Transformation in Plants: A Review

Ratna Preeti Kaur1, Sugani Devi2

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.

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.
In planta transformation in plants: A review

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*, *nptII*, 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.
In planta transformation in plants: A review

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°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
In planta transformation in plants: A review

In planta transformation involves the direct manipulation of the plant’s reproductive tissues to introduce foreign DNA, typically achieved through the use of Agrobacterium tumefaciens. This bacterium naturally infects plants, leading to the formation of crown gall tumors. Transgenic plant species can be generated by introducing desired genes into the plant cells using Agrobacteriummediated transformation. The T-DNA, which is a segment of the Agrobacterium plasmid, is transferred into the plant and integrated into the host genome.

The transformation process can be initiated through the application of Agrobacterium to the plants, either by spraying or vacuum infiltration. This method involves the direct application of the bacterial culture to the flower buds or to the ovary tissue, allowing the T-DNA to be transferred to the plant cells. Another approach is the floral dip method, where a flower is dipped in a solution of Agrobacterium and antibiotic in a Petri dish to induce transformation.

Various studies have demonstrated the effectiveness of these methods in transforming different plant species. For example, Liu et al. (2009) transformed radish (Raphanus sativus L. longipinnatus Bailey) using a floral dip method with the nptII marker gene, followed by selection on an antibiotic-containing medium. Also, Zale et al. (2009) carried out in planta transformation of Crocus spring wheat using simple floral dip at the early boot stage, obtaining transgenic plants with the nptII marker.

In planta transformation methods have been used to introduce various genes into plants, such as the gus and nptII marker genes. The presence and expression of these genes can be confirmed using histochemical staining and PCR analysis, respectively. The transformation efficiency can be calculated as the percentage of transformed plants out of the total number of kanamycin-resistant plants obtained from the transformed seeds. For instance, Zale et al. (2009) reported a transformation frequency of 13.3% using the floral dip method in Crocus spring wheat.

The optimization of transformation methods is crucial for improving transformation efficiency. Factors such as the timing of treatment, the type of Agrobacterium strain, and the selection pressure play a significant role in determining the success of transformation. Additionally, the integration of the T-DNA into the plant genome can be assessed through PCR analysis and Southern blot analysis to ensure the stability of the transgenic trait across generations.

Overall, in planta transformation has become a powerful tool for plant biotechnology, enabling the introduction of desired traits into plants for various applications, including herbicide resistance, disease resistance, and improved crop yield.
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. GA3 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 soybean 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 uidA 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 puncture 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 well 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 nptII 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 nptII 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 T1 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 T1 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°C for two days and after disinfection and vernalization treatment of 5°C for 25 days, were grown to maturity to set seeds. The transformation was confirmed in the T1 seeds by five different methods of altered phenotype, resistance to grow on selective media containing genetancin 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 T1 plants. The transformation frequency achieved was estimated to be 62% and 82% on selective agent genetancin and hygromycin,
In planta transformation in plants: A review

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

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 β-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.
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 (Fagopyrum 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 | | | Seeding 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 infiltration duration, vernalization | 9.4% (seedlings) | Vernalization treatment to induce flowering |
| Liu et al. (1998) | Brassica rapa L. ssp chinensis | Vacuum infiltration duration | 0.3 | Flowering stage most receptive |
| Qing et al. (2000) | Brassica rapa L. ssp chinensis | Vacuum infiltration duration | 0.3 | Flowering stage most receptive |
| Tague et al. (2001) | Arabidopsis laciocarpa | Vacuum infiltration, surfactant | 0.7% | Flowering stage most receptive |
| Curtis et al. (2002) | Raphanus sativus L. longipinnatus Bailey | Developmental stages, cultivars | 5% | Flowering stage most receptive |
| Wang et al. (2003) | Brassica napus | Vacuum pressure, duration, developmental stage, No. of treatments | 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* | 0.001-0.0004% 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 copy transformants |
| Mu et al. (2012) | Maize | Developmental stage, OD | 3.3% | gfp and PCR analysis |
| Ratnasut et al. (2017) | Rice | Agrobacterium strains, 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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### In planta transformation in plants: A review

| Author(s) (Year) | Plant | Treatment | Transformation Frequency | Notes |
|------------------|-------|-----------|-------------------------|-------|
| 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 | Timing of Agrobacterium inoculation | 2.4% | Floral spray or Floral dip |
| 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% | |
| Zale et al. (2009) | Wheat | Developmental stage | 0.44% | |
| **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 Germinating seeds | - | Transient expression |
| Feldmann and Marks (1987) | Arabidopsis thaliana | | 0.3% | |
| Gassama Dia et al. (2004) | Hibiscus sabdarnia | Embryo axes of mature seeds | 28% | |

