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Original Contribution

TRANSFORMATION EFFICIENCY ENHANCEMENT OF ARABIDOPSIS VACUUM INFILTRATION BY SURFACTANT APPLICATION AND APICAL INFLORESCENCE REMOVAL

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ABSTRACT
Efficiency of Arabidopsis in planta transformation is a major prerequisite for Success of Insertional-mutagenesis projects. Different parameters including Agrobacterium strain, surfactant application and mature siliques and apical inflorescence meristem removal treatments were comprehensively investigated to elucidate their impact on transformation efficiency. Results showed that plants infiltrated with Agrobacterium strain GV3850 produced the highest frequency of transformants (1.54%), while GV3101 and LBA4404 strains resulted in transformation efficiencies of 1.42 and 1.18%, respectively. Surfactants added to infiltration medium remarkably promoted transformants rate. Silwet L-77 caused 80% increase, while Tween-20 increased transformation efficiency up to 53%. Triton X-100 was found to be toxic for plant tissues. Transformation rate was increased 10% with mature siliques removal two days after infiltration. Apical inflorescence elimination promoted transformants ratio up to 39%. Acetosyringone was proved to have no significant effect on transformation efficiency. Modification of these factors for vacuum infiltration transformation of Arabidopsis results in an average transformation efficiency of 2.5-3%.

Key words: In planta transformation; apical inflorescence meristem; Agrobacterium strain; mature siliques;

INTRODUCTION

As a model plant species for plant molecular and cellular biology studies, Arabidopsis thaliana has been extensively adopted by researchers worldwide (1). Genome with a small size that has been completely sequenced (2), short generation time of maximum 8 weeks from seed to seed, small plant size with lots of seeds, easy growth conditions and availability of efficient transformation methods has made it suitable for a wide variety of plant biology experiments (3).

Expression of foreign genes and subsequent investigation of induced alteration of plant biology has extended the understanding of plant biology different aspects. Various strategies have been employed for genetic transformation of plants among them Agrobacterium-mediated gene transfer is the most frequent used method (4). Different forms of this method have been used for Arabidopsis transformation experiments that most of them have been superseded by so called In planta methods (5). Several advantages of In planta methods including: minimum tissue culture procedures, genetically uniform progenies, high rate of transformants obtained within a quite short time, and minimal labor and reagents required for transformation have made them desirable for many plant biology laboratories (5). These are especially suitable for research projects which require a large scale production of transformed lines. Large number of independent transgenic lines

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produced through In planta methods, has facilitated functional analysis of Arabidopsis genome. After completion of genome sequencing projects of different species, the post-genomic studies were prompted in order to elucidate function of different genes (6). Saturated T-tagging experiments, map-based gene cloning projects or site-specific gene replacements could not be achieved without a reliable-high-throughput transformation method that produces several thousands of independent transformant lines (7, 8, 9). Lack of a high throughput transformation strategy for most plant species is the main reason why insertional mutagenesis methods are not widely used in other species.

Individual genes function has been thoroughly established for less than 10% of Arabidopsis genes (10) and out of the ~26000 genes identified in *Arabidopsis*, the functions of only a few thousand have been defined with confidence (11). New T-DNA insertion collections are being prepared at different laboratories worldwide to elucidate function of other Arabidopsis genes.

Therefore, high transformation efficiency of In planta experiments is the main prerequisite for large scale functional analysis projects. Feldmann and Marks reported the first successful Arabidopsis in planta transformation through seed transformation (12). Afterwards several in planta transformation methods for Arabidopsis were designed to achieve higher transformation rates, including: clip and squirt method (13, 14) vacuum Infiltration (15), floral dip (16), floral spray transformation (17).Vacuum infiltration and floral dip methods have been used more extensively, because of their easiness, reproducibility and the large number of independent transformants generated (18).

Several factors contribute to Arabidopsis in planta transformation efficiency, such as plant developmental stage, infiltrations medium composition, surfactant concentration and plant growth conditions after vacuum infiltration of floral dip (16, 19). Some investigators suggested use of Agrobacterium strain GV3101 (15) while others reported successful use of various Agrobacterium strains for in planta transformation (16, 18). So far, there is no report of different Agrobacterium strains impact on transformation efficiencies nor any comparison of different strains has been performed. Furthermore, most of experiments so far used Silwet L-77 as unique surfactant for transformation procedures. This can cause some restrictions for researchers in small laboratories with limited source of reagents and budget. It would be so beneficial to scrutinize different surfactants and compare their effect on transformation efficiency. Clough and bent reported a remarkable increase in transformation efficiency of floral dip with increasing surfactant concentration up to 1%, while surfactant addition caused no significant increase in transformation efficiency for vacuum infiltration (16).

