Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Drought stress enhances the efficiency of floral dip method of Agrobacterium-mediated transformation in *Arabidopsis thaliana*

Estresse hídrico aumenta a eficiência do método de imersão floral de transformação mediado por Agrobacterium em *Arabidopsis thaliana*

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Abstract

The Agrobacterium-mediated floral dip protocol is the most extensively used transformation method for a model plant *Arabidopsis thaliana*. Several useful methods for *Agrobacterium tumefaciens*–mediated transformations of *Arabidopsis* are existing, but they are time consuming and with low transformation efficiency. Here, we developed a transgenic *Arabidopsis* lines *TET12p::TET12-RFP* in a short period of time and enhanced transformation efficiency by using a modified transformation method by applying drought stress after floral dip. In this protocol, *Agrobacterium* cells carrying *TET12p::TET12-RFP* recombinant vectors were resuspended in a solution of 5% sucrose, 0.05% (v/v) silwet L-77 to transform female gametes of developing *Arabidopsis* inflorescences. Treated *Arabidopsis* were then applied with different levels of drought stresses to stimulate plants for the utilization of maximum plant energy in seed maturation process. The applied stresses achieved the fast maturation of already treated inflorescences while stopped the growing of newly arising untreated inflorescence, thus decreased the chances of wrong collection of untransformed seeds. Consequently, the collected seeds were mostly transgenic with a transformation frequency of at least 10%, thus the screening for positive transformants selection was more advantageous on a selective medium as compared to a classical floral dip method. Within 2-3 months, two hundred of individual transgenic plants were produced from just 10 infiltrated plants. This study concludes that application of drought stresses in a specific stage of plant is a beneficial strategy for achieving the transgenic *Arabidopsis* in a short period of time with high transformation efficiency.

Keywords: *Arabidopsis thaliana*, drought stress, *Agrobacterium tumefaciens*, floral dip, transformation.

Resumo

O protocolo de imersão floral mediado por Agrobacterium é o método de transformação mais amplamente utilizado para uma planta-modelo *Arabidopsis thaliana*. Existem vários métodos úteis para transformações de *Arabidopsis* mediados por *Agrobacterium tumefaciens*, mas são demorados e com baixa eficiência de transformação. Aqui, desenvolvemos uma linha transgênica de *Arabidopsis* TET12p::TET12-RFP em um curto período de tempo e eficiência de transformação aprimorada usando um método de transformação modificado por meio da aplicação de estresse hídrico após o mergulho floral. Neste protocolo, células de *Agrobacterium* transportando vetores recombinantes TET12p::TET12-RFP foram ressuspensas em uma solução de 5% de sacarose, 0,05% (v/v) silwet L-77 para transformar gametas femininos de inflorescências de *Arabidopsis* em desenvolvimento. *Arabidopsis* tratadas foram então aplicadas com diferentes níveis de estresse hídrico para estimular as plantas a utilizar o máximo de energia da planta no processo de maturação das sementes. Os estresses aplicados alcançaram a rápida maturação

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Received: December 17, 2021 – Accepted: April 30, 2022

das inflorescências já tratadas enquanto paravam o crescimento de inflorescências não tratadas recém-surgidas, diminuindo, assim, as chances de coleta errada de sementes não transformadas. Consequentemente, as sementes coletadas eram principalmente transgênicas, com uma frequência de transformação de pelo menos 10%; portanto, a triagem para seleção dos transformantes positivos foi mais vantajosa em um meio seletivo em comparação com um método clássico de imersão floral. Dentro de 2-3 meses, 200 plantas transgênicas individuais foram produzidas a partir de apenas 10 plantas infiltradas. Este estudo conclui que a aplicação de estresse hídrico em um estágio específico da planta é uma estratégia benéfica para alcançar a *Arabidopsis* transgênica em um curto período de tempo com alta eficiência de transformação.

Palavras-chave: *Arabidopsis thaliana*, estresse hídrico, *Agrobacterium tumefaciens*, mergulho floral, transformação.

1. Introduction

Plant transformation is an approach of genetic manipulation by which foreign genes are introduced into plant genomes and stably integrated and the transformed cells are regenerated to obtain transgenic plants. The first technique to achieve in planta transformation comprised the use of tissue culture and plant regeneration (Feldmann and Marks, 1987). In *Arabidopsis thaliana*, the discovery of Agrobacterium-mediated transformation through vacuum infiltration of inflorescences successfully replaced tissue culture methods as it directly provides transformed seed and avoids complicated and lengthy tissue culturing steps (Bechtold and Bouchez, 1995). This method was further simplified into floral dip by relieving the need of vacuum infiltration step (Clough and Bent, 1998), distinctly advanced the ease of generating transformants in *Arabidopsis*, and remained the most efficient and extensively used transformation method to generate transgenic plants.

