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Minor modifications in obtainable *Arabidopsis* floral dip method enhances transformation efficiency and production of homozygous transgenic lines harboring a single copy of transgene

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ABSTRACT

Many researchers have developed various methods for *in-planta* or floral dip transformation of *Arabidopsis thaliana*, one of the simple protocol and widely used to produce transgenic *Arabidopsis*. As the efficiency and ease of getting a transformant is very much time consuming effort and less number of the transformants people get, we have developed a little modified transformation protocol to avoid the disparities. Four types of inoculums (inoculum-1, inoculum-2, inoculum-3 and inoculum-4) were used to check the transformation efficiency out of which Inoculum-3 showed the highest rate of transformation among the four types. 0.07% Twin-20 also acts in same manner as silwet L-77 to increase the rate of transformation efficiency and glucose instead of sucrose can be used in inoculum to transform *Arabidopsis*. After vacuum infiltration keeping the *Agrobacterium* infected plants for 7 - 8 hrs horizontally in low light at 28^{0}C temperature condition, considered best to get an increased number of transformed seeds. Modified protocol produced ~ 12% - 14% increase in transformants. Selection pots (kanamycin supplemented soil filled pots) in place of selection plates (Kanamycin supplemented Murashige and Skoog agar plates) proved beneficial as no MS medium and no aseptic condition is required for selection of transformed plants. This increase in transformation efficiency consequently increased the percentage of homozygous and single copied stable transgenic lines.

Keywords: *Arabidopsis Thaliana*; Floral-Dipping; Copy Number; Homozygous; Inoculum; Transgenic Plant

1. INTRODUCTION

Efficient study of plant gene function has been permissible through transgenic approaches. Plant transformation is a process of genetic operation by which foreign genes are introduced into plant genome and stably integrated and the transformed cells are regenerated to get transgenic plants. A plant transformation method that excludes the use of tissue culture and plant regeneration would greatly reduce the time required to produce transgenic plants, and such a method was first described as "*in planta*" transformation almost 20 years ago [1]. Much effort has been given to get stable transgenic lines. Feldmann and Marks [1] co-cultivated germinating seeds of *Arabidopsis thaliana* with an *Agrobacterium tumefacien* strain harboring a disarmed Ti plasmid and a binary vector, and remarkably, they got stable transgenic lines, although the transformation rates were very low. Several years later, the Pelletier group infiltrated flowering *Arabidopsis* plants with *Agrobacterium* and dramatically improved the transformation efficiency [2]. This account of transformation approach was frequently named as the "*Agrobacterium* vacuum infiltration method" [3]. Briefly, it involves uprooting of flowering *Arabidopsis* plants, vacuum infiltration of the plants using an *Agrobacterium* cell suspension, re-planting, harvesting of seed several weeks later, and screening for primary transformants on medium containing the appropriate selective agent (usually an antibiotic or a herbicide). These transformation procedures were later simplified and substantially improved [4]. The vacuum-aided infiltration of inflorescence [2] was substituted using a surfactant (Silwet L-77), which had already been shown, in the formulation of some pesticides, to help chemicals enter the plant tissues. All these modifications simplified the initial procedure. The *Arabidopsis* flower buds were simply dipped in an *Agrobacterium* cell suspension containing 5% sucrose (wt/vol) and 0.01% - 0.05% Silwet L-77 (vol/vol)

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to allow uptake of the *Agrobacteria* into female gametes [5,6]. This simple transformation plan was commonly known as "the floral dip method" [4]. It requires minimal labor, relatively inexpensive equipment and few specialized reagents, and can be successfully performed. The floral dip method easily maintains genomic stability in *Arabidopsis thaliana* transgenic plants, which can otherwise be harmed by the tissue culture–based transformation [7]. The success and popularity of this floral transformation procedure is reflected by its high citation index since its description [3,4]. Similar to floral dip, floral spray also works very well with *Arabidopsis* [8]. Using a similar protocol, Curtis and Nam reported the successful production of transgenic radish and *Brassica rapa* [9], *Arabidopsis lasiocarpa* [10] and rape seed *Brassica napus* [11].

The segregation test of transgene was useful to identify transgenic plants with single locus DNA integration and zygosity determination. Southern blot analysis determines the copy number of the transgene. But it is both time and labor consuming and precautions were required at the step of radioactive handling. The quantitative RT-PCR and single step PCR method could able to overcome those types of problems in identifying copy number analysis.

