REVIEW ARTICLE

In Planta Transformation in Plants: A Review

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Abstract

In planta transformation has been established as an innovative and simple technique involving a direct transformation of plant parts without involving the tedious tissue culture step. Methodologies of *in planta* transformation involve different plant parts and strategies. *Agrobacterium* strain carrying the gene of interest is targeted to the specific plant part either directly or in the induction medium. Vacuum infiltration is sometimes used to facilitate foreign gene integration. Initial studies of this novel technique involved treatment of whole plants with the inoculum, and later shifted to treatments of shoot tips and floral parts, and finally, the female reproductive parts have been targeted. Zygotes, embryos, and seeds have recently been used extensively yielding successful transformation. The method is simple, convenient, and overcomes the problem of tissue culture-induced genetic variability in the transformants. The review traces the origin and development in the in planta methodologies used over the past and the various parameters considered by the workers for increased effectiveness viz. developmental stages, *Agrobacterium* stain, surfactant, induction medium, etc., in the various crops compiled. Based on the review, it may be inferred that there is immense potential *in planta* transformation, and the ease of regeneration and selection of transformants in the methods described can be utilized for crop improvement.

Keywords: Floral dip, *In planta* transformation, Pollen tube pathway, Vacuum infiltration.

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Introduction

The rapid transfer of beneficial traits to plants using the gene transfer techniques is of much interest and value to plant breeders. *In planta*, transformation technique is an innovative and novel technique for achieving transformation. Genetic transformation of plants for specific genes started in the 1970s. Traditionally, it utilized mostly the *Agrobacterium*-mediated and biolistic techniques to carry out gene transfer (Birch, 1997; Hansen and Wright, 1999). Other lesser-used techniques included electroporation, microinjection, and virus-mediated delivery, etc. Most of these protocols require extensive tissue culture work of the plant species for efficient plant regeneration from a single transformed cell. This led to a slower pace of genetic transformation work. The *in planta* techniques efficiently escape the tissue culture step and target the meristems or the other tissues (germ-line) that ultimately lead to the formation of gamete (Chee and Slighton, 1995). The first reports of *in planta* transformation were by Graves and Goldman (1986) who reported that the *Agrobacterium* could infect the scutellar and mesocotyl cells of germinating corn cells. But, the resulting transformed plants were chimeras and transformation efficiency achieved was extremely poor. Feldmann and Marks (1987) utilized the strategy of germinating seeds of *Arabidopsis* in a suspension of *Agrobacterium tumefaciens* and planting out the seeds using selective agents. Some of the seeds produced by these plants on self-pollination gave rise to entirely transformed plants that could be selected easily by germination on selective medium (Feldmann and Marks, 1987; 1991). Since then, different methodologies and plant parts have been employed to achieve the transformation of crop species using this innovative technology. Chowrira *et al.* (1995; 1996) electroporated nodal axillary buds of pea, lentil, and cowpea *in-vivo* using *gas* gene for transformation. Chimeric branches were obtained, and stable inheritance of genes could be observed up to R2 generation.

The concept of *in planta* transformation has gained much impetus in the recent times, and several alterations of the basic methodologies are being developed with variations in not only the stage but also the technique used and its related parameters. The initial studies on *in planta* transformation were mostly related to the model crop *Arabidopsis*. However, new crops are now being considered, and *in planta*, techniques are modified for the specific crop species under consideration.

The various strategies utilized for achieving the *in planta* transformation are briefly described here. The review has been divided into four sections. The first discusses the various strategies that have been adapted to achieve *in planta* transformation. The second section covers the factors which have been reported to affect the *in planta* transformation frequencies. The last section covers the advantages and limitations of *in planta* transformation methods.

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In planta transformation methodologies/ targets

In planta transformation using shoot tips

This strategy of *in planta* transformation involves the application of *Agrobacterium* suspension to developing shoots which would finally give rise to floral buds. The method was utilized by Chang *et al.* (1994) and Katavic *et al.* (1994) and called the "clip and squirt method." Chang *et al.* (1994) clipped off reproductive inflorescences in *Arabidopsis* and applied *Agrobacterium* solution to the center of the plant rosette. The new inflorescences formed a few days later were again removed, and *Agrobacterium* was reapplied, the plants were then allowed to develop and set seed. In the experiments by Katavic *et al.* (1994), the primary and secondary inflorescences were cut, and the wound site was inoculated with *Agrobacterium* solutions. The treated plants were then grown to maturity and allowed to set seeds, which were collected and tested for gene integration, inheritance, and copy number of the inserted gene. More than 90% of the transformed progeny exhibited Mendelian segregation patterns of *nptII* and *gus* reporter genes. Of those, 60% contained one functional insert, 16% had two transfer deoxyribonucleic acid (T-DNA) inserts and 15% segregated for T-DNA inserts at more than two unlinked loci. The remaining transformants displayed non-Mendelian segregation ratios

The similar *in planta* transformation methodology involving the treatment of shoots was carried out by Kojima *et al.* (2000) in Buckwheat (*Fagopyrum esculentum*) and Kojima *et al.* (2004) in Kenaf (*Hibiscus cannabinus*). In their experiments, the apical meristems of the 10-day old seedlings were pricked with a needle and those of three-month-old seedlings were pricked after decapitation. These sites of pricking were used as the sites of giving the *Agrobacterium* inoculum which was applied with a cotton applicator. The inoculated plants were allowed to grow, and the fastest-growing bud was retained and allowed to set seeds. The plants raised from the seeds were confirmed to contain the transgenes based on the phenotype of the transformed progeny, detection of transgenes and rescue of plasmid T-DNA.

Weeks *et al.* (2008) used cold-treated alfalfa seedlings that were cut at the apical node for *in planta* marker-free all-native T-DNA comprising a silencing construct for the caffeic acid o-methyltransferase (*comt*) gene. Rigorous vortexing of seedlings with deoxyribonucleic acid (DNA) was found to enhance alfalfa transformation frequencies about 50-fold as compared to either gentle agitation or vacuum infiltration. The transformation was confirmed using polymerase chain reaction (PCR) based genotyping that identified 2.4% of plants to contain the modified phosphate deoxyribonucleic acid P-DNA. The utilization of this marker-free all native transfer DNA has implications in getting more biosafe and acceptable transgenic plants.

Transformation using whole plants

Another important *in planta* transformation strategy to generate transformants was of vacuum infiltration; the method was an improvement over the application of *Agrobacterium* directly to shoot tips. The application of vacuum enabled the *Agrobacterium* culture to interact with all the cells uniformly and achieve higher transformation rates and elimination of chimeric transformants to some extent. It required simple equipment and was comparatively less time-consuming. In this method, different plant parts were subjected to vacuum conditions to withdraw all internal solution and its replacement with *Agrobacterium* suspension.

Bechtold *et al.*(1993) used vacuum infiltration to transform uprooted *Arabidopsis* plants using a concentrated *Agrobacterium* suspension. The plants were placed *en masse* into a bell jar along with the *Agrobacterium* solution, and a vacuum was then applied and released. This caused the air trapped within the plant to bubble off and replaced with *Agrobacterium* solution. The plants were then transplanted back to the soil, grown to seeds. Stably transformed lines up to 1% of the seeds tested could be selected in the next generation using the appropriate selection agent.