Being a routinely used transformation method in genetics and molecular laboratories, several efforts were made in the past two decades to further simplify and improve the floral dip method with respect to reducing the time, cost and the required workload (Ali et al., 2022a; Davis et al., 2009; Logemann et al., 2006; Martinez-Trujillo et al., 2004; Yew et al., 2018; Zhang et al., 2006). However, advances in simplifying and improving the "floral dip" step of the transformation have been established, no considerable advances in improving or shortening the "after floral dip" stages, seed collection and transformants selection processes have been described. Here we used a drought stress after floral dip stage as an approach to enhance the efficiency of Agrobacterium-mediated transformation method by ten times as compared to classical methods however, the time, cost and the required workload were also reduced remarkably. By using this method, two hundred of individual transgenic plants (*TET12p::TET12-RFP*) were produced from just 10 infiltrated plants within 2-3 months.

2. Procedure

2.1. Gene cloning and vector generation

For construction of *Arabidopsis* transgenic line *TET12p::TET12-RFP*, the 1.1-kb native promoter, the full-length genomic coding regions of *TETRASPANIN12* (*TET12;* 888 bps) fused with red fluorescent protein (*RFP*) were cloned into a binary vector pCAMBIA1300 to label sperm cells membranes (TET12 specifically localized to sperm cells membranes) with red fluorescence marker (Figure 1A). Binary vector pCAMBIA1300 carrying a kanamycin resistance gene for bacterial selection and a hygromycin B resistance gene for plant selection. Promoter and coding regions of *TET12* were amplified by using a primer pairs (TET12-F and TET12-R; Table 1) from *Arabidopsis* genomic DNA, while *RFP* region was amplified by using primer pairs (RFP-F and RFP-R; Table 1) from already constructed vector *ACT11p::H2B-RFP* (Ali et al., 2022b).

The binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (Figure 1B) and positive transformed *Agrobacterium* colonies were selected on lysogeny broth (LB) plates with the kanamycin antibiotics (50 µg/mL). A single Agrobacterium colony was inoculated into 500-ml liquid LB medium (1% NaCl, 0.5% yeast extract, 1% tryptone) containing the kanamycin antibiotics (50 µg/mL) for binary vector selection and incubated for two days at 28 ^{0}C and 250 r.p.m. Agrobacterium cells were collected through centrifugation at 5,000g for 20 minutes at room temperature and then resuspended in 500 ml freshly prepared 5% (w/v) sucrose solution with 0.05% Silwet L-77 (i.e. 250ul/500ml).

2.2. Arabidopsis germination and healthy growth

The seeds (about 20–30) of *Arabidopsis thaliana* (ecotype Columbia-0) were germinated on wet soil (standard potting compost mixed with perlite) in four pots (4 in. × 4 in.) in short day conditions (8 h light/16 h dark, 20 ºC), and covered with a transparent sheet until small seedlings appeared above soil (about 7-10 days). The pots were transferred into long day conditions (16h:8h/light:dark, 22 ºC) and seedlings were grown in well-watered healthy conditions till they started to bolt and produced floral inflorescences (about 5-6 weeks).

2.3. Agrobacterium-mediated floral dip transformation

The Agrobacterium cell suspension (prepared in step 2.1) were added to a small beaker and *Arabidopsis* healthy plants (grown in step 2.2) were submerged into Agrobacterium suspension in a way that all aerial tissues were gently sunk for 1–3 minutes. The treated plants were kept in dark for 20-24 hours and then moved back to the growth chamber or the greenhouse.