This study was designed for comprehensive investigation of different parameters influencing efficiency of vacuum infiltration experiments as an effort to maximize transformant production rate. Different agrobacterium strains, different surfactants and siliques elimination treatments were analyzed. The modifications suggested by results of this research improve transformation efficiency, whilst eliminating some unnecessary steps or reagents used. This modified version of vacuum infiltration procedure enables higher transformant production required for T-DNA tagging or other insertional-mutagenesis experiments.

MATERIALS AND METHODS

Plant growth

Arabidopsis thaliana (Columbia) seeds were vernalized by keeping on moistened filter papers in Petri dishes at 4°C for 2 days and were then sown in individual pots (three plants per each 28 cm^2 plastic pot) in flats and grown under greenhouse conditions at 22°C with a photoperiod regime of 16 hours light and 8 h dark (long days). When reached 2 to 5 cm, the primary inflorescences were clipped for stimulation of secondary bolts emergence. Infiltration experiments were performed when most of secondary inflorescences were about 5 to10 cm with several young floral buds.

Binary vector and Agrobacterium strains

The recombinant binary vector used for Arabidopsis in planta transformation experiments, pBI121-ChiS, is a derivative of pBI121(Clontech, USA), which its β-glucuronidase *(uidA)* gene sequence was replaced by a 1.7 kb fragment encoding a bacterial Chitinase(ChiS) from B. Pumilus SG2. The orientation and correct alignment of

the fusion of the fragments in these constructs were verified by digestion with restriction enzymes and was successfully expressed in tobacco (unpublished results). This recombinant binary vector was introduced to Agrobacterium tumefaciens strains GV3101 (20), GV3850 (21), LBA4404 (22) by electroporation.

Agrobacterium growth and Infiltration medium

A single colony of Agrobacterium was used for inoculation of a 5 ml starter culture including YEP medium(10 gl^{-1} Bacto peptone, 10 gl^{-1} Yeast extract and 5 gl^{-1} NaCl) supplemented with 25 mgl^{-1} Rifampicin or 50 mgl^{-1} Gentamycin and 30 mgl^{-1} Kanamycin, incubated overnight at 28°C with vigorous shaking (180 rpm). Overnight grown starter culture was used to inoculate a new 400 ml YEP medium prepared and grown as above. Overnight cultured agrobacterium cells (with an OD_{600} of 1.6 to 2) were centrifuged at 5000 g for 15 min and the pellet was resuspended in 2 volumes of Infiltration medium (IM) containing 1/2 MS salts (23) and 5% sucrose. Another treatment of IM was supplemented with 50 mM Acetosyringone to assess its impact on transformation efficiency. The IM suspension was then incubated for a further 2-3 hours at 28°C with vigorous shaking.

Vacuum infiltration

Vacuum infiltration was performed according to standard protocols (15) with some modifications. Briefly, for vacuum infiltration, in a vacuum chamber (large glass desiccator) plastic vessels were filled with IM containing different surfactants including Silwet L-77, Triton X-100 and Tween-20 (0.02 %), as well as a treatment without any wetting agent. Plant inflorescences were immersed in horizontally at a position such that maximum inflorescences were covered by IM. Using a Pump, vacuum was gradually applied until air bubbles emerged on the surface of IM and the vacuum was held for 15-20 minutes until the IM started to boil. The vacuum was then released quickly to coerce the agrobacterium cells into the plant tissue. The infiltrated plants were briefly blotted on filter papers for seconds and were then horizontally placed in trays wrapped with transparent plastic covers to maintain high humidity. The trays were kept at greenhouse at a low light atmosphere for 24 h, and then they were set upright and were grown at normal greenhouse conditions for a further 4 weeks. To investigate the effect of pruning (mature siliques or apical inflorescence elimination) on transformants frequency, 3 treatments were investigated: mature siliques of first group of plants were clipped 2 days after infiltration, apical inflorescence meristem (top blossom) tips of plants in second group were cut 10 days after infiltration to stop new silique formation and plants of the third group were grown normally without any pruning. After the siliques were dry, seeds of plants in a pot were harvested separately and stored for further analysis.