Our wide use of the floral dip method over the years to generate transgenic *Arabidopsis* plants in the laboratory has allowed us to adjust its various steps. However, there is scope to improve this protocol. Many researchers have put their hands on modification of the previous protocols to improve transformation process [8,12-15]. There are some steps that we found to be both time-consuming and costly. We provide some modifications in steps of floral-dip method of *Arabidopsis* that eradicates the need to screen on sterile conditions as only agro-peat soil treated with kanamycin is a suitable alternative to an agar substrate during the seed selection process. In another development, we provide a description of modified inoculum preparation for infiltration that supports direct dipping and plant transformation, thereby enhancing the chance of more transformant production having homozygous and single copy of integrated target gene.

2. MATERIAL AND METHOD

2.1. Materials

Plant

Arabidopsis thaliana (Columbia-0) (**Figure 1(a)**)

Bacterium

. *Agrobacterium* strain: GV1301

Chemicals and Reagents

. agarose.
. MS medium
. Liquid LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter)
. sucrose
. glucose
. 0.025%, 0.05%, 0.075% and 0.1% (vol/vol) Silwet L-77 (Lehle Seeds)
. Selection plates (see reagent setup)
· Kanamycin
. ethanol
. Tween-20
. Ethidium bromide
. Standard PCR buffers and solutions.

2.2. Equipments

. Growth chambers or light room or greenhouse, adjustable to long-day condition of 16 h light/8h dark, 20 ± 2^{0}C
. 30^{0}C chamber
. Pots for plant growth
. Laminar-Air flow hood
. Acrodisc Syringe Filter with 0.2-mm membranes (Pall Life Sciences)
. Petri dishes: 150 × 150 × 25 mm (Falcon 3025)
. Agarose gel running system
. Thermal cycler

2.3. Reagent Setup

MS medium and selection plates

. Autoclaved MS medium (4.3 g Murashige & Skoog salts, 10 g sucrose, 0.5 g MES, 8 g agar per liter; pH 5.7), cooled to approximately 50^{0}C before pouring into Petri dishes (MS solid medium). To prepare selection plates 50mgL^{-1} kanamycin was added to MS medium.

Selection pots

. Autoclaved soil (agropeat)-To prepare selection pot, 50 mgL^{-1} kanamycin was supplemented to the pot filled with soil until rosette leaves appeared.

Buffers

. TAE buffer for agarose gel running.

Primers

. Kanamycin gene (transgene) specific and a known (control) single copy gene specific forward and reverse primers were used (**Table 2**)

2.4. Methodology

Step 1: Growing Arabidopsis plants

Arabidopsis seeds were kept for 3 days at 4^{0}C to break dormancy. Vernalized seeds were layered on agro-peat filled pots. Pots were placed and well watered and allowed to grow in growth chamber or green house in long day (16hL/8hD) condition up to inflorescence or floral stage comes up (**Figure 1(a)**).

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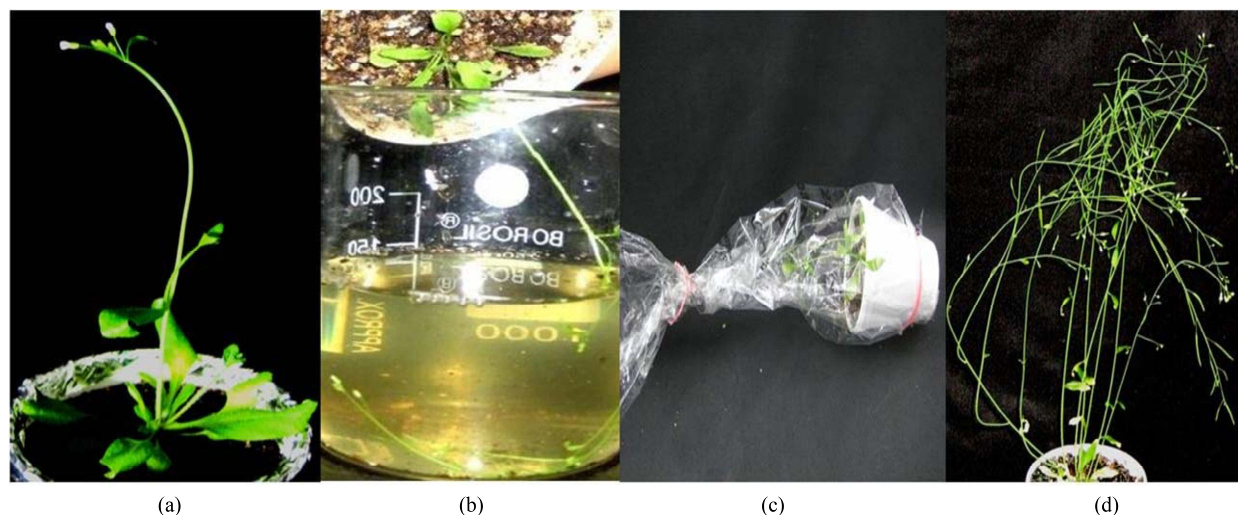
Table 1. Composition of inoculums.