*Contd...*
In planta transformation in plants: A review

Yasmeen et 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 the 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., 2009).
In planta transformation in plants: A review

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 minutes either 1 week apart or consecutively. 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, in these particular conditions. Seol *et al.* (2008) reported that the use of simple vacuum infiltration method in cactus *Notocactus 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 affects 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 optimum 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 minutes 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°C 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 °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°C (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 calcitrant 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; Arensibia *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.

Addressing 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; Wu 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 in 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.

**References**

Ahmad, M., Mirza, B. (2005) An efficient protocol for transient transformation of intact fruit and transgene expression in Citrus. Plant Molecular Biology Reports, 23:419-420.

Arencibia, A., Gentinetta, E., Cuzzoni, E., Castiglione, S.; Kohli, A., Vain, P., Leech, M., Christou, P., Salà, F. (1998) Molecular analysis of the genome of transgenic rice (Oryza sativa L.) plants produced via. particle bombardment or intact cell electroporation. Molecular Breeding, 4:99-109.

Bao, P.H., Castiglione, S., Giordani, C.L., Wang, W., Datta, S.K., Datta, K., Potrykus, I., Sala, F. (1993) State of the foreign gene and of the genome in transgenic rice (Oryza sativa L.). Cytotechnology, 11:123-125.

Barakat, A., Gallois, P., Raynal, M., Mestre-Ortega, D., Sallaud, C., Guiderdoni, E., Deseny, M., Bernadri, G. (2000) The distribution of T-DNA in the genomes of transgenic Arabidopsis and rice. FEBS Letters, 471:161-164.

Bechtold, N., Ellis, J., Pelletier, G. (1993) In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. Life Science C R Academic Science Paris, 316:1194-1199.

Bechtold, N., Jaudreau, B., Jolivet, S., Maba, B., Vezon, D., Voisin, R., Pelletier, G. (2000). The Maternal Chromosome Set Is the Target of the T-DNA in the In Planta Transformation of Arabidopsis thaliana. Genetics, 155:1875-1887.

Bechtold, N., Jolivet, S., Voisin, R., Pelletier, G. (2003) The Endosperm and the Embryo of Arabidopsis thaliana are Independently Transformed through Infiltration by Agrobacterium tumefaciens. Transgenic Research, 12:509-517.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R.L., Giraudat, J., Leung, J.L., Staskawicz, B.J. (1994). RPS2 of Arabidopsis thaliana: A Leucine-Rich Repeat Class of Plant Disease Resistance Genes. Science, 265:1856-1860.

Bered, F., Barbosa, N.J.F., Rocha, B.M., da Carvalho, F.I.F. (2002). Genetic variability in wheat (Triticum aestivum L.) germplasm revealed by RAPD markers. Crop Breeding and Applied Biotechnology, 2:499-505.

Birch, R.G. (1997). Plant transformation: problems and strategies for practical application. Annual Review in Plant Physiology and Plant Molecular Biology, 48:297-326.

Brunaud, V., Balzer, B., Dubreucq, B., Aubourg, S., Samson, F., Chauvin, S., Bechtold, N., Cruaud, C., DeRose, R., Pelletier, G. et al. (2000). T-DNA integration into the Arabidopsis genome depends on sequences of preinsertion sites. EMBO Reports, 3:1152-1157.

Budziszewski, G.J., Lewis, S.P., Glover, L.W., Reineke, J., Jones, G., Ziemnik, L.S., Lonowski, J., Nyfeler, B., Aux, G., Zhou, Q. (2001) Arabidopsis genes essential for seedling viability: isolation of insertion mutant plants and molecular cloning. Genetics, 159:1765-1778.