Variations of this method were found to succeed in several other crops, including *Medicago truncatula* (Trieu *et al.*, 2000) *Petunia hybrid* (Tjokrokusumo *et al.*, 2000), *Brassica rapa* L. ssp. *Chinensis* (Liu *et al.*, 1998; Qing *et al.*, 2000), *Arabidopsis laciocarpa* (Tague, 2001) and in *Raphanus sativa* L. *longipinnatus* Bailey (Curtis and Nam, 2001; Curtis *et al.*, 2002). The major limitation of the technique was that it relied on the morphology and size of plants and therefore could not be applied to large-sized plants.

Trieu *et al.* (2000) used *Medicago truncatula* flowering plants and young seedlings that yielded 2.9–36% transformation in seeds tested for transformation. A major intriguing finding of the study was the formation of transformants homozygous for the transgene. In further experiments using vacuum infiltration, Trieu *et al.* (2000) subjected the seedlings to a 4°C/14d vernalization treatment for two weeks which induced early flowering. Mean transformation efficiency of 36% was observed in flowering plants and 9.4% in the seedlings.

Wang *et al.* (2003) reported *in planta* transformation of two cultivars of *Brassica napus* plants. *Agrobacterium*-mediated inoculation of plants was performed at various developmental stages along with vacuum infiltration. The transformation was attempted for *gus*, *npt*II, and protoporphyrinogen oxidase (PPO) genes, for resistance to PPO-inhibiting herbicides such as Butafenicol. The aerial parts of whole plants or seedlings were fully immersed in the *Agrobacterium* suspension and placed in vacuum chambers. The vacuum was held for 2–5 min with a pressure ranging from 25–27 inches Hg. Plastic domes covered these treated parts, or Saran wraps for 3–7 days and after that allowed to set seeds. The transformation was recorded using histochemical staining, ELISA, PCR, and southern analysis. The flowering stage was found to be the most receptive stage for transformation and production of transgenic plants. Transient

gus expression was observed on all floral parts after 20 days, and in seeds after 5 days of inoculation. A transformation frequency of 5% was achieved in these experiments.

Seol *et al*. (2008) used vacuum infiltration for the transformation of cactus bulb of the species *H. trigonus, N. scopa* cv. Soonjung using four methodologies in which vacuum (30, 40, and 50 cm Hg) for 30 minuts each was applied in a tightly sealed vessel to intact, precut, post cut and pricked cactus bulbs soaked in a transformation buffer mixture containing *Agrobacterium*. The degassing process released vacuum pressure from the container and completed the forceful uptake of *Agrobacterium* into the cactus tissue. All treated cactus bulbs were dried under dark conditions for 24 hour and compelled to produce roots (25–30^{o}C for 10–14 hour). The reasonable transformation was achieved in all the methods. However, the low survival rate of transformants was attributable to the severe damage caused by vacuum infiltration.

Xu *et al.* (2008) performed vacuum infiltration of 45–50 days old Pakchoi (*Brassica rapa* ssp. *Chinensis*) plants having inflorescence. The transformation was detected in both upper and lower parts of the plants, and stained ovules and pollen grains were recovered from an unopened flower. The transformation frequency was observed to be higher for floral tissues as compared to leaves and stem.

Transformation using floral parts

Since many of the researchers reported floral tissues to be more receptive to transformation, the research shifted in favor of simple dipping of inflorescence into a solution containing *Agrobacterium tumefaciens* (Wang *et al*., 2003). The floral tissues are the sites carrying the germline cells which form the next generation of transformed progeny. The treatment targeted to the whole plant was cumbersome and non-practical for larger plants. In treatment of floral parts transformation was reported to occur at a very early stage of embryogenesis (Feldmann and Marks, 1987; Bent *et al*., 1994; Bechtold *et al*., 2000 and Desfeux *et al*., 2002). The efficiency of this method utilizing the simple dipping of inflorescence became popular due to the increase in transformation frequencies up to 3% (Clough and Bent, 1998; Budziszewski *et al*., 2001; McElver *et al*., 2001). Although, the cellular targets for the *planta* transformation are not well characterized, but various lines of evidence seemed to indicate that the ovules or cells giving rise to ovules or more specifically the female germline cells were the primary targets of *in planta* transformation, as reported in case of *Arabidopsis* (Ye *et al*., 1999; Bechtold *et al*., 2000; Desfeux *et al*., 2002; Bechtold *et al*., 2003 and Chumakov *et al*., 2006).

A major question that arose with regards to the egg cell being the primary targets of transformation was its complete covering by several layers of ovule which prevented DNA or *Agrobacterium* entry into the ovule tissue. The pollen tube pathway explained this, i.e., the introduction of foreign DNA through the path taken by the pollen tube to enter into the ovule for fertilization to occur, this has been confirmed by the microscopic studies where the nucelli was observed to degenerate and become a part of the pollen tube pathway. The pollen-tube transformation approach was put forward based on the hypothesis of Zhou *et al.* (1979), in which it is assumed that the heterologous DNA segment injected at a certain time could be transferred into the ovary by following the nucellus pathway. The pathway was larger than the pollen tube itself and between pollen tube formation and closing, heterologous DNA could enter the embryo sac and integrate into the zygote cell and the forepart embryo cells through this way (Russell, 1993). Also, the enzymatic activity causing degradation of the cell wall carbohydrates and acid polysaccharides contained in the extracellular matrix of the central septum released by the growth of pollen tube acts as stimulants for the *vir* genes required for the T-DNA transfer (Wu *et al*., 1995; Lennon *et al*., 1998 and Smagur *et al*., 2009). This method was therefore called the pollen tube pathway, which gained much popularity and was observed to yield positive results.

Desfeux *et al*. (2002) used a floral dip method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. They performed transformation of pollen donor parent and female reproductive tissue separately using manual outcrossing. From their study, they inferred that the ovules are the site of productive transformation in the floral dip method and a higher transformation frequency of 4% could be achieved by this method. Similar reports in Arabidopsis have been observed by Das and Joshi (2011).

Similarly, successful vacuum infiltration of inflorescences developed on auxiliary branches supplemented with *Agrobacterium* culture medium of 0.8 and 1 OD with different surfactants was reported in Arabidopsis by Dehestani *et al.* 2010.

Ye *et al.* (1999) also used vacuum infiltration of pollen grains for conducting experiments on *Arabidopsis thaliana* using GUS visual marker. Confirmation of transformation was carried out using southern analysis, progeny segregation and the microscopic analysis of GUS expression in ovules of infiltrated plants. GUS expression frequencies of up to 1% of pollen were observed 3–5 days post-infiltration, whereas frequencies of up to 6% were detected with ovules of unopened flowers, 5–11 days post-infiltration. They reported that the transgenic seeds could be obtained only from genetic crosses using infiltrated plants as the pollen recipient and not the pollen donor, demonstrating *Agrobacterium* transformation occurred through the ovule pathway. High transformation frequencies of up to 394 transgenic seeds per infiltrated plant were achieved.

Tjokrokusumo *et al.* (2000) used the vacuum infiltration to transform pollen of *Petunia hybrida* that was later applied on stigma, simultaneously suspension of *Agrobacterium* was placed directly on the stigma of a style before pollination. The experiments resulted in 9% and 7.5% of plants showing resistance to selectable marker genes, respectively.