Composition	Inoculum-1	Inonulum-2	Inoculum-3	Inoculum-4
MS salt	0.5X	0.5X	0.5X	0.5X
B_5 vitamins	1X	1X	1X	1X
Glucose	---	---	5%	5%
Sucrose	5%	5%	---	----
Silwet L-77	0.03%	----	0.075%	0.1%
Tween-20	---	0.075%	-----	----



(a) (b) (c) (d)

Figure 1. Steps of floral dip transformation of *Arabidopsis thaliana*. (a) Plant at budding stage; (b) Floral Dip in *Agrobacteria* culture by inverting the plant; (c) Incubation of plant horizontally to ground after dipping; (d) Matured T_0 plants.

Modifications in Step 1:

No net or nylon screen was used which proved economically reliable and less labor consuming. The tightness of soil was considered adequate to keep it as such at the time of floral dip. A very little loss of soil was occurred at the time of vacuum infiltration (**Figure 1(b)**). Clipping of primary inflorescence is not required as this reduces the time to get ready with the floral buds for infiltration.

Step 2: *Agrobacteium* culture and inoculum preparation

. *Agrobacterium* strains that harboring gene of interest in a binary vector (pCAMBIA 1304) were inoculated in a 5 ml culture tube and incubated at 28^0C and 200rpm/min rotation for 48hrs. This is the primary culture.

. Secondary culture was prepared by inoculating 1 ml of primary culture into 1000 ml of LB medium supplemented with specific antibiotic and allowed to grow up to ~ 2.4 - 2.5 OD at 600nm (stationary phage), in same condition as primary culture.

. Bacteria cells were collected by centrifugation at 5000xg and the inoculum was prepared by re-suspending the pellet in infiltration medium which is composed of 0.5X MS salts, 1X B5 vitamins, 5% sucrose or glucose, 0.004 M BAP, 0.02% - 0.1 % (vol/vol) silwet L-77 or 0.1% tween-20 and pH adjusted to 5.7.

Modifications in Step 2:

.We went up to ~ 2.5 OD of the bacterial culture to check its effect on the transformation rate.

.We used glucose rather than sucrose in one of the inoculums (**Table 1**).

.We used 0.7% (vol/vol) tween-20 rather than silwet L-77 in one of the inoculums (**Table 1**).

. Four types of inoculums were used in this study (**Table 1**)

Step 3: Floral dipping and vacuum infiltration

. Plants in the stage of budding without clipping of primary inflorescence were taken and dipped in the inoculums by upturning. One plant (**Figure 1(b)**) or multi plants per pot can be used to do the experiment. The entire set up was kept in a bell jar and the vacuum (400 mm Hg) was applied till bubbles were formed on the leaves and stem. The vacuum was rapidly released and plants were left in liquid for 5 minutes.

. The plants were removed from the bacterial suspension; the inflorescence part was covered with poly bag (to maintain humidity) and placed parallel to ground (**Figure 1(c)**) for 7 - 8hrs at 28^0C. The plants were uncovered after 7 - 8 hrs and rinsed with water to wash the excess silwet L-77 or tween-20 and allowed to grow photoperiodically (16h L/8h D) in plant growth chamber

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in 100 μmoles photons m^{-2} s^{-1} of light intensity.

. Watering was stopped at the starting time of pod maturation (**Figure 1(d)**). Seeds were collected after the pods turned to brown.

. Seeds can be stored at room temperature up to 3 - 4 months or in refrigerator up to 1 year.