Bundock, P., Hooykaas, P.J.J. (1996) Integration of Agrobacterium tumefaciens T-DNA in the Saccharomyces cerevisiae genome by illegitimate recombination. Proceedings of National Academy of Sciences, 93(26):15272-15275.

Chang, S.S., Park, S.K., Kim, B.C., Kang, B.J., Kim, D.U., Nam, H.G. (1994) Stable genetic transformation of Arabidopsis thaliana by Agrobacterium inoculation in planta. Plant Journal, 5:551-558.

Chee, P.P., Slighton, J.L. (1995) Transformation of soybean (Glycine max) via Agrobacterium tumefaciens and analysis of transformed plants in KMA Gartland. In: Agrobacterium Protocols: Methods in Molecular Biology. Edited by Davey M., vol. 44. Totowa, NJ: Humana Press 101-119.

Chong, K., Tan, K.H. (1995) Function analysis of ver 203 gene through antisense transgenic winter wheat plants. Plant Physiology, 108:475-485.

Chowira, G.M., Akella, V., Fuerst, P.E., Lurquin, P.F. (1996). Transgenic grain legumes obtained by in planta electroproporation-mediated gene transfer. Molecular Biotechnology, 5(2):85-96.

Chowira, G.M., Akella, V., Lurquin, P.F. (1995) Electroporation-mediated gene transfer into intact nodal meristems in planta. Generating transgenic plants without in-vitro tissue culture. Molecular Biotechnology, 3(1):17-23.

Chumakov, M.I., Rozhok, N.A., Velikov, V.A., Tyrova, V.S, Volokhina, I.V. (2006) In planta transformation of maize through inoculation of Agrobacterium into the silks. Genetika, 42(8):1083-1088.

Chumakov, M.I., Kurbanova, I.V., Solovova, G.K. (2002) Agrobacterium-Mediated Transformation of Undamaged Plants. Fiziologiya Rastenii., 49(6):898-903.

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

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

Curtis, I.S., Nam, H.G. (2001) Transgenic radish (Raphanus sativus L. longipinnatus Bailey) by floral-dip method - Plant development and surfactant are important in optimizing transformation efficiency. Transgenic Research, 10(4):363-371.

Curtis, I.S., Nam, H.G., Yun, J.Y., Seo, K.H. (2002) Expression of an antisense GIGANTEA (GI) gene fragment in transgenic radish causes delayed bolting and flowering. Transgenic Research, 11:249-256.

Das, P., Joshi, N.C. (2011) Minor modifications in obtainable Arabidopsis floral dip method enhances transformation efficiency and production of homozygous transgenic lines harbouring a single copy of transgene. Advances in Biosciences and Biotechnology, 2:59-67.

Dehestani, G., Ahmadian, G., Salmanian, A. H., Jerold, N. B., Kazemtabar, K. (2010) Transformation efficiency enhancement
of Arabidopsis vacuum infiltration by surfactant application and apical inflorescence removal. Trakia Journal of Sciences, 8(1):19-26.

Desfeux, C., Clough, S.J., Bent, A.F. (2002) Female reproductive tissues as the primary target of Agrobacterium mediated transformation by the Arabidopsis floral-dip method. Plant Physiology, 123:895-904.

Dolezel, J., Novak, F.J. (1984) Effect of plant tissue culture media on the frequency of somatic mutations in Tradescantia stamen hairs. Zeitschrift für Pflanzenphysiologie, 114:51-58.

Feldmann, K. (1991) T-DNA insertion mutagenesis in Arabidopsis: mutational spectrum. Plant Journal, 1:71-82.

Feldmann, K.A., Marks, M.D. (1987) Agrobacterium mediated transformation of germinating seeds of Arabidopsis thaliana: A non-tissue culture approach. Molecular Genomics and Genetics, 208(1-9).

Fullner, K.J., Nester, E.W. (1996) Temperature Affects the T-DNA Transfer Machinery of Agrobacterium tumefaciens. Journal of Bacteriology, 178:1498-1504.