Selection of putative transformants

For selection of transformants, aliquots of seeds from each plant were surface sterilized by immersion in 75% ethanol for 1 min, then were immersed in 20% commercial bleach (1% active sodium hypochlorite)solution containing 0.05% Tween-20 for 12 min, followed by four rinses in sterile distilled water before being placed on selection plates. Selection medium was consisted of MS salts (23) at half strength supplemented with 50 mgl^{-1} kanamycin monosulfate and solidified by 8 gl^{-1} agar. Disinfected seeds were plated on kanamycin selection plates, stratified for 48 h and then transferred to 24°C greenhouse. They were incubated at dark for 3 days and were then transferred to a photoperiod of 16 h lights (50-100 microEinsteins) and 8 h dark. After 3 to 4 days, green seedlings with small dark green secondary true leaves were identified as putative transformants and 50 randomly selected transformed plants were transplanted into pots containing a water saturated soil mixture (peat: perlite: vermiculite, 1:2:1) for further analysis by polymerase chain reaction (PCR) . Transformation efficiencies were calculated as follows: (number of kanamycin resistant seedlings/total number of seedlings tested) × 100.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed to determine if the T-DNA region has been integrated into the plant genome. Small leaf segments were collected from plants two weeks after transplantation and Genomic DNA was isolated using a quick DNA Miniprep procedure (24). About 1 µl of the isolated DNA (5-10 ng) was used as template in PCR reactions.PCR was performed to amplify a 1729 bp fragment of ChiS gene in the T-DNA region. The primers were as follows: forward primer, Chi-F1 (5'

GGGGGTCTAGAATGAGTTCTGACAAAA GTTA-3') with an *XbaI* restriction enzyme site (underlined) and reverse primer, Chi-R3 (5'-GGGGGGAGCTCGAGCCCACTCTCTCTTT A-3') with a *SacI* site. PCR reactions were prepared in a total volume of 25 µl containing 5-10 ng of DNA template, 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs (Cinnagen, Iran), 0.25 pmol each of primers and 1.5 U of Taq DNA polymerase (Cinnagen, Iran). The following conditions were used for PCR: 94°C for 5 min and 30 cycles of 94°C for 45 s, 52°C for 1min and 72°C for 2 min; followed by a final extension at 72°C for 8 min. A wild type Arabidopsis DNA sample was included as negative control. PCR products were loaded on 1% agarose gel and stained with ethidium bromide and then photographed by a UVP gel documentation system (UVP, England).

Data analysis

The data were analyzed using Microsoft office Excel and MSTATC. Statistical estimations and mean comparisons were performed using the LSD test at $p<0.05$.

RESULTS

Polymerase Chain Reaction analysis of the 50 randomly selected plants indicated all of them were true transformants **Figure1** shows the 1729 bp band of ChiS gene amplified from DNA of transformed seedlings, which is absent in non-transformed wild type seedling. Results revealed no significant increase in transformation rate by applying Acetosyringone in IM compared to IM without Acetosyringone (Data not shown).

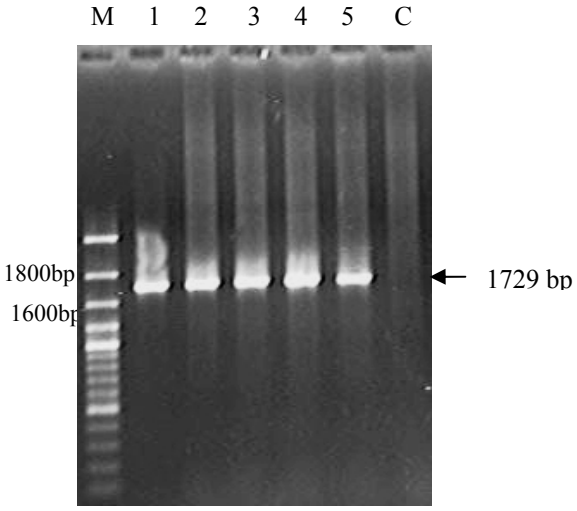

Fig. 1- PCR analysis of DNA samples from selected putative transformants using specific primers for ChiS gene produced the 1729 bp band in transformed seedlings (1-5), M: Molecular weight marker, C: wild type control.

Agrobacterium strains

Different transformation efficiencies were obtained with use of each agrobacterium strain **(Figure2).** The highest transformation rate was recorded when plants were infiltrated with GV3850 suspension, with a transformation efficiency of 1.54% when Silwet L-77 applied as wetting agent and with no post-infiltration plant pruning. At similar conditions, GV3101 inoculated plats produced 1.42% of transformants. The lowest transformation efficiency was recorded for plants inoculated with LBA4404 with an average of 1.18%. No obvious Agrobacterium growth was observed when seeds were plated on selection medium, although some colonies of GV3850 appeared when the incubation time exceeded 8-9 days.

