Mohsenpour M. (2010): Effective factors in cotton (*Gossipium spp.*) transformation using *Agrobacterium*. Journal of Agricultural Biotechnology, 2: 1–24. (in Farsi, Abstract in English)

Tohidfar M., Khosravi S. (2015): Transgenic crops with an improved resistance to biotic stresses. A review. Biotechnologie, Agronomie, Société et Environnement, 19: 62–70.

Tohidfar M., Ghareyazie B., Mosavi M., Yazdani Sh., Golabchian R. (2008): *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a synthetic *cry1Ab* gene for enhanced resistance against *Heliothis armigera*. Iranian Journal of Biotechnology, 6: 164–173.

Trick H.N., Finer J.J. (1997): SAAT: sonication-assisted *Agrobacterium*-mediated transformation. Transgenic Research, 6: 329–336.

Tripathi L., Singh A.K., Singh S., Singh R., Chaudhary S., Sanyal I., Amla D.V. (2013): Optimization of regeneration and *Agrobacterium*-mediated transformation of immature cotyledons of chickpea (*Cicer arietinum* L.). Plant Cell, Tissue and Organ Culture, 113: 513–527.

doi: 10.17221/177/2016-CJGPB

Trujillo M., Briones V., Ponce J.L., Estrella L. (2004): Improving transformation efficiency of *Arabidopsis thaliana* by modifying the floral dip method. Plant Molecular Biology Reporter, 22: 63–70.

Tzfira T., Citovsky V. (2002): Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends in Cell Biology, 12: 121–129.

Wang M., Zhang B., Wang Q. (2013): Cotton transformation via pollen tube pathway. In: Zhang B (ed.): Transgenic Cotton: Methods and Protocols, Methods in Molecular Biology. New York, Humana Press: 71–77.

Xu K., Huang X., Wu M., Wang Y., Chang Y., Liu K., Zhang J., Zhang Y., Zhang F., Yi L., Li T., Wang R., Tan G., Li C. (2014): A rapid, highly efficient and economical method of *Agrobacterium*-mediated *in planta* transient transformation in living onion epidermis. PLoS ONE, 9: e83556.

Zalabák D., Pospíšilová H., Šmehilová M., Mrízová K., Frébort I., Galuszka P. (2013): Genetic engineering of cytokinin metabolism: Prospective way to improve agricultural traits of crop plants. Biotechnology Advances, 31: 91–117.

Zale J.M., Agarwal S., Loar S., Steber C.M. (2009): Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. Plant Cell Reports, 28: 903–913.

Zhang L., Cheng L., Xu N., Zhao N., Li C., Yuan J., Jia S. (1991): Efficient transformation of tobacco by ultrasonication. Nature Biotechnology, 9: 996–997.

Zhou G.Y., Weng J., Zeng Y., Huang J., Qian S., Liu G. (1983): Introduction of exogenous DNA into cotton embryos. Methods in Enzymology, 101: 433–481.

Zupan J., Muth T.R., Draper O., Zambryski P.C. (2000): The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. The Plant Journal, 23: 11–28.

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