Gao, X.R., Wang, G.K., Su, Q., Wang, Y., An, L.J. (2007) Phytase expression in transgenic soybeans: stable transformation with a vector-less construct. Biotechnology Letters, 29(11):1781-1787.

Gassama-Dia Y.K., Sane, D., N’Doye, M. (2004) Direct genetic transformation of Hibiscus sabdariffa L. African Journal of Biotechnology, 3(4):226-228.

Gelvin, S.B. (2000) Agrobacterium and plant genes involved in TDNA transfer and integration. Plant Molecular Biology, 51:223-256.

Geng, H.W., Zhang, Q.Z., He, Z.H., Xia, L.Q., Chen, X.M., Wang, D.S., Qu, Y.Y. (2006) Molecular characterization of transgenic wheats with ectopic puroindoline genes. Scientia Agricultura Sinica, 39(9):1751-1755.

Graves, A.C., Goldman, S.L. (1986). The transformation of Zea mays seedlings with Agrobacterium tumefaciens: detection of T-DNA specific enzyme activities. Plant Molecular Biology, 7:43-50.

Hansen, G., Wright, M.S. (1999) Recent advances in the transformation of plants. Trends in Plant Sciences, 4:226-231.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., Cottage, A. (2006) A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. Plant Methods, 2:19.

Hasan, M., Khan, A.J., Khan, S., Shah, A.H., Khan, A.R., Mirza, B. (2008) Transformation of tomato (Lycopersicon esculentum mill.) with Arabidopsis early flowering gene apetala1 (api) through Agrobacterium infiltration of ripened fruits. Pakistan Journal of Botany, 40(1):161-173.

Holme, I.B., Brinch-Pedersen, H., Lange, M., Holme, P.B. (2006) Transformation of barley (Hordeum vulgare L.) by Agrobacterium tumefaciens infection of in-vitro cultured ovaules. Plant Cell Reports, 25:1325-1335.

Hou, W.S., Guo, S.D., Lu, M. (2003) Development of transgenic wheat with Galanthus nivalis agglutinin gene (sgna) via the pollen tube pathway. Chinese Bulletin of Botany, 20:198-204.

Hu, C.Y., Wang, L. (1999) In planta transformation technologies developed in China: procedure, confirmation and field performance. In-vitro Cellular and Developmental Biology of Plants, 35:417-420.

Jeon, J.S., Lee, S., Jung, K.H., Jun, S.H., Jeong, D.H., Lee, J., Kim, C., Jang, S., Yang, K., Nam, J. An, K., Han, M., Sung, R., Choi, H., Yu, J., Choi, J., Cho, S., Cha, S., Kim, S., An, G. (2000). T-DNA insertional mutagenesis for functional genomics in rice. Plant Journal, 22:561-570.

Katavic, V.C., Haughn, G.W., Reed, D., Martin, M., Kunst, L. (1994). In planta transformation of Arabidopsis thaliana. Moecular Genome and Genetics, 245:363-370.

Kaur, R.P. (2008) Agrobacterium mediated and in planta transformation methods for engineering insect resistance gene (cry1Ac) in Chickpea (Cicer arietinum L). Ph.D. Pantnagar, India: G.B. Pant University of Science and Technology.

Kojima, M., Shoiihi, H., Nogawa, M., Nozue, M., Matsumoto, D., Wada, A., Saiki, Y., Kiguchi, K. (2004) In planta Transformation of Kenaf Plants (Hibiscus cannabinus var. Aokawa No. 3 by Agrobacterium tumefaciens. Journal of Biosciences and Bioengineering, 98(2):136-139.

Kojima, M., Shoiihi, H., Nogawa, M., Nozue, M.; Matsumoto, D.; Wada, A., Saiki, Y., Kiguchi, K. (2000) Development of a Simple and Efficient Method for Transformation of Buckwheat Plants (Fagopyrum esculentum) Using Agrobacterium tumefaciens. Biosciences Biotechnology and Biochemistry, 64:845-847.

Koncz, C., Nemeth, K., Redei, G.P., Schell, J. (1992) T-DNA insertional mutagenesis in Arabidopsis. Plant Molecular Biology, 20:963-976.