Chumakov *et al.* (2006) carried out the integration of T-DNA into the maize genome by treatment of silks with a cell

suspension of *Agrobacterium* cells, containing activated *vir* genes. The cell suspension was applied onto the previously isolated silks, which were afterward pollinated with the pollen of the same cultivar. Integration of T-DNA into the maize genome was confirmed by PCR (the *npt*II and *gus* reporter genes) and histochemical staining of the seedling tissues, obtained from the transformed seeds. Amplification of the *npt*II gene showed the presence of about 60.3% of PCR-positive plants out of the total number of kanamycin-resistant seedlings examined, or 6.8% of the total number of seedlings**.**

Other approaches for achieving the transformation of floral tissues included the spraying, inoculating, dripping, and injecting of the *Agrobacterium* suspension directly into the female reproductive tissues to effect transformation. Several reports are also available on direct injection of exogenous naked DNA into naked ovaries to yield transformed progeny in several crops such as cotton (Zhou *et al.*, 1983; 1988, Li *et al.*, 1999; 2000; Zuo *et al.*, 2002; Li *et al.*, 2005; Liu *et al.*, 2007), rice (Luo and Wu, 1988 and Zhao, 2003), wheat (Zeng *et al.*, 1994 and Hou *et al.*, 2003), maize (Chong and Tan, 1995; Zhang *et al.*, 2005; Geng *et al.*, 2006 and Wu *et al.*, 2008), soybean (Zhao *et al.*, 1995; Hu and Wang, 1999 and Gao *et al.*, 2007) and tobacco (Li *et al.*, 2000). Chung *et al.* (2000) demonstrated that spraying of *Agrobacterium* culture into immature *Arabidopsis* flower could yield high transformation frequencies. In their study, floral spray and floral dip methods were used to carry out the transformation of a superoxide dismutase (SOD) gene into *Arabidopsis*. The primary inflorescence shoots of *Arabidopsis* plants were removed when they started to initiate flower buds and transformed by floral spray or floral dip. A transformation frequency of 2.4% was reported for the floral spray method. The floral spray method can be used for *in planta* transformation of plant species which are too large for dipping or vacuum infiltration. A similar study on a floral dip and floral drip for rice has been reported by Ratanasut *et al.* (2017). The transformed plants could be easily screened by a selection of the seeds on selection medium containing respective antibiotic (Harrison *et al.* 2006)

Bechtold *et al*. (2000) performed floral dip *in planta* transformation of *Arabidopsis thaliana* by inoculating its different floral stages with *Agrobacterium* containing the *gus* marker gene. They examined the gametophytic expression and reported that transformation occurred in flowers where treatment was given at a stage of the third division of the embryo sac. No *gus* expression was observed in embryo sacs or pollen of plants infiltrated with an *Agrobacterium* strain bearing a *gus* gene under the control of a male gametophyte-specific promoter. The genetic target was identified using an insertion mutant in which a gene essential for male gametophytic development had been disrupted by a T-DNA bearing a Basta resistance gene (B(R)). In this mutant, the B(R) marker is transferred to the progeny only by the female gametes. The study of 193 progeny of these transformants revealed 25 plants in which the two resistance markers were linked in coupling and only one plant where they were linked in repulsion. These results point to the chromosome set of the female gametophyte as the main target for the T-DNA. A similar floral dip method was also adapted by Curtis and Nam (2001) to generate transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey). They reported plant development stage and use of surfactant were important parameters for the optimization of transformation efficiency.

Zale *et al.* (2009) carried out *in planta* transformation of Crocus spring wheat using simple floral dip at the early boot stage, i.e., at the early, mid or late uninucleate microspore stage. For giving the treatment, the spikes were unsheathed and the developing spikes carefully removed. After the removal of the sterile terminal and inner florets, the remaining florets are clipped to the end of the glumes. Best results were obtained when the spikes were approximately 6–7 cm in length and had not emerged from the sheath. The duration of the floral dip in *Agrobacterium* infiltration medium was 1-2 min and was repeated twice in one of the experiments. The three transgenics developed 3B2, 14C1 and 1CR1 were characterized for stability over three to six generations. A transformation frequency of 0.44% was observed in their experiments.

The floral dip and vacuum infiltration methods have also been compared for their efficiencies by Smagur *et al.*(2009) in *Arabidopsis thaliana*. They reported that transformants were obtained only when floral buds were inoculated with *Agrobacterium* between the 5th and 10th day, before formation of the flower, when the pistil is open, this allows the bacteria to use the natural gate of the pollen tube to enter the ovary and transfer T-DNA to the egg cell, like a sperm cell reaches an ovule. They reported that irrespective of the method utilized the highest efficiency of *Agrobacterium*-mediated transformation of *A. thaliana* was obtained with unopened flower buds of young inflorescences.

Kaur (2008) carried out floral dip transformation in flowers of *Cicer arietinum* L. using 5 µL basal medium containing Agrobacterium (pelleted at 0.5 OD and dissolved in 100 mL medium) carrying Agrobacterium strain LBA 4404 containing the binary vector *pBinBt* was used containing *cry1Ac* gene under CaMV35S promoter and nos-terminator with *nptII* as plant selectable marker and acetosyringone (100 µM). Out of the 2855 flowers treated, 545 seeds were obtained which were selected on selection medium containing Hygromycin. The kanamycin concentration of 100 mg/l in S1 and 200 mg/L an S2 and S3 selection cycles were considered for selection of the transformed embryos. The putative transformed embryos were cultured on the selective medium for the identification of the transformants yielding a transformation frequency of 13.3%.

A modification of floral dip involving direct dripping the solution of exogenous DNA called as the floral drip method was performed by Liu *et al.* (2009a and b). FITC-labeled minimal linear gene cassette without selectable marker was directly dripped onto the wound site created on the ovaries of soybean in the form of DNA solution containing short

linear DNA fragments. The pods were individually harvested to analyze the efficiency of transformation achieved. The simultaneously pollen-tube pathway was also utilized for transformation, and the two methods were compared. The fluorescence signals in the ovary-drip transformation were observed in the basal part of the ovary while they were observed in the topmost part of the ovary in the pollen-tube pathway. From experiments, it was inferred that in the ovary-drip transformation, exogenous DNA had more chances to penetrate the embryo sac compared to the pollen-tube pathway transformation.

Furthermore, the ovary-drip transformation gave higher transformation frequency (3.0%) utilizing a shorter pathway passageway. The differences in transformation frequency observed between the two transformation methods may be due to the differences in the cutting sites along with the style, the time of DNA application at post-pollination, and the components of the DNA solution with respect to the concentration of surfactant. The highest transformation frequency was achieved in flower drip with the removal of the whole style by incorporating the surfactant Silwett L-77 into the DNA solution.

Zhang *et al.* (2009) reported transformation *via.* pollen-tube pathway in cotton using DNA injection. Transformation was targeted into the flowers located at the basal position of the second and tenth fruit branch, at 10–20 hours after pollination. The DNA was injected into the center of the ovary after adjustment of the appropriate DNA concentration. GA_3 was applied to prevent abscission. In their experiments, the effects of genotype, the concentration of the injected target DNA, and the effect of flower position on transformation efficiency was evaluated. They reported that the higher transformation frequencies via. pollen tube pathway could be affected by considering high and good quality DNA of suitable length for integration into the plant genome. The genotype, selection of reproductive organs at suitable fruit branches and planting density were also reported to affect the transformation efficiency.