Step 4: Screening of T_0 transformed seeds

. Seeds were surface sterilized under laminar hood with 70% ethanol, washed 4 times with sterile water and plated by pipetting method on MS plate (selection plate) containing specific antibiotic (kanamycin in our case) or vernalized seeds were directy layered on antibiotic containing agopeat (soil) .

. ~ 150 seeds were plated for antibiotic selection on each plate or pot.

. Plates were dried up under laminar flow, sealed and kept three days in 4^0C for vernalization.

. Following vernalization they were moved to growth chamber having long day condition and 22^0C temperature for germination and growth.

Modifications in Step 4:

. T_0 seeds (~ 150 seeds) were vernalized and layered on an agropeat filled soil which was pretreated with antibiotic solution, without any surface sterilization.

. Soil was regularly treated with antibiotic solution until rosette leaves come up.

Step 5: Genomic DNA isolation

Genomic DNA was isolated by CTAB method [16] .Small piece of leaf tissue (1cm X 1cm) of primary transformants was ground with genomic DNA extraction buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-Cl and 0.1% β-ME) at room temperature and kept at 60^0C in a water bath for 30 min. To this phenol: chloroform: isoamyl alcohol (24 : 24 : 1) was added and mixed by vortexing followed by centrifugation at maximum speed for 5 min in a microfuge at room temperature. The aqueous layer was transferred to another fresh tube. To the aqueous phase 2/3 volume of isopropanol was added, mixed properly and kept at room temperature for 5 - 10 min to precipitate the genomic DNA. After precipitation of genomic DNA samples were centrifuged at maximum speed for 5 min at room temperature in a microfuge. The pellet was washed 3 times with 70% ethanol, dried and dissolved in 30 μl sterile water containing 20 μg/ml RNase and incubated at 37^0C for 30 min and used for analysis.

Step 6: Confirmation of T-DNA integration in *Arabidopsis* genome

To detect the integration of T-DNA, PCR was performed with kanamycin specific forward and reverse primers (**Table 2**) amplifying 800bp.

Step 7: Segregation analysis and detection of homozygous stable transgenic lines

. Segregation analysis was performed to estimate the number of copies of transgene that integrated into the genome of transgenic *Arabidopsis*. Genomic DNA was isolated from the leaves of T_1, T_2 and T_3 generation plants, produced from T_0 lines dipped in inooculum1 and inoculum-3. Gene specific primers were used for PCR analysis and thermal cycling conditions were similar, as described by Fu *et al* [17].

Step 8: Identification of Single copied transformants

Transgenic lines carrying single copied transgene were identified by single step genomic PCR method described by Kihara *et al* [11]. About 20 μg of genomic DNA was used as templates in a total volume of 20 μl of PCR mixture in individual PCR tube. The PCR mixture consisted of Taq DNA polymerase, dNTPs and 0.5 μM each of primer pairs targeting the transgene and a known single copy control gene (4-hydroxyphenylpyruvate deoxygenase-[4HPPD;At4g03280]). The PCR reaction was carried out with up to 22 cycles of 94^0C for 30s, 60^0C for 30s and 72^0C for 1 min. After PCR, samples were loaded onto regular agarose gel (1.5% w/v) and visualized by ethidium bromide staining. Picture of gel was taken by gel documentary system-(Bio-Rad) and the band intensity was quantified using NIH image software (http://rsb.info.nih.gov/nih-image). Ratio of the band intensity of target and control was calculated to determine the copy number of the transgene.

3. RESULT

Inoculum type for vacuum infiltration and antibiotic screening of T_1 transformants:

Table 2. Sequences and Tm values used in Quantitative Dual Target PCR for determination of copy number of T-DNA integrated into the *Arabidopsis thaliana* genome. 4HPPD, represents 4-hydroxyphenylpyruvate dioxygenase.