Kumar, M.A., Reddy, K.N., Manjulath, M., Arellano, E.S., Sreevaths, R., Ganeshan, G. (2011) A rapid, novel and high-throughput identification of putative bell pepper transformatants generated through in planta transformation approach. Scientia Horticultrae, 129(4):898-903.

Kumar, M.A., Reddy, K.N., Sreevaths, R., Ganeshan, G., Kumar, M.U. (2009) Towards crop improvement in bell pepper (Capsicum annuum L.): transgenics (uid A::hpt II) by a tissue culture independent Agrobacterium mediated in planta approach. Scientia Horticultrae, 119:362-370.

Labra, M., Vannini, C., Grassi, F., Bracale, M., Balsemin, M., Basso, B., Sala, F. (2004) Genomic stability in Arabidopsis thaliana transgenic plants obtained by floral dip. Theoretical and Applied Genetics, 109:1512-1518.

Lennon, K.A., Roy, S., Helper, P.K., Lord, E.M. (1998) The Structure of the Transmitting Tissue of Arabidopsis thaliana (L.) and the Path of Pollen Tube Growth. Sexual Plant Reproduction, 11:49-59.

Li, F.G., Cui, J.J., Liu, C.L., Wu, Z.X., Li, F.L., Zhou, Y., Li, X.L. (2000) The study of insect-resistant transgenic cotton harbouring double-gene and its insect-resistance. Scientia Agricultura Sinica, 33(1):46-52.

Li, F.G., Guo, S.D., Liu, C.L., Li, F.L., Cui, H.Z., Zhou, Y., Li, X.L. (1999) The study on the transformation and selection of insect-resistant cotton harboring double-gene. Acta Gossypii Sinica, 11(2):106-112.

Li, J., Han, X.L., Shen, F.F., Liu, L. (2005) Study on promoting the rate of pollen-tube pathway transformation in cotton. Cotton Science, 17(2):67-71.

Li, J., Tan, X., Zhu F., Guo, J. (2010) A rapid and simple method for Brassica napus floral-dip transformation and selection of transgenic plantlets. International Journal of Biology, 2:127-131.
In planta transformation in plants: A review

Li, J., Todd, T.C., Trick, H.N. (2010) Rapid in planta evaluation of root expressed transgenes in chimeric soybean plants. Plant Cell Reproduction, 29:113-123.

Liu, F., Cao, M.Q., Yao, L., Li, Y., Robaglia, C., Tourneur, C. (1998) In planta transformation of pakchoi (Brassica campestris L. ssp. Chinensis) by infiltration of adult plants with Agrobacterium. Acta Horticulturae, 467:187-192.

Liu, J.F., Su, Q., An, L.J., Yang, A.F. (2009a) Transfer of a minimal linear marker-free and vector-free smGFP cassette into soybean via ovary-drip transformation. Biotechnology Letters, 31(2):295-303.

Liu, M., Yang, J., Cheng, Y., An, L. (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.J., Liu, Y.H., Wang, Z.X., Wang, X.J., Zhang, Y.Q. (2007) Generation of glyphosate-tolerant transgenic tobacco and cotton by transformation with a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. J Agricultural Biotechnology, 15(6):958-963.

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

Lurquin, P.F., Cai, Z., C.M., S., Fuerst, E.P. (1998) Half-embryo cocultivation technique for estimating the susceptibility of pea (Pisum sativum L.) and lentil (Lens culinaris Medik.) cultivars to Agrobacterium tumefaciens. Molecular Biotechnology, 9(2):175-179.

Martinell, B.J., Julson, L.S., Emler, C.A., Huang, Y., McCabe, D.E., Williams, E.J. (2002) Soybean Agrobacterium transformation method, US Patent . In. United States.

Mayavan, S., Subramanyam, K., Arun, M., Rajesh, M., Kapil Dev, G., Martinell, B.J., Julson, L.S., Emler, C.A., Huang, Y., McCabe, D.E., Williams, E.J. (2002) Soybean Agrobacterium transformation method, US Patent . In. United States.

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(10), 1557-1574.