Ming *et al.* (2009) utilized a minimal *gus* reporter gene cassette to transform the ovary of the soyabean plants after cutting the style at various degrees. In the experiments it was revealed that the complete removal of the style without ovary wounding resulted in a maximum transformation frequency. An average transformation frequency of 8.2% was obtained from the transformation of three soybean cultivars. The ovary-drip transformation method was reported genotype independent and optimization of the length of the cut style can improve the efficiency and reproducibility of transformation in soybean.

Yang *et al.* (2009) carried out *in planta* transformation on maize where the styles were completely removed, and DNA solution was directly applied to the ovaries. The movement of the exogenous DNA was monitored using fluorescein isothiocyanate-labeled DNA, which showed that the time taken by the exogenous DNA to enter the ovaries was shortened in this method as compared to that of the pollen-tube pathway. A transformation frequency of 3.38% was observed on PCR analysis. Further, they reported an increase in transformation frequency on use of surfactant Silwett L-77. Mayavan *et al.* (2013) reported stable transformation using seed imbibition supplemented with different parameters like vacuum infiltration, sonication, and use of surfactant and acetosyringone for transformation of five varieties of sugarcane.

In planta treatment of zygotes and embryos

The methods of direct transformation of developing embryos were also evolved. The general strategy was to target the embryo either directly by imbibition or by injury. The embryos were targeted after isolation or attached to the cotyledons. The first report of this *in planta* transformation technique dates back to 1987 by Feldmann and Marks (1987) who reported G418 herbicide-resistant transgenic *Arabidopsis* plants obtained by cultivating germinating seeds with *Agrobacterium*, containing a primary plasmid vector with *npt* II selectable marker gene in its T-DNA region. These seeds were grown on selective media to select for the transformed plants. Penza *et al.* (Penza *et al.*, 1992) reported direct gene transfer to cowpea embryos *gus* reporter gene. Differences in gene transfer efficiency were observed by Lurquin *et al.* (1998) in longitudinally sliced embryonic axes of pea and lentil mature seeds for different Agrobacteria strains, cultivars and cocultivation conditions when co-cultivated with *A. tumefaciens* carrying a *gus* reporter gene in its T-DNA. Expression of transgenic gene was evaluated for an average number of transgenic sectors observed in positive plants.

Holme *et al.* (2006) performed experiments on isolated embryos of barley variety Golden Promise. Two methods of transformation were used. The first method comprised pipetting a droplet of inoculum on top of each ovule. In the second method, a hole was punched with a fine needle (0.4 mm × 9 mm) in the ovule wall near the micropyle, and a droplet of inoculums was placed on top of the perforated area to facilitate *Agrobacterium* invasion into the interior of the ovule. They reported that the perforation in the ovule provided better opportunity to achieve higher transformation frequencies as compared to intact ovules as it contained the developing embryo. Transformation frequency of 3.1% was achieved in these experiments.

Senaratna *et al.* (1991) used dry somatic embryos of *Medicago sativa* and imbibed them in a solution containing a plasmid carrying the *uid*A gene. The embryos were found to uptake the DNA directly without an *Agrobacterium* vector. Transient expression of the *gus* gene was observed visually in germinating embryos and seedlings. However, the technique was not found to be reproducible and is a less efficient method since naked DNA lacks the machinery associated with an *Agrobacterium* T-DNA in terms of *vir* genes, mobilization and integration proteins.

In experiments by Rohini and Rao (2000a), zygotic embryos of Safflower with one cotyledon removed were

pricked with sterile needle and given 10 min agitation with *Agrobacterium* inoculums and 24 hour cocultivation period and allowed to germinate. The plantlets derived were tested for transformation using histochemical, PCR, and Southern analysis. Transformation frequencies of 1.3 and 5.3% were reported for the two cultivars 'A-1' and 'A-300' used in their studies.

Rohini and Rao (2000b) conducted similarly *in planta* transformation experiments on peanut (*Arachis hypogea* L. cv. TMV-2) by random pricking of the embryos with a sterile needle infected by immersion in an *Agrobacterium* suspension containing wounded tobacco extract. The explants of mature seeds with one cotyledon excised were given 16-hour cocultivation and subsequent decontamination, and kept for germination on soilrite for 16 days under growth room conditions and subsequently transferred to the greenhouse. Histochemical assay and PCR analysis showed that 3.3% of the seedlings were *gus* positive.

Kaur (Kaur, 2008) carried out dehydration imbibition of *Cicer arietinum* L. embryos and embryos with one cotyledon attached, where embryos were dehydrated for 6, 24 and 48 hrs before before imbibition in Agrobacterium suspension (LBA 4404 containing the binary vector *pBinBt* was used containing *cry1Ac* gene under CaMV35S promoter and nos-terminator with *nptII* as plant selectable marker) and Acetosyringone at 100µM. The imbibed embryos were dried on sterilized filter paper and kept for cocultivation. After two days of co-cultivation, the explants were directly transferred to selection medium containing selective agent kanamycin up to in 200 mg/l in subsequent three selection cycles of 2 weeks duration each. The survival percent for the embryo explant was reported to be1.5 % and that for the embryo with one cotyledon attached was 2.6 %. Transformation of seeds was also carried out first dipping the seeds in water and making puncure marks near the embryo to allow direct interaction of embryo with Agrobacteria. The seeds were then air-dried and imbibed in Agrobacterium suspension consisting of a liquid medium with *Agrobacteium* pellet made to an OD600 of 0.5 and 1.0 and Acetosyringone at 100µM. After two days of cocultivation, the seeds were kept for germination in a welled tray, irrigated with Hoagland solution containing 750mg/l kanamycin. Marked differences were observed on selection between control (non-transformed) and transformed. Based on the visual observations seedlings attaining (more than 15 cm shoot length) the transformation frequency of 8.5 and 2.4% were reported for 1.0 and 0.5 OD *Agrobacterium* treatments.

Kumar *et al.* (2009) carried out *in planta* transformation of bell pepper, the procedure used was similar to that used by Rohini and Rao (2000a and b). Seedlings with just emerging plumule were infected by pricking the meristem with a sterile sewing needle and subsequently dunking the seedlings in *Agrobacterium* culture for 40 min. The seedlings were then placed on moistened soilrite for germination. Sixty percent of seeds could be transferred to greenhouse for growth. Histochemical staining showed that these were essentially chimeras. Further testing was performed in the T1 and T2 generations. Transformation in the later generations was mostly stable. In similar experiments on bell pepper, Kumar *et al.* (2011) reported an overall transformation frequency of 27.4% using GFP gene.

Martinell *et al.* (2002) transformed soybean embryos by co-cultivating the exposed meristematic tissue of an embryo with *Agrobacterium* after the tissue had been wounded by ultrasonic waves, a plasma blast discharge, or by puncturing the tissue with a sharp object. In addition to transformation of zygotic embryos, the transformation of somatic embryos using naked DNA was also performed.

