

FLORAL DIP: A SIMPLE AND EFFICIENT AGROBACTERIUM MEDIATED TRANSFORMATION METHOD IS USED IN A MODEL PLANT *ARABIDOPSIS THALIANA*

***Sushanta Kumar Barik**

National Botanical Research Institute, Rana Pratap Marg, Lucknow, 226001, India

**Author for Correspondence*

ABSTRACT

“Floral dip” is a successful *Agrobacterium* transformation method achieved in *Arabidopsis thaliana*. This method of *Agrobacterium* mediated transformation in *Arabidopsis thaliana* is providing the knowledge on basic science research in the area of plant biotechnology. Most of the laboratories in the world are getting successful results in the area of genomics by transforming genes and promoters of *Arabidopsis thaliana*. This method helps to researcher to know about the basic function of a gene and promoter in *Arabidopsis thaliana*. Many developed countries are funding projects on transgenic research on *Arabidopsis thaliana* due to a valid protocol available for transformation process.

Key Words: *Arabidopsis Thaliana, Floral dip, Agrobacterium, Promoters, Genes*

INTRODUCTION

Arabidopsis thaliana belongs to a member of cruciferae family and is familiar with basic research in plant science as a model plant (Meinke *et al.*, 1998). The *Arabidopsis thaliana* “floral dip” method is chosen as a best method for plant transformation because this method requires minimal labor, no plant tissue culture and can be performed successfully by nonspecialists. *Arabidopsis thaliana* flowering plants are simply dipped or sprayed with a particular strain of *Agrobacterium* carrying the gene of interest. After transformation process, seeds are collected from T0 plants and again under gone selection process by presence of antibiotics for next T1 individuals.

This method has been used in hundreds of laboratories in the world due to result of transformation process is atleast 1% efficiency (Logemann *et al.*, 2006).

The *Arabidopsis thaliana* research information is available in www.arabidopsis.org and transgenic lines information is available in (<http://signal.salk.edu/cgi-bin/tdnaexpress> (Alonso *et al.*, 2003). The dominant technology used for the production of transgenic plants is based on *Agrobacterium* mediated machinery. The interaction between *Arabidopsis* –*Agrobacterium* mechanism is a magic attraction in the field of plant molecular biology for insertion of foreign DNA with a suitable vector carrying T-DNA.

A brief important description of *Agrobacterium* mediated transformation events:

Agrobacterium tumefaciens is commonly used as a transformation vehicle for the production of transgenic plants, help to basic science research in the field of agriculture biotechnology. The main role of *Agrobacterium* transformation is mediated by VirE2 interacting protein VIP1 (Liu *et al.*, 2010).

However, the increase in efficiency of transformation in *Arabidopsis thaliana* is due to histone H2A-1 encoded by HTA1 gene (Tenea *et al.*, 2009). A most significant event mediated by *Agrobacterium* in T-DNA (direct repeats in both left and right ends) insertion in to plant cells is mediated by two bacterial proteins VirD2 and VirE2 (Ballas *et al.*, 2009; Tzfira *et al.*, 2001).

The mechanism of transfer of T-DNA by *Agrobacterium tumefaciens* is unidirectional (Miranda *et al.*, 1992). Both VirE1VirE2 complexes are responsible for transfer of single stranded DNA from *Agrobacterium tumefaciens* to nucleus of plantcells by formation of channels (Duckely *et al.*, 2005). Experimental evidence suggests the low frequency of transformation depends on the number of T-DNA copies during floral dip transformation process (Debuck *et al.*, 2009).

Review Article

Use of agrobacterium Strains in Transformation Process

Ti plasmid	<i>Agrobacterium</i> strain
1. Octopine	LBA4404, GV2260
2. Nopaline	C58C1, GV3100, GV3101, GV3850, A136, EHA101
3. Agropine	A281, A543
4. Succinamopine	EHA105, AGL1

Agrobacterium strains containing different Ti plasmids and opine utilization used in transformation process in *Arabidopsis thaliana* (Dessaux *et al.*, 1992).