McElver, J., Tzafrir, I., Aux, G., Rogers, R., Ashby, C., Smith, K., Thomas, C., Schetter, A., Zhou, Q., Cushman, M.A. et al. (2001). Insertional mutagenesis of genes required for seed development in Arabidopsis thaliana. Genetics, 159:1751-1763.

Ming, L., Yang, J., Cheng, Y.Q., Li-Jia (2009) An Optimization of soybean (Glycine max (L.) Merrill) in planta ovary transformation using a linear minimal gus gene cassette. Journal of Zhejiang University, 10(12):870-876.

Miyashita, N.T., Kawabe, A., Innan, H. (1999) DNA variation in the wild plant Arabidopsis thaliana revealed by amplified fragment length polymorphism analysis. Genetics, 152:1723-1731.

Mu, G., Chang, N., Xiang, K., Sheng, Y., Zhang, Z., Pan, G. (2012) Genetic Transformation of Maize Female Inflorescence Following Floral Dip Method Mediated by Agrobacterium Biotechnology,11:178-183.

Naseri, G., Sohani, M.M., Pourmassalehgou, A., Allahi, S. (2012) In planta transformation of rice (Oryza sativa) using thaumatin-like protein gene for enhancing resistance to sheath blight. African Journal of Biotechnology, 11(31):7885-7893.

Orzaez, D., Mirabel, S., Wieland, W.H., Granell, A. (2006) Agroinjection of Tomato Fruits. A Tool for Rapid Functional Analysis of Transgenes Directly in Fruit. Plant Physiology, 140:3-11.

Penza, R., Akella, V., Lurquin, P.F. (1992) Transient expression and histological localization of a gus chimeric gene after direct transfer to mature cowpea embryos. Biotechnology, 13(4):576-580.

Pooran, C., Singh, S.P. (2003) Genetic diversity maintenance of cross pollinated germplasm. Progressive Agriculture, 3:1-7.

Pradeep, T., Sumalini, K. (2003) Impact of mating systems on genetic variability in segregating generations of Asiatic cotton (Gossypium sp.). Ind. J. Genet. Plant Breeding, 63:143-147.

Qing, C.M., Fan, L., Lei, Y., Bouchez, D., Tourner, C., Yan, L., Robagalia, C. (2000) Transformation of Pakchoi (Brassica rapa L. ssp. chinensis) by Agrobacterium in-filtration. Molecular Breeding, 6,67-72.

Radchuk, V.V., Van, D.T., Klocke, E. (2005) Multiple gene co-integration in Arabidopsis thaliana predominantly occurs in the same genetic locus after simultaneous in planta transformation with distinct Agrobacterium tumefaciens strains. Plant Science, 168:1515-1523.

Ratansut, K., Rod-In, W., Sujipuli, K. (2017) In planta Agrobacterium-Mediated Transformation of Rice. Rice Science 24(3):181-186.

Razaq, A., Hafiz, I.A., Mahmod, I., Hussain, A. (2011) Development of in planta transformation protocol for Wheat. African Journal of Biotechnology, 10(5):740-750.

Rohini, V.K., Rao, K.S. (2000) Embryo Transformation, A Practical Approach for Realizing Transgenic Plants of Safflower (Carthamus tinctorius L.). Annals of Botany, 86:1043-1049.

Rohini, V.K., Rao, S. (2000) Transformation of peanut (Arachis hypogea L.) A non-tissue culture based approach for generating transgenic plants. Plant Science, 150:41-49.

Russell, S.D. (1993). The egg cell: development and role in fertilization and early embryogenesis. Plant Cell, 5:1349-1359.

Sala, F., Arecibia, A., Castiglione, S., Yифан, H., Labra, M., Savini, C., Bracale, M., Pelucchi, N. (2000) Samoclonal variation in transgenic plants. Acta Horticulturae, 530:411-419.

Sebastiani, L., Lenzi, A., Pugliesi, C., Fambrini, M. (1994) Samoclonal variation for resistance to Verticillium dahliae in potato (Solanum tuberosum L.) plants regenerated from callus. Euphytica, 80:5-11.