Transgenic *Hibiscus sabdariffa* plants were produced by Gassama-Dia *et al.* (2004) by using a tissue culture-independent method using *A. tumefasciens*. Embryo axes of mature seeds with one cotyledon excised were infected by immersion in a suspension of *Agrobacterium* LBA 4404 strain culture with pBal plasmid containing the *gus* and *npt*II genes. On the selective medium containing killer concentration of kanamycin (100 µg/ml), 54.3% of the seedlings grew well and 68% of the explants excised from putative transformed plants were found to be GUS positive. The assessment of the transformation by PCR revealed that the lines tested, carried the *npt*II gene.

Supartana *et al.* (2005) inoculated the embryos of 2 days dipped seeds of rice, by piercing its embryonic apical meristem with a needle dipped in *Agrobacterium* inoculum. The inoculated seeds were grown to maturity to set seeds. The transformation was confirmed in the T_1 seeds based on their altered phenotype, histochemical detection of GUS activity, resistance to grow on selective media containing hygromycin B, detection of gene by PCR and rescue of plasmid consisting of the integrated T-DNA and flanking rice genome DNA from T_1 plants. The transformation frequency achieved was estimated to be 40% and 43% by PCR and *gus* histochemical assay, respectively. In an experiment on rice seeds for sheath blight resistance using piercing and dipping in *Agrobacterium* inducing solution 24% transformation frequency for regenerated plants using PCR was reported by Naseri *et al.* 2012.

On the same lines, Supartana *et al.* (2006) carried out *in planta* transformation experiments on wheat. The embryos of overnight dipped seeds of wheat were inoculated by piercing its embryonic apical meristem with a needle dipped in *Agrobacterium* inoculum. The inoculated seeds were incubated at 22^0C for two days and after disinfection and vernalization treatment of 5^0 C for 25 days, were grown to maturity to set seeds. The transformation was confirmed in the T_1 seeds by five different methods of altered phenotype, resistance to grow on selective media containing geneticin or hygromycin B, detection of gene by PCR and Southern analysis and rescue of plasmid consisting of the integrated T-DNA and flanking wheat genome DNA from T_1 plants. The transformation frequency achieved was estimated to be 62% and 82% on selective agent geneticin and hygromycin,

33% by PCR, 75% by Southern hybridization and 40% by plasmid rescue. Stable transformation of pierced and imbibed seeds has been reported in tomato (Shah *et al.* 2014), wheat (Razzaq *et al.* 2011). The imbibed seeds were directly tested on selection medium by Razzaq *et al.* 2011, reporting transformation frequency of up to 27%.

In planta treatment of fruits

The *in planta* transformation of fruits have been carried out for both transient and stable gene expression. The general methodology involves the injection of *Agrobacterium* into the fruits, and expression is studied either in the tissues of the fruit or the seeds extracted from the fruit after maturity. The *in planta* transformation of fruits have been reported for apple, pear, peach, strawberry (Spolaore *et al.*, 2001), citrus (Spolaore *et al.*, 2001; Ahmad and Mirza, 2005), tomato (Spolaore *et al.*, 2001; Orzaez *et al.*, 2006; Hasan *et al.*, 2008; Yasmeen *et al.*, 2009)

Yasmeen *et al.* (2009) carried out *in planta* transformation experiments by *in-vitro* fruit injection, *in-vivo* fruit injection, and floral dip methods. Inoculation of the fruit was carried out using sterile hypodermic syringes and fruits were incubated at 28°C for different time intervals. In *in-vivo* method the tomatoes attached to the mother plant were injected twice on two consecutive days. The seeds were collected on ripening of the fruit and selected on selective agent and tested for stable integration of genes. The best transformation rates were observed for *in-vivo* fruit injection where mature red fruits were found to yield higher transformation rates. Transformation frequencies upto 42% were reported using histochemical and PCR analysis.

Orzaez *et al.* (2006) used agroinjection of fruits for transient gene expression studies. They observed that agroinjection of tomato fruits (cv Micro Tom) with *Agrobacterium* solution through the fruit stylar apex resulted in complete fruit infiltration and rendered high levels of 35S Cauliflower mosaic virus-driven b-glucuronidase and yellow fluorescence protein transient expression. The expression levels were observed to be highest around the placenta and moderate levels in the pericarp. The usefulness of fruit agroinjection was assayed in three case studies: (1) the heat shock regulation of an *Arabidopsis* (*Arabidopsis thaliana*) promoter, (2) the production of recombinant IgA antibodies as an example of molecular farming, and (3) the virus-induced gene silencing of the carotene biosynthesis pathway.

Spolaore *et al.* (2001) carried out transient transformation of commercially important fruits viz. apple, pear, strawberry, peach, tomato, and orange using *gus* intron gene fused with 35S promoter. The fruits were injected with the *Agrobacterium* suspension and were assayed after 2 days incubation. The transformation patterns in the tissues were observed to be different in each case and attributable to the way the injection was made and the anatomy of the tissue targeted. The study was proposed to offer a cheap and efficient method to carry out promoter analysis in a simple and inexpensive way.

Hasan *et al.* (2008) infiltrated tomato fruits by injecting *Agrobacterium tumefaciens* harboring plasmid pROKII carrying *Arabidopsis* early flowering gene *APETALA1* (*API*). Fruits were incubated for 2 days and seeds collected were germinated on kanamycin selection medium. The plants raised were analyzed for stable transformation using histochemical and PCR analysis. Mature fruits injected with *Agrobacterium* culture were sampled for transformation efficiency in various tissues after different incubation periods. Transient *GUS* expression was observed in transverse section of fruits. An overall transformation frequency of 68% was reported.

In a report by Zia *et al.* (2011) the soybean pods were directly injected with *Agrobacterium* strain, and out of the three developmental stages the most mature one was identified to depict maximum transformation frequency.

Factors affecting Transformation

Plant part, Stage and Methodology

The stage of development at which *in planta* transformation is carried out is one of the most crucial factors determining the frequency of transformation achieved. The technique of *in planta* transformation generally varies with the stage or the part of the plant targeted for carrying out the transformation. An overview of the transformation frequencies obtained by different workers has been presented in Table 1.

As compared to the germinating seedlings, the floral stage has been determined to be the most effective stage to achieve transformation (Trieu *et al.*, 2000; Wang *et al.*, 2003; Xu *et al.*, 2008). Bechtold *et al.* (2003) reported that in *Arabidopsis* transformation occurs at the stage of the third division of the embryo sac. Smagur *et al.* (2009) reported that in *Arabidopsis* transformation occurred only when the floral buds were inoculated between 5th and 10th day before the formation of a flower when pistil is open. Clough and Bent (1998) demonstrated that the highest transformation efficiency was achieved when the floral shoots of *Arabidopsis* were 2–10 cm and had many closed flower buds. Chung *et al.* (2000) reported that in *Arabidopsis* the best transformation efficiencies were achieved when the *Agrobacterium* infection occurred prior to locule closure. In Alfalfa, best transformation results were achieved when partly open flower buds were present. In *Brassica rapa* ssp. *chinensis* the floral shoots of 50–60 cm with few open flowers were reported to be best for carrying out transformation (Liu *et al.*, 1998; Qing *et al.*, 2000). In maize the maximum transformation frequency is observed when the corn silk size is 5-10 cm, using a floral dip method as reported by Mu *et al.* (2012).