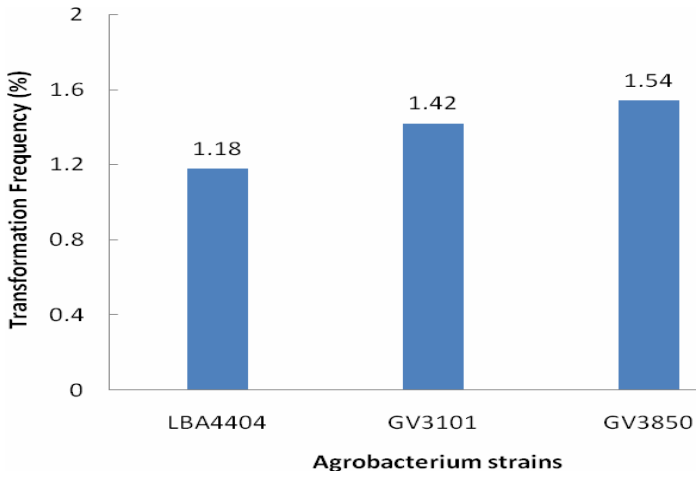

Fig. 2- Different transformation efficiencies were obtained by three Agrobacterium strains, LBA4404, GV3101 and GV3850.

Effect of Different surfactants

All of the 3 different surfactants used, resulted a remarkably higher transformation efficiency in comparison with infiltrations performed without any surfactants. Different surfactants influence on transformation efficiency and the plant stress they caused are summarized in **table1.** Plants infiltrated with IM free of surfactant resulted in a transformation efficiency of 0.79%, when inoculated with GV3101 and with no post-infiltration plant pruning. The highest transformation rate of 1.42% was achieved when infiltration medium was supplemented with 0.02% Silwet L-77, showing 80% growth in comparison with plants infiltrated with IM without surfactants. A transformation efficiency of 1.21% was recorded for IM containing Tween-20 (53% increase in transformation efficiency) and the lowest efficiency of 0.91% (showing 15% growth in transformation) was obtained when Triton X-100 was applied. The plants infiltrated with Triton X-100 supplemented IM showed excessive stress with some burned inflorescences indicating high toxicity. Furthermore, some siliques were damaged and failed to seed. This toxic effects were observed when even lower concentrations of this detergent (as low as 0.005%) was used. No adverse effects were observed with use of Silwet L-77 or Tween-20 at a concentration of 0.02% and plants were recovered 1-2 days after infiltration. Seed weight of plants treated with different surfactants was calculated to determine whether the surfactants applied (at a concentration of 0.02%), have caused any loss of seed production or not. An average of 74.06 mg of seeds was produced by plants inoculated with IM free of surfactants. Plants treated with IM containing Silwet L-77 and Tween-20 yielded 70.57 and 68.31 mg seed respectively. A seed weight of 54.19 for plants inoculated with Triton X-100 indicated a considerable loss in seed production due to siliques and inflorescences damage.

Table 1*- Different surfactants influence on transformation efficiency and on the severity of the post-Infiltration stress. Mean seed weight per plant is reduced by post-Infiltration stress. Means with the same letter did not differ significantly at $p<0.05$.*

Surfactant	No. of scored seedlings	No. of transformed seedlings	Mean Transformation efficiency (%)	mean Seed weight per plant(mg)	stress severity
No surfactant	1396	11	0.79a	76.06a	No stress
Silwet L-77	1482	21	1.42c	70.57b	Medium
Tween-20	1315	16	1.21b	68.31b	Medium
Triton X100	1424	13	0.91a	54.19c	High

Top inflorescence and Silique removal

Transformants ratio showed a considerable increase when pruning practices were applied **(Table2)**. An average percentage of 1.42 transformants were obtained when no pruning treatment was applied, while plants were inoculated with IM containing GV3101 and 0.02% Silwet L-77. With removal of mature siliques 2 days after infiltration, the infiltrated plants produced a bulk of seeds containing 1.57% of transformants. This shows a 10% increase in Transformation rate. Transformants frequency experienced more increase when apical inflorescence meristems were cut 2 weeks to stop new siliques formation. For this group of plants a transformation rate of 1.98% was recorded that indicates a 39% increase in transformant frequency. These plants became mature in a shorter period of time and the siliques were dry and ready for seed collection approximately one week sooner than normal plants.