Senaratna, T., McKersie, B.D., Kashia, K.J., Procunier, J.D. (1991). Direct DNA uptake during the imbibition of cells. Plant Science, 79:223-228.

Seol, E., Jung, Y., Lee, J., Cho, C., Kim, T., Rhee, Y., Lee, S. (2008) In planta transformation of Notocactus scopa cv. Soonjung by Agrobacterium tumefaciens. Plant Cell Reporsts, 27:1197-1206.

Shah, S.H., Ali, S., Jan, S.A., Jalal-u-uddin, 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, 6:67-72.

Smagur, W., Konka, K.H., Kononowicz, A.K. (2009) Flower Bud Dipping or Vacuum Infiltration-Two Methods of Arabidopsis thaliana Transformation. (4), 560-568. Russian Journal of Plant
In planta transformation in plants: A review

Physiology, 56(4):560-568.

Spolaore, S., Trainotti, L., Casadoro, G. (2001) A simple protocol for transient gene expression in ripe fleshy fruit mediated by Agrobacterium. Journal of Experimental Botany, 52:845-850.

Supartana, P., Shimizu, T., Nogawa, M., Shioiri, H., Nakajima, T., Haramoto, N., Nozue, M., Kojima, M. (2006) Development of simple and efficient In planta transformation method for wheat (Triticum aestivum L.) using Agrobacterium tumefaciens. Journal of Biosciences and Bioengineering, 98(3):162-170.

Supartana, P., Shimizu, T., Shioiri, H., Nogawa, M., Nozue, M., Kojima, M. (2005) Development of simple and efficient In planta transformation method for rice (Oryza sativa L.) using Agrobacterium tumefaciens. Journal of Biosciences and Bioengineering, 100:391-397.

Szabados, L., Kovacs, I., Oberschall, A., Abraham, E., Kerekés, I., Zsigmond, L., Nagy, R., Alvarado, M., Krasovskaia, I., Gal, Berente, M. A., Redei, G. P., Haim, A. B., Koncz, C. (2002) Distribution of 1000 sequenced T-DNA tags in the Arabidopsis genome. Plant Journal, 32:233-242.

Tague, B.W. (2001) Germ-line transformation of Arabidopsis lasiocarpa. Transgenic Research, 10:259-267.

Thomas, C.M., Jones, D.A., English, J.J., Carroll, B.J., Bennetzen, J.L., Harrison, K., Burbidge, A., Bishop, G.J., Jones, J.D. (1994) Analysis of the chromosomal distribution of transposon-carrying TDNAs in tomato using the inverse polymerase chain reaction. Molecular Genomics and Genetics, 242:573-585.

Tjokrokusumo, D., Heinrich, T., Wylie, S., Potter, R., McComb, J. (2000) Vacuum infiltration of Petunia hybrida pollen with Agrobacterium tumefaciens to achieve plant transformation. Plant Cell Reports, 19:792-797.

Trieu, A.T., Burleigh, S.H., Kardalsky, I.V., Maldonado-Mendoza, I.E., Versaw, W.K., Blaylock, L.A., Shin, H., Chiou, T.J., Katagi, H., Dewbre, G.R. et al. (2000) Transformation of Medicago truncatula via infiltration of seedlings or flowering plants with Agrobacterium. Plant Journal, 22:531-541.

Wang, T.L., Domoney, C., Hedley, C.L., Casey, R., Grusak, M.A. (2003) Can we improve the nutritional quality of legume seeds? Plant Physiology, 131:886-891.

Weeks, J.T., Ye, J., Rommens, C.M. (2008) Development of an in planta method for transformation of alfalfa (Medicago sativa). Transgenic Research., 17:587-597.

Wu, H.M., Wang, H., Cheung, A.Y. (1995) A Pollen Tube Growth Stimulatory Glycoprotein Is Deglycosylated by Pollen Tubes and Displays a Glycosylation Gradient in the Flower. Cell, 82:395-403.

Wu, W., Su, Q., Xia, X.Y., Wang, Y., Luan, Y.S., An, L.J. (2008) The Suaeda liaoautensgus Kitag betaine aldehyde dehydrogenase gene improves salt tolerance of transgenic maize mediated with minimum linear length of DNA fragment. Euphytica, 159(1-2):17-25.