The female gametophyte was found to be the actual target for such floral tissue transformations (Ye *et al.*, 1999; Bechtold *et al.*; 2000, Desfeux *et al.*, 2002; Chumakov *et al.*, 2006). The strategies were thereafter developed for targeting the ovule to effect transformation by the methods of pollen tube pathway, clipping of styles or ovule perforation. Zale *et*

Table 1: *In planta* transformation methodologies

Methodologies and Workers	*Crop/Species*	*Factors studied*	*Transformation Frequency*	*Other relevant information*
Transformation using shoot tips				
Katavic *et al.* (1994)	*Arabidopsis thaliana*	Occurrence of transformation	-	Clip and squirt method
Chang *et al.* (1994)	*Arabidopsis thaliana*	Occurrence of transformation	-	Clip and squirt method
Kojima *et al.* (2000)	Buckwheat (*Fagopyrun esculentum*)	*Agrobacterium* strains	Not reported	Treatment of apical or lateral bud meristems of 10-day old seedlings
Kojima *et al. (2004)*	Kenaf (*Hibiscus cannabinus*)	*Agrobacterium* strains	Not reported	
Weeks *et al.* (2008)	Alfalfa		2.4%	Seedlings vacuum infiltration, gentle agitation and vigorous vortexing. Marker free approach.
Transformation using whole plants				
Dehestani *et al.* (2010)	*Arabidopsis*	*Agrobacterium* strain, surfactant application and mature siliques and apical inflorescence meristem removal	upto 1.54 %	
Bechtold *et al.* (1993)	*Arabidopsis thaliana*	*Arabidopsis*	1%	
Trieu *et al.* (2000)	*Medicago truncatula*	Vacuum infiltarion duration, vernalization	9.4%(seedlings) 36% (flowering plants)	Vernalization treatment to induce flowering Flowering stage most receptive
Liu *et al.* (1998)	*Brassica rapa* L. ssp *chinensis*	Vacuum infiltarion duration	0.3	
Qing *et al.* (2000)	*Brassica rapa* L. ssp *chinensis*	Vacuum infiltration duration	0.3	
Tague *et al.* (2001)	*Arabidopsis laciocarpa*	Vacuum infiltration, surfactant	0.7%	
Curtis *et al.* (2002)	*Raphanus sativus* L. *longipinnatus* Bailey	Developmental stages, cultivars	5%	
Wang *et al.* (2003)	*Brassica napus*	Vacuum pressure, duration, developmental stage, No. of tratments	5%	Flowering induction using cold and planting density
Seol *et al.* (2008)	*N. scopa* cv Soonjung, *H. trigonus*	Vacuum pressure, duration, frequency of pin pricking, methodology of treatment	Upto 90%	Pin pricking combined with vacuum infiltration
Xu *et al.* (2008)	*Brassica rapa ssp. chinensis*	Stem region, persistence of *Agrobacterium* in tissues post infiltration	.0001-0.0004% of seeds	Vacuum infiltration
Treatment of floral parts				
Das and Joshi (2011)	*Arabidopsis thaliana*	Floral dip method, carbon source, surfactant	upto 12-14 %	kanamycin resistance and single step genomic PCR for identification of single cop[y transformants
Mu *et al.* (2012)	Maize	Developmental stage, OD	3.3%	*gfp* and PCR analysis
Ratnasut *et al.* (2017)	Rice	Agrobacterium stains, OD,	1.4%	gus expression and PCR
Desfeux *et al.* (2002)	*Arabidopsis thaliana*	Timing of *Agrobacterium* Infectin	0.48%	Floral Dip
Clough and Bent (1998)	*Arabidopsis thaliana*	Flower heights, various densities of the *Agrobacterium* inoculum, concentrations of sugar and surfactant, time intervals between repeated applications	0.1–3.0%	Floral dip
Ye *et al.* (1999)	*Arabidopsis thaliana*	Site of *in planta* transformation	6%	Vacuum infiltration

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Liu *et al.* (2009b)	Soybean	Partial style and ovary removal	8.2%	
Tjokrokusumo *et al.* (2000)	*Petunia hybrida*	Application of transformed pollen (TP) or *Agrobacterium* to stigma (Ag)	9.0% (TP) 7.5% (Ag)	
Chung *et al.* (2000)	*Arabidopsis thaliana*	Timing of *Agrobacterium* inoculation	2.4%	Floral spray or Floral dip
Bechtold *et al.* (2000)	*Arabidopsis thaliana*	Floral stages		
Curtis and Nam (2001)	*Raphanus sativus* L. *longipinnatus* Bailey	Surfactant, plant development stage, inoculation time (5-15 sec)	0.1–3%	
Chumakov *et al.* (2006)	Maize	Temperature	6.8%	
Samagur *et al.* (2009)	*Arabidopsis thaliana*	Developmental stage, surfactant, inoculation time	Upto 2.01% for FD and upto 1.55% for VI	Floral dip (FD) and vacuum infiltration (VI)
Zhang *et al.* (2009)	Cotton	Quality of DNA, floral position, exogenous DNA concentration injected	1.04–3.63%	Pollen-tube Pathway, transformation frequency depended on fruit branch no.
Liu *et al.*(2009a)	Soybean	Cutting of style, exogenous DNA, Surfactant Silwet L-77	3.18%	Ovary drip
Ming *et al.* (2009)	Soybean	Ovary drip	8.2%	
Yang *et al.* (2009)	Maize	Removal of styles, Silwet L-77 with sucrose 5%,	3.38% 6.47%	Ovary Drip, FITC-labeled DNA to estimate transformation time, creation of vector- and selectable marker-free transgenic maize
Zale *et al.* (2009)	Wheat	Developmental stage	0.44%	Floral dip
Treatment of seeds, zygotes and embryos				
Shah et al.. (2015)	Tomato	Piercing and incubation of 3 day old seedlings apical meristem	-	selective medium for shoot regeneration and RT-PCR
Razzaq et al. (2011)	Wheat	imbibition of seeds	27% for GUS and 26% for kanamycin resistance	
Naseri et al. (2012)	Rice	Piercing of Seed embryonic region and dipping	24% of regenerated seeds were PCR positive	leaf sheath testing using inocum and PCR analysis
Mayavan *et al.* (2013)	Sugarcane	pre-culture duration, acetosyringone concentration, surfactants, co-cultivation, sonication and vacuum infiltration duration	45.4%	*gus*, *gfp*, PCR and southern hybridization
Senaratna *et al.* (1991)	Alfalfa *(Medicago sativa)*	Dry somatic embryos using naked DNA	-	Transient expression
Feldmann and Marks (1987)	*Arabidopsis thaliana*	Germinating seeds	0.3%	
Gassama Dia *et al.* (2004)	*Hibiscus sabdarrifa*	Embryo axes of mature seeds	28%	