Floral dip is a Magic Protocol that Avoids Tissue Culture

Plant transformation technology is knowledge based provides basic scientific research in the area of plant molecular biology and genetic engineering. Initially the transgenic plants are generated through plant cells or tissues, introduction of DNA using *Agrobacterium tumefaciens* or particle bombardment and propagation of plants. Therefore, this kind of transformation methods depends on three matters like skilled labor, time and expensive laboratory facilities (Christou *et al.*, 1996). In contrast the transformation of *Arabidopsis thaliana* is well developed by “*Arabidopsis* vacuum infiltration” method. In this method *Arabidopsis* grown up to flowering stage, uprooting of plants, then mount of *Agrobacterium* to whole plants via vacuum infiltration in a sucrose or hormone medium, replantation and collection of seeds and progeny are identified on media containing antibiotic or herbicide (Bechtold *et al.*, 1993). In present research in laboratory, vacuum infiltration protocol was eliminated by “Floral dip” method by simple dipping inflorescences in to a solution containing *Agrobacterium tumefaciens*, 5% sucrose and surfactant silwet L-77 (concentration varies in range .01% to .1%) (Clough *et al.*, 1998). Then this method is widely used in all accessions of *Arabidopsis thaliana* and the transformation frequency is at least 1% generally obtained (Zhang *et al.*, 2006). In another protocol of floral dip method, the transformation efficiency of *Arabidopsis thaliana* increased to 2-3% when the infected inoculum OD 600 was .8 to >2 (Martinez-Trujillo *et al.*, 2004).

Description of Current Protocols Used in Laboratory in Transformation Process

A. (Clough and Bent, 1998)

1. *Arabidopsis thaliana* plants were grown up to flowering stage in a green house at 20 degree celcius in a long day condition.
2. The primary bolts were clipped off for obtaining more flower bud
3. *Agrobacterium tumefaciens* strain GV3101 carrying the binary plasmid pBINm-gfp5-ER were grown up to stationary phase in liquid media at 25-28 degree celcius, 250 rpm in a sterilized liquid broth and initial dilution of culture was 1:100.
4. Then *Agrobacterium* cells were harvested and pelleted at RT 5500 g for 20 minute and resuspended in infiltration medium to a final OD 600 of approximately 0.80. The inoculation medium contained 5.0% sucrose and 0.05% silwet L-77.
5. The whole solution were prepared in a beaker containing inoculum and plants were inverted in to this suspension for 3-5 sec of gentle agitation after then dipped plants were removed from beaker and covered with plastic dome to covered to maintain humidity in a plastic tray.
6. The *Arabidopsis* plants were kept in a dark location overnight in a green house and domes were removed approximately 12-24 hours.
7. Plants were grown up to 3-5 weeks until siliques were brown and dry seeds were harvested by gentle handling of inflorescences over a piece of clean paper.
8. Seeds were stored at 4 degree celcius under desiccation.
9. Selection of transformants using an antibiotic marker.

Transformants selection through Antibiotic markers:

Two methods are used to sterilize the seeds.

1. Liquid sterilization

Review Article

2. Vapor-phase sterilization.

1. In liquid sterilization, seeds were first treated with 95% ethanol for 30-60 seconds add with 50% bleach containing 0.05% tween 20 for 5 minute followed by three rinses with sterile water.

2. In vapor phase sterilization, seeds were placed in to a 10 liter desicator jar, placed in a fume hood. Add 100 ml bleach and 3 ml concentrated HCL carefully on to the desicator jar. The chlorine fumes within the desicator remained sealed 4 to 15 hours.

A. Transformed seeds were suspended in a 0.1% sterile agarose with kanamycin or other antibiotics selection plates at a density of approximately 3000 seeds per 150 X 15 mm square agar (½ MS medium, 0.8% Agar, 50microgram /ml kanamycin monosulfates) plate. Keep the plates for 2 days at 4 degree celcius and then grown in green house for 7 to 10 days in a controlled environment at 24 degree celcius under 23 hour light 50-100 micro einsteins m⁻² s⁻¹.

B. Petriplates were sealed with surgical tape during first week of growth. The plates containing some transformants with 3-5 adult leaves and then transferred in to moistened potting soil.

Transformation Rate

Transformation rate expressed as ‘percentage of transformation’. It is calculated as:

Number of kanamycin or other resistant seedlings /total number of seedlings tested X 100

B. (Zhang et al., 2006)

1. This procedure is divided in to three parts:

(A) Growth of *Arabidopsis thaliana* plants up to inflorescens stage.

(B) Floral dipping transformation

(C) Transformants screening.

(A) Growth of *Arabidopsis Thaliana* Plants Up to Inflorescens Stage

1. *Arabidopsis thaliana* seeds were grown in 0.05% agar and keep darkness for 3days to break dormancy. This short period allows maximal seed germination.

2. *Arabidopsis thaliana* 20-30 seeds spread on wet soil in each 4X4 inch. pot.