Table2*- Effect of different pruning practices on final transformation efficiency. Mean of Transformation efficiencies with the same letter did not differ significantly at $p<0.05$.*

Pruning Treatment	No. of total germinated seeds	No. of transformants	Mean Transformation efficiency (%)
without Pruning	1482	21	1.42
Mature siliques	1396	22	1.57
Apical inflorescence	1465	29	1.98

DISCUSSION

Transformation efficiency of Arabidopsis in planta experiments is especially important for large scale insertional mutagenesis projects such as T-DNA tagging, map-based gene cloning and site-specific gene replacement (7, 9, 25). Several modifications have been applied to preliminary methods to increase the transformants frequency. Different factors have been reported to affect the transformation efficiency (16, 26). In our study, Acetosyringone addition caused no increase in transformation efficiency and IM with or without Acetosyringone was not significantly different. IM supplemented with different surfactants, remarkably increased the vacuum infiltration efficiency. Clough and Bent reported first use of Silwet L-77 for Arabidopsis floral dip method. Addition of Silwet L-77 to Agrobacterium suspension caused a considerable increase in transformants obtained from dipped plants, but surfactant addition had no significant effect on transformation efficiency of Vacuum infiltration experiments. Comparison of different amounts of surfactant in the inoculation medium showed that levels of Silwet L-77 between 0.02% and 0.1% gave about 20-fold greater rates of transformation than did 0.005% when plants were inoculated by dipping. They recommended use of 0.05% Silwet L-77 for floral dip experiments, while it caused just a low increase in transformation efficiency of vacuum infiltrations (16). In preliminary studies we found 0.05% of Silwet L-77 in IM is too high for vacuum infiltration experiments, due to extensive damage of plants infiltrated for a relatively long time. A concentration of 0.02% Silwet L-77 did not show any adverse effects after a 15-20 minute infiltration (Data no shown). To date, all reports of Arabidopsis in planta transformation used Silwet L-77 as the unique wetting agent (16, 26, 18) and there is no report of other wetting agents been used. Silwet L-77 is not used as a general reagent in most laboratories, so it could be an inhibiting factor for some researchers. We assessed two non-ionic detergents that are generally used in molecular biology labs, Tween-20 and Triton X-100. They are inexpensive and easy to find in most molecular biology laboratories. Results proved that Tween-20 can be successfully used as an alternative to Silwet L-77 to improve transformation efficiency of vacuum infiltration experiments. Compared with

Vacuum infiltration without addition of any wetting agents which produced only 0.79 % of transformants, addition of Silwet L-77 increased transformation efficiency to1.42%, whileTween-20 addition resulted in a transformation rate of 1.21%. Although Triton X-100 increased the efficiency to 0.91%, its application is not recommended due to its adverse effects on plants which reduce the vigor and seed productivity. Most of previously reported studies implied no preference in use of Agrobacterium strains for in planta transformations (16, 18). In our study GV3850 produced the highest transformant ratio (1.54%) when GV3101 and LBA4404 resulted in a transformation efficiency of 1.42 and 1.18%, respectively. While there was no evidence of GV3101 or LBA4404 infection in selection plates, Some GV3850 colonies appeared when plated were incubated 8-9 days after plating.

Mature siliques and apical inflorescence meristem removal was remarkably helpful for elimination of untransformed seeds, therefore the seed bulk volume to be selected was lower and transformants rate increased. While plants without any pruning treatments showed a transformation efficiency of 1.42%, Plants which their mature siliques were removed 2 days after infiltration produced 1.57 of transformants (10% more than normal plants without pruning). Removal of apical inflorescence meristem strikingly increased transformants ratio to 1.98% which indicates a 39% growth in transformation efficiency.

This study proved surfactants can increase transformation efficiency of vacuum infiltration experiments as well as floral dip. Tween-20 can be successfully used for Arabidopsis transformation experiments as an alternative for Silwet L-77. Different agrobacterium strains used in this study showed variations. Although GV3850 resulted in the highest transformation efficiency, GV3101 and LBA4404 produced high rates of transformants as well. Mature siliques and apical florescence meristem removal remarkably increased the transformants ratio, Furthermore the volume of seeds to be selected was much lower allowing screening of seeds in a shorter period of time with minimal labor. In order to achieve highest transformation efficiencies for vacuum infiltration experiments, we strongly suggest addition of Silwet L-77 or Tween-20 to IM along with mature siliques and apical inflorescence meristem removal. These modifications will increase the vacuum infiltration efficiency and a transformation efficiency of 2.5 -3 % will be achievable.

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