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Holme *et al.* (2006)	Barley	Pricked ovules, duration, surfactant, acetosyringone	3.1%	
Rohini and Rao (2000)	Safflower	Effect of acetosyringone and wounded tobacco leaf extracts	1.3-5.3%	*Agrobacterium* infection to pricked cotyledonary node
Rohini and Rao (2000)	Peanut (*Arachis hypogea* L.)	Effect of acetosyringone and wounded tobacco leaf extracts	3.3%	*Agrobacterium* infection to pricked embryonic axes
Supartana *et al.* (2005)	Rice	*Agrobacterium* strains	43%	needle pricking at embryonic apical meristem
Supartana *et al.* (2006)	Wheat	*Agrobacterium* strains	33%	needle pricking at embryonic apical meristem
Kumar *et al.* (2009; 2011)	Bell pepper	Needle pricking of germinated seedlings	27.4%	Needle pricking at apical meristem of plumule
Treatment of fruits				
Spolaore *et al.* (2001)	Apple, Pear, Tomato, Peach, Strawberry	*Agrobacterium* injection	-	Transient expression, variation in expression observed
Yasmeen *et al.* (2009)	Tomato	Injection to Ripe and unripe fruits and fertilized and unfertilized flowers	42%(Fruit injection) 23% floral dip	Fruit injection and floral dip
Orzaez *et al.* (2006)	Tomato	*Agrobacterium* injection		Transient expression
Hasan *et al.* (2008)	Tomato	*Agrobacterium* injection	68%	Transient expression
Zia *et al.* (2011)	Soybean	*Agrobacterium* injection to pods, developmental stage	6.45-14.2	Transient expression of *gus*

al. (2009) observed that the transformation by floral dip was completely dependent on the developmental stage of the flowers dipped. Mid to late uninucleate stage when spikes have not emerged from the sheath (4–7 days prior to anthesis) was found to be the most receptive stage of transformation in wheat. Holme *et al.* (Holme *et al.*, 2006) reported that most likely window for transformation may range from the zygotic stage until an early stage of embryo formation where a transformed cell from early-stage embryos develop into a new partly or completely individualized embryo. Yasmeen *et al.* (2009) reported that the higher frequency of transformation was achieved when ripe fruits of tomato were used for injecting the vector as compared to unripe tomato fruits. The developmental stage of the seed in pods of soybean has been reported as a parameter governing transformation by Zia *et al.* (2011)

Ionic detergents

The incorporation of surfactant Silwett L-77 has been observed to increase the uptake of exogenous DNA into the cell due to the reduction of surface tension and thereby permitting more intensive penetration of DNA into the plant tissues. Silwett L-77 was also reported to improve the overall yield of Arabidopsis transformants approximately two-fold in the Agrobacterium-mediated transformation involving the floral-dip method (Clough and Bent, 1998; Liu *et al.*, 2009; Yang et al., 2009). Clough and Bent (1998) reported a 40-fold increase in the transformation frequency when surfactant Silwett L-77 were incorporated in the inoculation medium.

Yang *et al.* (2009) and Das and Joshi (2011) reported that the presence of surfactant Silwett L-77 and 5% sucrose played an important role in transporting DNA molecules into ovaries. It was reported to be vital in the floral dip transformation of *Arabidopsis* for facilitating the T-DNA delivery by decreasing the tensile force of the cell surface (Clough and Bent, 1998) and also function to protect the linear gene cassette from degradation improving the capacity and shortening the time of the exogenous DNA entry into the cells. Similar favourable effects of Silwett L-77 have also been reported by Rohini and Rao (2000a &b), Curtis and Nam (2001), Holme *et al.* (2006), Liu *et al.*, (2009), Smagur *et al.*, (2009), Mayavan *et al.* (2013), Dehestani *et al.* 2010).

Holme *et al.* (2006) reported increase in transformation frequency by inclusion of Acetosyringone and Silwett L77 in inoculation medium. Zale *et al.* (2009) observed that incorporation of high concentration of Silwett L77 in inoculation medium decreased transformation by reduction in seed setting.

Vacuum pressure and duration

In the studies utilizing the vacuum infiltration to facilitate DNA uptake by the cells lower pressure has been found to be inadequate to affect transformation whereas a higher pressure harms the cells and tissues resulting in their low survivability. Effect of vacuum pressure and duration on *in planta* transformation rates have also been reported by (Liu *et*

al., 1998; Qing *et al.*, 2000; Wang *et al.*, 2003; Seol *et al.*, 2008, Mayavan *et al.* 2013).

Wang *et al.* (2003) reported that the successful conditions for the vacuum infiltration in case of *Brassica napus* were a pressure of 25–27 inches Hg applied twice for longer than 5 minuts either 1 week apart or consecutively. Vacuum infiltration at an intensity lower than 25 in. Hg or submersion of plants into the *Agrobacterium* inocula were not sufficient to recover transgenic plants, in these particular conditions. Seol *et al.* (2008) reported that the use of simple vacuum infiltration method in cactus *Notocarpus scopus* caused the lowest survival rates as compared to when it was combined with methods involving pricking and top cutting. However, vacuum infiltration ensured the delivery of the *Agrobacterium* to the totipotent cells resulting in transformation event. Out of all the treatments considered, it has been proposed that the most promising in *in planta* transformation involved pin-pricking combined with vacuum infiltration (10 and 20 cm Hg for 15 min) prior to bulb top-cutting off. They also reported that the genotypic differences between different cactus species led to differences in their tolerance to vacuum pressure applied, and thereby survivability. Higher vacuum pressure resulted in higher transformation rates but lower survivability.

Samagur *et al.* (2009) reported that the maximum transformation efficiency in case of *Arabidopsis* was obtained at a vacuum pressure of 530 HPa when inoculation time was kept for 4 min. They reported that the vacuum pressure was able to limit the effect of surface tension encountered by the *Agrobacterium* cells when they came in contact with the plant cells only as long as the vacuum pressure was applied. They proposed that the surfactants like Silvet L-77 could be used in its place as they have a much prolonged effect causing higher transformation efficiency.

Duration of treatment

The duration of treatment directly effects the transformation frequencies with longer durations providing greater opportunity for the transforming *Agrobacterium* cells to interact with the plant cells. However, if the duration is prolonged it effects the viability of the plant cells treated especially for treatments involving vacuum. Moreover, longer durations can also lead to higher infection by the *Agrobacterium* leading to loss of viability. Clough and Bent (1998) in their floral dip experiments reported that longer dipping resulted in a higher transformation rate and whereas an excessive prolongation of inoculation time led to a necrosis and destruction of flower buds.

Wang *et al.* (2003) reported optimium conditions for *in planta* transformation using vacuum infiltration of *Brassica napus* plants. Vacuum pressure of 25–27 inches Hg applied twice for longer than 5 minuts either 1 week apart or consecutively was found to yield highest number of transformants. Vacuum infiltration at an intensity lower than 25 inches Hg or submersion of plants into the *Agrobacterium* inocula were not sufficient to recover transgenic plants. No transgenic plants were recovered when germinating seedlings were used as targets. Kumar *et al.* (2009) reported that increase in infection time in bell pepper seedlings led to reduced survival rates indirectly affecting transformation efficiency.

Other factors

The transformation process is highly sensitive to temperature depending upon transformation stages and plant species. In particular, the temperature of 28^0C is critical for the excretion and assemblage of *vir* -dependent extracellular proteins of *Agrobacterium* (pili), which are essential for the successful T-DNA transfer (Fullner and Nester, 1996). The temperature interval of 19 to 22 ^{0}C has been observed to be optimal for the expression of virulence genes of *Agrobacterium* for the T-DNA transfer to tobacco seedlings, while total suppression of these *vir* genes were observed at temperatures higher than 31^0C (Chumakov *et al.*, 2002). In addition to the temperature, the frequency of T-DNA transfer and integration can be influenced by the conditions of the virulence genes induction (acetosyringone concentration and induction time), and pH of medium (Li *et al.*, 2010). Tobacco leaf extract was included in the inoculation medium by Rohini and Rao (2000) for *vir* gene induction in their experiments to yield transformants in safflower and peanut.