Or

Arabidopsis thaliana sterilize seeds placed on MS medium. Then keep the seeds in darkness at 4 degree celcius for 3 days. Keep the seeds for germination up to 2 weeks in long day conditions (16 h light /8hour dark, 20 degree celcius) and transfer them to wet soil. Nylon screen or a piece of cheese cloth were used to cover the soil tighten by a rubber band.

3. *Arabidopsis thaliana* plants are grow in a growth chamber or a green house under short days for 3-4 weeks gradually shift them to long day conditions to induce flowering.

4. Before flowering stage make constructs ready. *Arabidopsis thaliana* plants with more immature flower clusters, then remove the first bolts to allow proliferation that can allow proliferation of many secondary inflorescens. Enhance the nutrient supply to plants.

(B) Floral Dipping Transformation

1. Preparation of *Agrobacterium* strain containing the gene of interest in a binary vector. Then inoculate this strain in to a 5 ml liquid LB medium containing the appropriate antibiotics and incubate it at 28 degree celcius for 2 days.

2. Inoculation of feeder culture in a 500 ml liquid LB medium containing appropriate antibiotics and grow the culture up to stationary phase (OD 1.5-2.0) at 28 degree celcius for 16-24 hours.

3. *Agrobacterium* cells collect by centrifugation at 4,000g for 10 minute at RT (room temperature).

4. *Agrobacterium* cells are gently resuspended in 1 volume of freshly made 5% sucrose solution with a stirring bar. A concentration of 0.02% silwet L-77 is use in the mixing solution and *Agrobacterium* cell suspension transfer to a 500 ml beaker (Silwet L-77 is generally use up to 0.02%(vol/vol). Higher concentration might be toxic. *Agrobacterium* cell suspension quantity up to 400-500 ml required for transformation of at least six pots of *Arabidopsis thaliana*).

5. *Arabidopsis thaliana* plants are invert and dip in to the *Agrobacterium* cell suspension for 10 seconds with gentle agitation (Dip both inflorescens and rosette to soak shorter axillary inflorescens).

Review Article

(Before disposal, the remaining *Agrobacterium* cell suspension is treating with 1 volume of Clorox bleach to kill bacteria before disposal of the suspension).

6. *Arabidopsis thaliana* plants are removed from the solution and wash excess liquid on the plants.
7. Plants are wrapping with plastic cover or plastic film. Plants are laydown on their sides for 16-24 hours to maintain high humidity (Treated plants are not expose to high temperatures or excessive light).
8. On the next day, remove the cover on the plants and keep the plants in a green house or the growth chamber. Plants are growing normally for one month by supplying water when siliques turn brown.
9. Plant dry seeds collect using a sieve mesh (for screening, keep the harvested dry seeds at room temperature or at 4 degree celcius).

C. Transformants Screening

1. Prepare selection MS- Agar plates containing carbenicillin and the appropriate antibiotics or herbicide (Carbenicillin is used for decontamination of harvested seeds covered with agrobacteria).

Composition of MS Agar (Murashige and Skoog salts):

- A. Murashige and Skoog salts -4.3 g., 10 g sucrose ,0.5 g MES,8 g Agar/ liter, PH 5.7
- B. Different concentrations of antibiotics for selection: (Kanamycin 50 mg/l, Phosphinothricin or Basta 10mg/l ,Hygromycin 25 mg/l and Carbenicillin 100 mg/l).
2. Seeds are sterilized by treating with 50X volume of 70% ethanol for one minute and well mix the seeds.
3. Then again sterilize by treating the seeds with a 50X volume of 50% bleach /50% water/0.05% Tween for 10 minute and every two minute vortex the suspension.
4. Seeds are rinse three times with sterile water (The wash should appear clear without any yellow color).
5. Sterilized seeds are resuspended in previously made agar plates.
6. Seeds are vernalizing by placing them at 4 degree celcius for 3 days.
7. Then keep the plates in a tissue culture room or a growth chamber under continuous light (50-100 microeinsteins m⁻¹ s⁻¹) and transformants are readily distinguished as seedlings with healthy green cotyledons and true leaves , roots are extend in to medium after 7-10 days.
8. Once leaves are emerge from transformants then only transfer to a fresh selection plate.
9. Plantlets are transplant to water –saturated soil and tray cover with a plastic film to maintain high humidity for 2 days. Grow the plants in a green house or growth chamber under continuous light fo seed collection (Gently remove the plantlets root from the agar to avoid breakage).