Gene	Template	Primer name	Primer sequence (5' - 3')	Tm(^{0}C)
Control	Genomic DNA	4HPPDF'	ATGTGTCTATCGTTAGCTTCTACAGCT	59.20
Control	Genomic DNA	4HPPDR'	CTCTGCGAATTGGTGAAAACC	60.0
Transgene	Genomic DNA	KanamycinF'	TCGACCATGGGGATTGAAC	60.10
Transgene	Genomic DNA	KanamycinR'	TCGACCATGGGGATTGAAC	60.10

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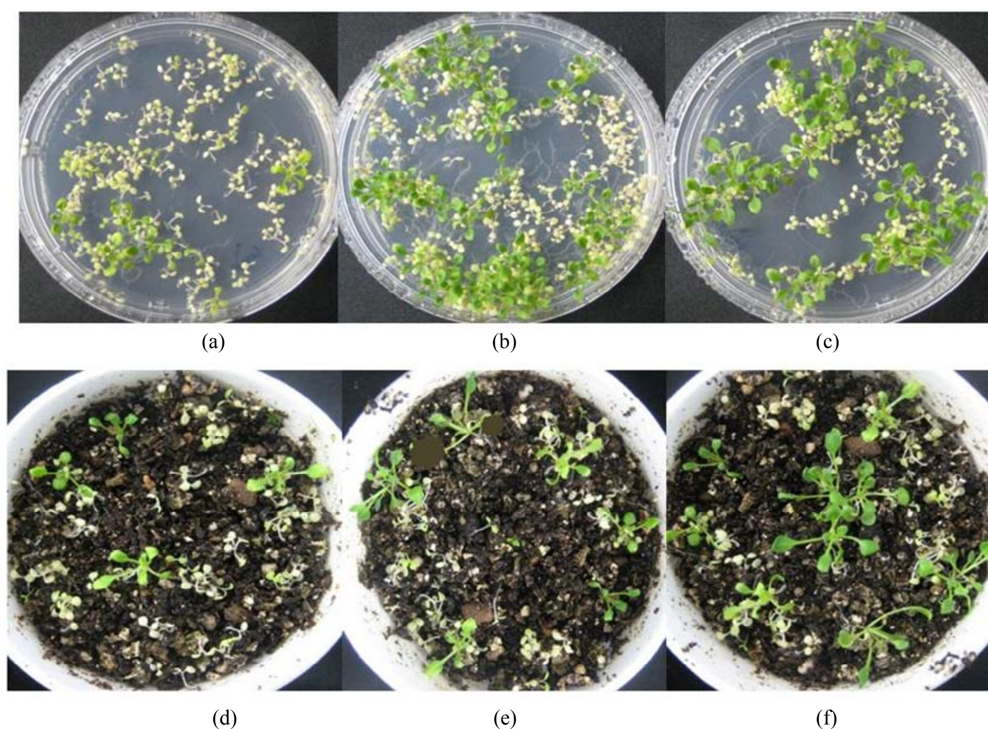

Figure 2. Screening of kanamycin resistant T_1 plants. (a), (b) and (c) showing screening of T_1 transformants of inoculum-1, 2 and 3 dipped plants respectively, on kanamycin containing MS agar plates. (d), (e) and (f) showing screening of inoculum-1, 2 and 3 dipped transformants respectively, on antibiotic irrigated soil filled pots.

Out of four types of inoculums, inoculum-3 gave best result to produce increased number of transformants. 12% - 14% (18 ± 4 antibiotic resistant plants from 100 seeds) increase in the number of transformants on kanamycin plate was observed than the standard inoculums used (**Figure 2(c)**). 0.075% silwet L-77 was considered best to get increased number of tranformants. ~ 150 seeds were taken from each type for screening on kanamycin plates. Only 2% - 3 % seeds from standard inoculum (inoculum-1) dipped T_0 plants were germinated on kanamycin plate. Inoculum-2 Dipped plants resulted 10% - 12% (14 ± 4 antibiotic resistant plants from 100 seeds) increase in transformants (**Figure 2(b)**). Inoculum-4 dipped T_0 plants were wilted after 7 - 8hrs of incubation in 28^0C. Screening of T_0 seeds on containing kanamycin agropeat filled pot gave similar result as on kanamycin plates. Increase in transformation efficiency was observed by screening on kanamycin containing agro-peat filled pots (**Figures 2(d)**, **(e) and (f)**).