Xu, H., Wang, X., Zhao, H., Liu, F. (2008) An intensive understanding of vacuum infiltration transformation of pakchoi (Brassica rapa ssp. chinensis). Plant Cell Reports, 27:1369-1376.

Yang, A.F., Su, Q., An, L. (2009) Ovary-drip transformation: a simple method for directly generating vector- and marker-free transgenic maize (Zea mays L.) with a linear GFP cassette transformation. Planta, 229(4):793-801.

Yang, A.F., Su, Q., An, L.J., Liu, J.F., Wu, W., Qiu, Z. (2009) Detection of vector- and selectable marker-free transgenic maize with a linear GFP cassette transformation via the pollen-tube pathway. Journal of Biotechnology, 139(1):1-5.

Yasmeen, A., Mirza, B., Inayatullah, S., Saifdar, N., Jamil, M., Ali, S., Choudhry, M.F. (2009) In Planta Transformation of Tomato. Plant Molecular Biology Reports, 27:20-28.

Ye, G.N., Stone, D., Pang, S.Z., Creely, W., Gonzalez, K., Hinchee, M. (1999) Arabidopsis ovule is the target for Agrobacterium in planta vacuum infiltration transformation. Plant Journal, 19:249-257.

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(6):903-913.

Zeng, J.Z., Wang, D.J., Wu, Y.Q., Zhang, J., Zhou, W.J., Zhu, X.P., Xu, N.J. (1994) Transgenic wheat plants obtained with pollen tube pathway method: Science China (37):319-325.

Zhang, H., Zhao, F., Zhao, Y., Guo, C., Li, C., Xiao, K. (2009) Establishment of transgenic cotton lines with high efficiency via pollen-tube pathway. (4), 359-365. Frontiers in Agriculture of China, 3(4):359-365.

Zhang, Y.S., Yin, X.Y., Yang, A.F., Li, G.S., J.R., Z. (2005) Stability of inheritance of transgenenes in maize (Zea mays L.) line produced using different transformation methods. Euphytica, 144:11-22.

Zhou, G.Y., Wang, C.L., Zong, S.Y., Huang, J.L., Gong, Z.Z. (2003) Herbicide resistant transgenic rice (Oryza sativa L.) transformed by pollen tube pathway method and its inheritance. Chinese Journal of Biotechnology, 23:92-97.

Zhao, L.M., Liu, D.P., Sun, H., Yun, Y., Huang, M.A. (1995) Sterile material of soybean gained by introducing exogenous DNA. Soybean Science, 14:83-87.

Zhou, G.Y., Gong, Z.Z., Wang, Z.F. (1979) The molecular basis of remote hybridization: an evidence of the hypothesis that DNA segments of distantly related plants may be hybridized. Acta Genetica Sinica, 6(4):405-413.

Zhou, G.Y., Weng, J., Gong, Z., Zeng, Y., Yang, W., Shen, W. (1988) Molecular breeding of agriculture: a technique for introducing exogenous DNA into plants after self-pollination. Scientia Agriculturae Sinica, 21:1-6.

Zhou, G.Y., Weng, J., Zeng, Y.S., Huang, J.G., Qian, S.Y., Liu, G.L. (1983) Introduction of exogenous DNA into cotton embryos. In: Methods in Enzymology. Edited by Wu R., Grossman L., Moldave K., vol. 101. New York: Academic press 433-481.

Zia, M., Arshad, W., Bibi, Y., Nisa, S., Chaudhry, M. F. (2011) Does Agro-injection to soybean pods transform embryos? Plant Omics Journal, 4(7): 384-390.

Zuo, K.J., Zhang, X.L., Nie, Y.C. (2002) Sequence analysis of Bt endotoxin genes in Bt cotton. Acta Genetica Sinica, 29:735-740.

Zupan, J., Muth, T.R., Draper, O., Zambrysky, P. (2000) Transient gene expression in ripe fleshy fruit mediated by Agrobacterium tumefaciens. Journal of Biotechnology, 81:407-419.