Advantages of *in planta* approaches of transformation

The above review show that several methods for performing the *in planta* transformation have been developed. The major advantages of the technique are as follows:

Easy and Convenient

Since, the *in planta* method of transformation is carried out in intact plants and tissues, it completely eliminates the cumbersome and time consuming tissue culture step requiring great deal of protocol refinement and focused efforts of expert practitioners. Moreover in recalcitrant species like legumes which are not amenable to tissue culture and the monocots species which lack the genetic principle for carrying out the T-DNA transfer, the *in planta* transformation methodologies offer additive advantage. It is therefore referred to as a theoretically practical method to transform all sexually reproducing plants.

Reduction in genetic variability in the transformants

In methods where tissue culture is a prerequisite for transformation, epigenetic and somaclonal variations may result in development of varied transformants in spite of being regenerated from the same transformation event. Such kind of variability in transgenics have been reported by several workers (Bao *et al.*, 1993; Arencibia *et al.*, 1998; Sala *et al.*, 2000; Labra *et al.*, 2004). The genomic variability in transgenic plants may arise due to several reasons which have been documented into the following categories (1) tissue culture induced mutagenesis (Dolezel and Novak, 1984; Sebastiani *et al.*, 1994), (2) pre-existing genomic variability

in the original plant population, as documented in cross pollinating (Pooran and Singh, 2003; Pradeep and Sumalini, 2003), self-pollinating (Miyashita *et al*., 1999; Bered *et al*., 2002) and cloned plants (Sala *et al*., 2000) and (3) insertion mutagenesis, whereby the foreign gene may disrupt the gene it is inserted into or close to (Koncz *et al*., 1992; Jeon *et al*., 2000). Labra *et al*. (2004) investigated genomic DNA changes in *in planta* derived transgenics, callus derived *Arabidopsis thaliana* plants and control plants using AFLP and RAMP analysis. They observed that the transgenic *Arabidopsis* plants derived by *in planta* method showed low level of genomic polymorphism comparable to that of control plants whereas substantial polymorphism was observed in plants derived from callus. They concluded that somaclonal variation was correlated with stress imposed by the *in-vitro* cell culture and not by integration of a foreign gene into the plant genome. Therefore the *in planta* transformation derived plants tend to escape from such alterations in their genome arising due to prolonged tissue culture procedures under the *in-vitro* conditions as applied to the conventional *Agrobacterium* mediated and biolistic methods of transformation.

The mechanism of *Agrobacterium*-mediated gene transfer and integration in plants has been extensively studied to understand the integration mechanisms (Gelvin, 2000; Zupan *et al*., 2000). However, the distribution pattern of T-DNA integration in plants and its correlation with the integration mechanisms and genomic rearrangement are still not explicit. Studies suggest that T-DNA integration is a random process (Thomas *et al*., 1994; Bundock and Hooykaas, 1996; Barakat *et al*., 2000), but recent studies in *A. thaliana* have shown that transgene insertions preferentially occur in the 3′ and 5′ regulatory regions of genes and in introns rather than in exons (Brunaud *et al*., 2000; Szabados *et al*., 2002; Radchuk *et al*., 2005) in their experiments on cotransformation of *Arabidopsis* using three different *Agrobacteria* each carrying different genes reported higher frequency of cotransformants with all three genes as compared to those carrying single or two genes. The three genes were found to show linked inheritance and were located at a single locus in either head to head or tail to tail orientation. The high frequency of co-transformation indicates that multiple gene transfer is not a random event. It appeared that the integration of one T-DNA into a specific site of the plant genome mediates the insertion of other genes into the same locus of the genome, resulting in the higher frequency of plants carrying several transgenes showing coupled inheritance in the following generations.

Addresses the biosafety issue

The major issue related to biosafety of transgenics concerns the utilization of regulatory sequences of viral origins and selectable marker genes that pose a potential harm to the human health Therefore the general consensus for the best solution to the problem of biosafety for transgenic plants is to totally avoid the use of selectable marker genes and vector backbone sequences from the beginning of the transgenic plant generation. There have been reports of the utilization of the *in planta* method where the vector- and selectable marker-free linear gene cassettes were transformed into the plants to overcome the obstacles of biosafety limits (Gao *et al*., 2007; Weeks *et al*., 2008; Liu *et al*., 2009). The pollen tube pathway allows for a suitable method to achieve the transfer of such genomic DNA and plasmid DNA into the plants. The *in planta* protocol by this means make the procedure simple, biosafe and practical for all sexual reproducing plants. Direct creation of vector- and selectable marker-free transgenic plants by linear gene cassettes transformation via the ovary-drip method has been reported as an effective method to carry out biosafe transformation (Weeks *et al*., 2008).

Limitations

The major difficulties observed in the *in planta* methodologies involve the non-persistence of the transformed sectors into gametes, low frequencies of transformation and non–reproducibility of protocols. The method of transformation somewhat crude and unreliable, depending much on the plant under consideration, method of *in planta* technique, physiological stage of the plant and other parameters governing transformation.

Future Prospects

The *in planta* technique has gained wide popularity and acceptance by the researchers for its simplicity, innovativeness, reliability and cost effectiveness especially in the last one decade. Several patents have also been filed on successful transformation experiments carried out. The general trend observed in the study is towards targeting the vector to the appropriate site (embryos, floral parts, shoot meristems etc.) using a suitable mechanism (vacuum infiltration, pollen tube pathway etc.) and development of methods for easy isolation and identification of transformants. The most recent trends in *in planta* transformation more so plant genetic transformation relates to complete removal of selectable marker genes and vector backbone sequences since these are both superfluous and undesirable in DNA transfer procedures. Generation of such vector- and maker-free transgenic plants would enable production of more biosafe plants, as the major concerns in utilization of genetically modified plants relates to the presence of these undesirable sequences which are transformed along with the target gene. Recently, direct transformation of a minimal linear gene cassette by stigma or ovary-drip without using bacteria has been reported in corn and soybean (Gao *et al*., 2007; Wu *et al*., 2008; Liu *et al*., 2009; Yang *et al*., 2009; Yang *et al*., 2009). This simple direct transformation method does not require the *Agrobacterium* Ti plasmid or vector, and does not result in any unnecessary backbone or selective marker in the transgenic products. It offers a new revolution for conducting transformation experiments on crop species for incorporating the traits of interest. Further, the development of methodologies that would dispense with the use of

Agrobacterium as well as vector with potential integration of backbone and undesirable DNA sequences would be ideal for wider acceptance of this innovative transformation technology. Overcoming the shortcomings in this innovative technology will be the new trend in the enhancement of application of this technology to the crops showing considerable recalcitrance to the different regeneration protocols.

Thus we can conclude that the i*n planta* transformation methods are still in early stages of development and a sure shot protocol for achieving *in planta* transformation is lacking in any crop considered till date. However, the *in planta* transformation methods hold much scope for research and offers immense possibilities for crop improvement in the new era.

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