Floral Dip Method is used to Transform Genes and Promoters in *Arabidopsis Thaliana*

The comparasion between the vaccum infiltration and floral dip method was developed during transformation of a superoxide dismutase gene in *Arabidopsis* and floral dip method was efficiently generated transgenic plants rather than other methods (Chung *et al.*, 2000). Transformation frequencies of *Agrobacterium tumefaciens* strain ABI and GV3101 harboring CCR2-LUC-HygR Trans gene varies using LB and YEBs media. Robust transformation by *Agrobacterium tumefaciens* strain ABI and GV3101 in *Arabidopsis thaliana* transgenics was observed in YEBs media containing 25 gram per liter sucrose and 200 microliter silwet L-77 per liter of culture, lower transformation efficiency in LB media (Davis *et al.*, 2009) and the *Arabidopsis* transformed seeds germinate within 3.25 days rather than 7-10 days had been proved in one of protocol containing *Agrobacterium* GV3101 strain carrying pBIN plus confers kanamycin resistance containing nptII gene, pSKI015 confers phosphinothricin resistance via bar gene and pBIG-HYG confers hygromycin B resistance via hpt gene (Harrison *et al.*, 2009). A modified method of plant transformation of *Arabidopsis thaliana* is based on preparation of *Agrobacterium cells*. By using this protocol successful *Arabidopsis* ecotypes col-0 and ler transformants are obtained through pKWS05 vector containing FLS2 gene (At5g46330) in *Agrobacterium* strain GV3101 (Logemann *et al.*, 2006). In Floral dip method, *Arabidopsis thaliana* ovules are primary target for *Agrobacterium* which was examined through 35s, LAT52 and ACT11 promoters fused with Gus A gene encoding B-glucuronidase *Agrobacterium* GV3101 strain (Desfeux *et al.*, 2000). The promoter sequences of three anther genes such as lipid transfer protein (LTP12), Xyloglucan endotransglucosylase / hydrolase (XTH3) and

Review Article

polygalacturonase (PGA4) well characterized in *Arabidopsis thaliana* by using this method (Ariizumi *et al.*, 2002). The *Arabidopsis thaliana* FAE1 (fatty acid elongation) gene and FAE1 promoter activity was studied in seeds using this suitable method through transformation by *Agrobacterium tumefaciens* strain GV3101 (Rossak *et al.*, 2001). In *Arabidopsis thaliana*, the alternative oxidase (AOX) genes provide a good model system for studies of the nuclear response to mitochondrial perturbations. The *Arabidopsis* AOX1 gene promoter mitochondrial retrograde regulation (MRR) region have been well characterized by *Agrobacterium tumefaciens* C58C1 (Dojcinovic *et al.*, 2005).

The *Arabidopsis thaliana* Genes and Promoters are transformed by floral dip method.

Strain	Vector	Gene	Promoter	References
GV3101	pBI121	Super oxide dismutase(SOD)	-----	Chung <i>et al.</i> , 2000
ABI,GV3101		CCR2	-----	Davis <i>et al.</i> , 2009
G V3101	pAL145,pBI,pCD1301, pCAMBIA2301	Gus A	LAT52,ACT11,ACT11, 35s	Desfeux <i>et al.</i> , 2000
GV3101	pBINPLUS,pSKI015, pBIG-HYG	CA1,uidA,mGFP4	-----	Harrison <i>et al.</i> , 2009
GV3101	pKWS05	FLS2	-----	Logemann <i>et al.</i> , 2006
EHA105	pBI101	-----	LTP12,XTH3,PGA4	Arrizumi <i>et al.</i> , 2002
GV3101	pBI101	FAE1	FAE1	Rossak <i>et al.</i> , 2001
C58C1	pBS1.2ALE	-----	AOX1	Dojcinovic <i>et al.</i> , 2005

Conclusion

Erwin smith was the first person to begin his carrier in *Agrobacterium* but later this bacterium is used in plant molecular breeding. *Agrobacterium* was a major tool for plant genetic engineering. The golden years of *Agrobacterium* research focus on bacterial biological processes, mechanisms etc. Now a days the *Agrobacterium* strains were designed and constructed for binary plasmids specifically tailored for each host species. The foundation for “*Agrobacterium*” like technologies was recently showing the gene transfer to plant species.

ACKNOWLEDGEMENT

Thanks to CSIR, Govt. of India provided me research opportunity in NBRI, Lucknow under Dr Samir V.Sawant, Principal Scientist. Also thanks to Dr C.S. Mohanty, Dr S.N. Jena in NBRI, Lucknow, Uttarpradesh and Dr R.K. Hazra, Regional Medical Research Centre, Bhubaneswar, Odisha.