Segregation and copy number analysis of transgene in inoculum-3 dipped T_2 plants compared to inoculum-1 dipped T_2 transenics:

PCR analysis of genomic DNA from T_1 and T_2 generation plants revealed a single gene segregation pattern of kanamycin registant gene in the *Arabidopsis* genome. Kanamycin registant gene was not detected in 3 (50%) out of 6 from inoclum1 dipped T_1 progeny where as only 3 (20%) out of 17, showed kanamycin negative from inoculum-3 dipped T_1 progeny. Only one line out of 3 of T_2 generation from inoculum-1 dipped transgenic progeny gave homozygous condition (**Figure 3(a)**). 9 plant out of 17 from T_1 generation of inoculum-2 dipped T_0 plants considered kanamycin positive and 4 plants were detected homozygous in T_2 generation (**Figure 3(b)**). T_1 generation of inoculum-3 dipped plants gave 14 transgenic lines detected kanamycin positive and 9 out of them were detected homozygous (**Figure 3(c)**).

The only transgenic #2 of T_1 generation, resulted by inoculum-1 dipping was considered, having 4 copies of transgene and the ratio of the PCR band intensity of the transgene and control gene was found to be 4.2 (**Figure 4(a)**). The other two (#1 and#3) were considered heterozygous as the PCR band intensity varies between target and control gene. From nine transgenic (T_1) (#1, #2, #3, #5, #8, #9, #10, #14 and #15) lines of inoculum-2 dipped plants, 4 lines (#1, #2 and #10) considered, having single copy of transgene (**Figure 4(b)**). The ratio between transgene and control gene were found to be 1.2, 0.9, and1.1 respectively. From forteen transgenic (T_1) (#2, #3, #4, #5,#7, #8, #9, #10, #11, #12, #14, #15, #16 and #17) lines of inoculum-3 dipped plants, 4 lines (#3,

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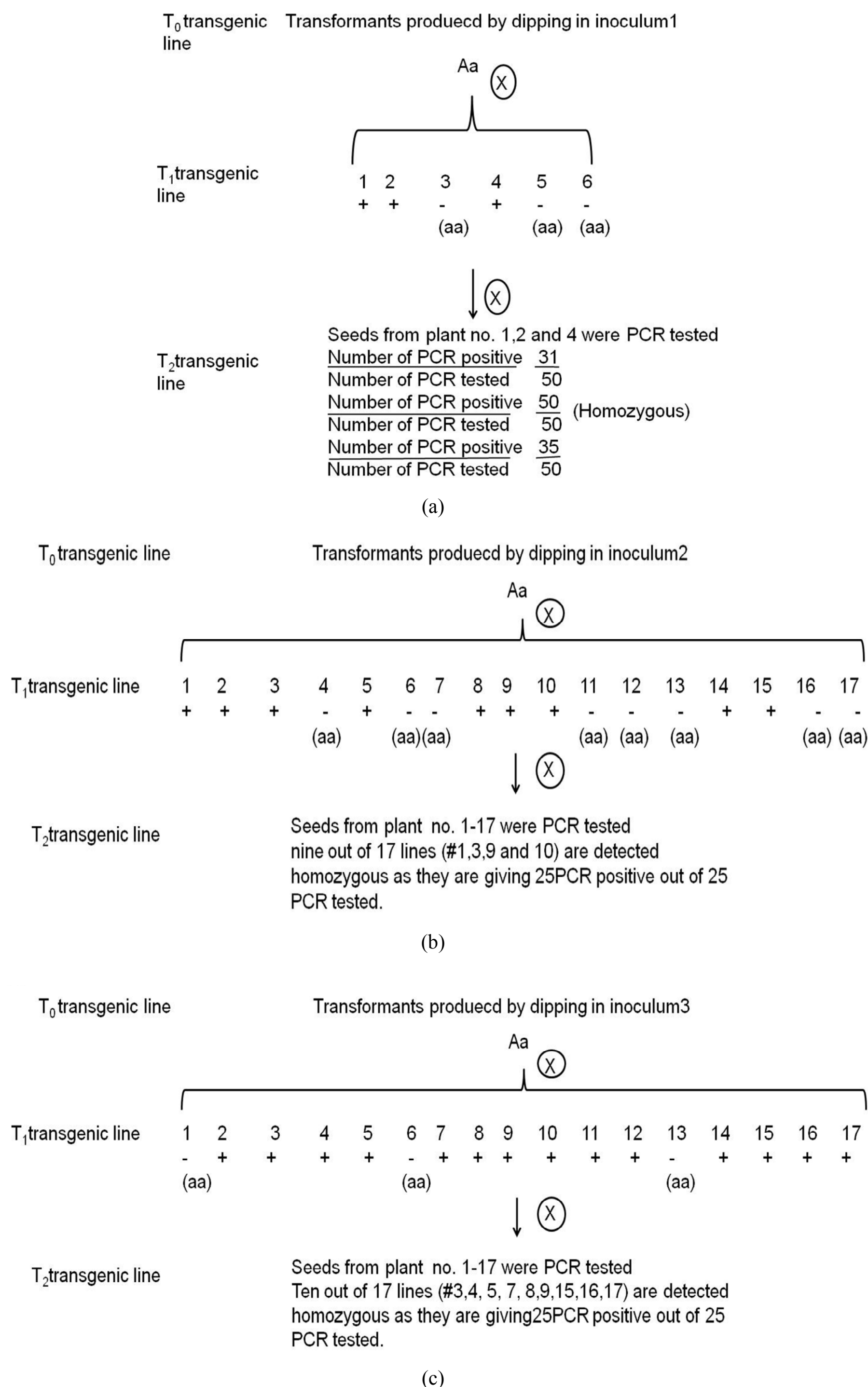

Figure 3. Segregation (survival) analysis of kanamycin resistant gene in transgenic plants developed using Inoculum-1 (a), inoculum-2 (b) and inoculum-3 (c). Zygosity of transgenic plants, were identified in T_2 generation by PCR amplification of integrated gene. X indicated self pollination. '+' indicated PCR positive and '-' indicated PCR negative transgenic plants.

#4, #5, #7, #8 and #9) considered, having single copy of transgene (**Figure 4(b)**). The ratio between transgene and control gene were found to be 0.9, 1.3, 1.1, 0.9, 1.0 and 1.1 respectively.

4. DISCUSSION

Here we confirmed the crucial role of silwet L-77 in the inoculum in enhancement of rate of transgenic produc-

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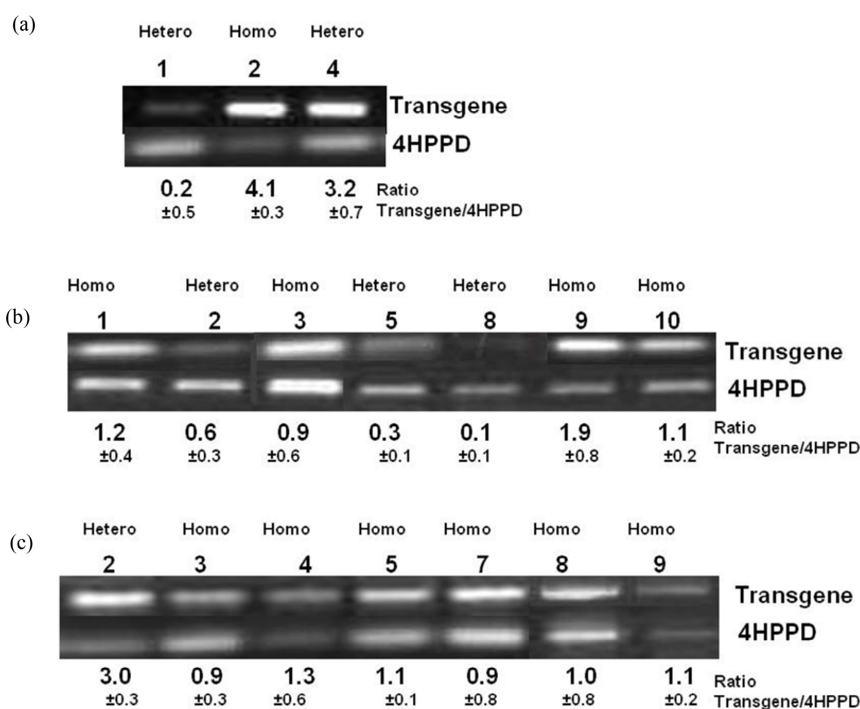

Figure 4. Identification of transgenic Arabidopsis plants carrying a single copy of integrated T-DNA resulted by dipping in inoculum-1 (a), inoculum-2 (b) and inoculum-3 (c) by single step PCR. The ratios of transgene to control (4HPPD) genes were obtained using gel images (±SD indicated). The upper bands represent the transgene amplicons. From inoculum-1 dipped T_1 lines, no single copy was detected. From inoculum-2 dipped T_1 lines #1, 2 and 10 detected as single copy transgenic lines. From inoculum-3 dipped T_1 lines #3, 4, 5, 7, 8 and 9 detected as single copy transgenic lines.