Review Article

REFERENCES

- Alonso JM, Stepanova AN and Leisse TJ (2003).** Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301** 653–657.
- Ariizumi T, Amagai M, Shibata D, Hatakeyama K, Watanabe M and Toriyama K (2002).** Comparative study of promoter activity of three anther-specific genes encoding lipid transfer protein, xyloglucan endotransglucosylase/hydrolase and polygalacturonase in transgenic *Arabidopsis thaliana*. *Plant Cell Reports* **21** 90–96.
- Ballas N and Citovsky V (1997).** Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. *Proceedings of the National Academy of Sciences USA* **94** 10723–10728.
- Bechtold N, Ellis J and Pelletier G (1993).** In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *Comptes Rendus de l'Académie des Sciences Paris, Life Sciences* **316** 1194–1199.
- Chung MH, Chen MK and Pan SM (2000).** Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. *Transgenic Research* **9** 471–476.
- Clough SJ and Bent AF (1998).** Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**(6) 735–743.
- Christou P (1996).** Transformation technology. *Trends in Plant Science* **1** 423–431.
- Davis AM, Hall A, Millar AJ, Darrah C and Davis SJ (2009).** Protocol: Streamlined sub-protocols for floral-dip transformation and selection of transformants in *Arabidopsis thaliana*. *Plant Methods* **5** 3.
- De Buck S, Podevin N, Nolf J, Jacobs A and Depicker A (2009).** The T-DNA integration pattern in *Arabidopsis* transformants is highly determined by the transformed target cell. *The Plant Journal* **60** 134–145.
- Duckely M, Oomen C, Axthelm F, Gelder PV, Waksman G and Engel A (2005).** The VirE1VirE2 complex of *Agrobacterium tumefaciens* interacts with single-stranded DNA and forms channels. *Molecular Microbiology* **58**(4) 1130–1142.
- Dojcinovic D, Krosting J, Harris AJ, Wagner DJ and Rhoads DM (2005).** Identification of a region of the *Arabidopsis AtAOX1a* promoter necessary for mitochondrial retrograde regulation of expression. *Plant Molecular Biology* **58**(2) 159–175.
- Desfeux C, Clough SJ and Bent AF (2000).** Female Reproductive Tissues Are the Primary Target of *Agrobacterium*-Mediated Transformation by the *Arabidopsis* Floral-Dip Method. *Plant Physiology* **123** 895–904.
- Dessaux Y, Petit A and Tempe J (1996).** Opines in *Agrobacterium* biology. In: Verma DPS (ed) *Molecular signals in plant-microbe communications*, CRC Press, Boca Raton 109–136.
- Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC and Cottage A (2009).** A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings. *Plant Methods* **2006** **2** 19.
- Liu Y, Kong X, Pan J, Li D (2010).** VIP1: linking *Agrobacterium*-mediated transformation to plant immunity? *Plant Cell Reports* **29** 805–812.
- Logemann E, Birkenbihl RP, Ulker B and Imre E Somssich LE (2006).** An improved method for preparing *Agrobacterium* cells that simplifies the *Arabidopsis* transformation protocol. *Plant Methods* **2** 16.
- Meinke DW, Cherry M, Dean C, Rounsley SD and Koornneef M (1998).** *Arabidopsis thaliana*. A model plant for genome analysis, *Science* **282** 662–282.
- Miranda A, Janssen G, Hodges L, Ernest G, Peralta EG and Ream W (1992).** *Agrobacterium tumefaciens* Transfers Extremely Long T-DNAs by a Unidirectional Mechanism. *Journal of Bacteriology* **228** 2288–2297.
- Martinez-Trujillo M, Limones Briones V, Carbreponce JL and Herrera-estrilla L (2004).** Improving transformation efficiency of *Arabidopsis thaliana* by modifying the floral dip method. *Plant Molecular Biology Reporter Journal* **22** 63–70.

Review Article

Rossak M and Smith M (2001). Expression of the FAE1 gene and FAE1 promoter activity in developing seeds of *Arabidopsis thaliana*. *Plant Molecular Biology* **46**(6) 717-25.

Tenea GN, Spantzel J, Lee Ly, Zhu Y, Lin K, Johnson SJ and Gelvin SB (2009). Overexpression of Several *Arabidopsis* Histone Genes Increases *Agrobacterium*-Mediated Transformation and Transgene Expression in Plants. *The Plant Cell* **21** 3350–3367.

Tzfira T, Vaidya M and Citovsky V (2001). VIP1 ,An *Arabidopsis* protein that interact with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. *The EMBO Journal* **20**(13) 3596-3607.

Zhang X, Henriques R, Lin S-S, Niu QW and Chua NH (2006). *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols* **1**(2) 947–953.