tion. We also found the replacement of sucrose by using glucose in the inoculum. We compared the traditional inoculation method of submerging inflorescences in the bacteria suspension [4] with the modified protocol (**Figure 1(a)**). Cough and Bent [4] described that the flowering stage is suitable for transformation. Desfeux *et al* [5] demonstrated that ovules are the target for transformation with *Agrobacterium*. During ovary development, an open structure exists until 3 days before anthesis, when locules are sealed by the stigma. Thus, applying *Agrobacterium* inoculum to closed floral buds and not opening flowers is important. Martinez-Trujillo *et al*. [18] developed a modified method *i.e.* drop by drop application of *Agrobacterium* suspension to the closed floral buds. This is quite labour consuming. Here, we would like to say that the budding stage can result more transformants by modifying a little in the inoculums and only single plant (**Figure 1(a)**) or multiple plants per pot can be used for infiltration for efficient transformant production. Dipping of Primary floral buds can be done to get transformants earlier as it avoids clipping of primary inflorescence and lessen the days to get transformed seeds. 500nm OD value of the agrobacteria culture can be increased upto ~ 2.5 to get better result of infection. Curtis [19] explained that the surfactant plays an important role in optimizing transformation efficiency of radish. We optimized the transformation efficiency of Arabidopsis by altering the concentration of silwet L-77. Rinsing of T_0 plants after 7 - 8 hrs of infiltration avoids any toxic effect of excess surfactant. This modification produced 8% - 10% enhanced number of T_0 transformants than the conventional method. Cough [20] said about the use of other surfactants rather than silwet L-77.We also tested another surfactant i. e. tween-20 instead of silwet L-77 which could also able to produce efficient number of transformants (**Figure 3(b)**). This can help us from economic view point and in case of unavailability of silwet L-77. Use of Glucose in place of sucrose in the inoculum proved better to give efficient transformant.

One limitation in the identification of transgenic *A. thaliana* lines after floral dip is that the seeds are often internally contaminated with the *A. tumefaciens* line used. Furthermore, there is often fungal contamination within the seed. Davis *et al* [21] developed a non-asceptic condition to avoid fungal contamination at the time of screening of T_0 transformants by using chromatography papers treated with antibiotics. We desired to establish a protocol for antibiotic selection that could be used under non-aseptic and in soil conditions. As an added return, this would eliminate the time needed to surface-sterilize seed prior to agar culture and also there would no need of chromatography papers. We were successful in identifying transformants on soil by treating the agro-peat with kanamycin in a concentration of 50µg/µl every day up to rosette leaves come up (**Figure 2**). However, we were convinced that it was possible to screen antibiotic resistance plants under non-sterile soil conditions.

Transgenic plants derived from *Agrobacterium* mediated transformation often carry multiple copies of integrated T-DNA [22] consequently, integrated gene can become unstable due to enhanced gene silencing as result of multiple copies of the ectopic gene [23] and zygosity is also a factor for stable transgenic line [6].We compared the zygosity and copy number of integrated transgene following Fu and Ristic [24], taking conventional (inoculum-1 dipped) and modified (inoculum-3 dipped) T_1 and T_2 transformants. Inoculum-3 dipped T_0 plants produced enhances number of transformants consequently produced more number of homozygous and single copied transgenic lines (**Figure 4**) than the inoculum-1 dipped T_0 plants. Only one PCR positive (T_1) plant produced by conventional method proved homozygous in T_2 generation and it was harboring 4 copies of transgene. From 9 PCR positive T_1 lines of inoculun2 dipped plants 4 were considered homozygous T_2 generation and from 9 plants 7 were tested for copy number analysis and 3 were proved harboring single copy of transgene (**Figure 4(b)**). Similarly 14 PCR positive T_1 lines of inoculum-3 dipped plants 9 were considered homozygous T_2 generation and from 14 plants 7 were tested for copy number analysis and 6 were proved harboring single copy of transgene (**Figure 4(c)**).

Here we have presented a very simplified method that is strongly efficient in the generation and selection of homozygous, single copied and stable transgenic Arabidopsis plants, which is less time consuming, economically efficient and avoids aseptic condition.

5. ACKNOWLEDGEMENTS

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