2.4. Drought stress after floral dip

After the floral dip method, the plants were treated by two ways. In the first way, half of the plants (two pots containing ten plants) were maintained in well-watered

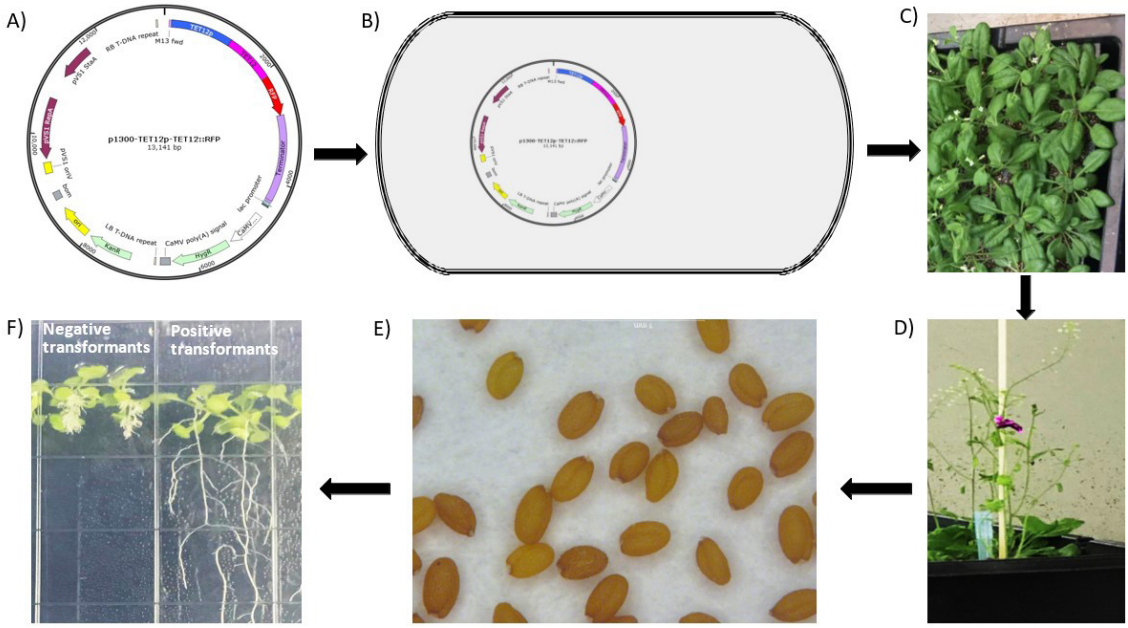

Figure 1. Construction of *Arabidopsis* transgenic line *TET12p-TET12::RFP* through modified floral dip transformation method. The 1.1-kb native promoter, the full-length coding regions of *TETRASPANIN12* (*TET12*) and red fluorescent protein (*RFP*) were inserted into a binary vector pCAMBIA1300 to make a construct *TET12p::TET12-RFP* (A). The construct vector was introduced into *Agrobacterium tumefaciens* strain GV3101 (B). The healthy plants with few floral inflorescences (about 5-6 weeks) were used for floral dip transformation method to insert foreign DNA into *Arabidopsis* (C). The transformed plants were treated with different levels of drought stresses by reducing water contents gradually to enhance the ripening of seeds quickly (D). The ripened seeds were collected on a piece of a paper from all treated plants (E). Positive transformants were selected based on true leaves and roots or tall green seedling as compared to small yellowish seedling without true roots in case of non-transformants (F).

Table 1. Primers sequences used in the PCR to amplify promoter and coding regions of *TET12* and coding region of *RFP*.

Primers	Primer sequence	Product size	Annealing Temperature
TET12-F	5′-GACGGCCAGTGCCAAGCTTATAGTCATATGGAAATTATTTGTCC	1980	58 ºC
TET12-R	3′-CGTCCTCGGAGGAGGCCATGAAGAACCGGCGCTTCCA		
RFP-F	5′-TGGAAGCGCCGGTTCTTCATGGCCTCCTCCGAGGACG	678 bp	56 ºC
RFP-R	3′-GGAAAACAAATGGAAAGAGTTGTTAGGCGCCGGTGGAGTGGCG		

healthy conditions till normal seeds maturation (about 8 weeks). The healthy conditions enabled the plants to produce many fresh inflorescences on new branches and many of them competed with the transformed inflorescences for seed maturation (dryness) and therefore huge numbers of seeds were obtained during seed collection. In the second way, remaining half of the plants (other two pots containing ten plants) were treated with different levels of drought stresses by reducing water contents gradually to enhance the ripening of seeds quickly while stop the arising of new flowers (about 4 weeks). In the first week after floral dip, the treated plants were provided with half amount (50%) of the normal amount of water which was needed for keeping plants in healthy condition (To maintain *Arabidopsis* plants in well-watered healthy conditions, they are watered daily as needed to avoid water stress). In the second week, the water contents were reduced to 70% while in the third week, watering plants was entirely stopped. Furthermore, after one week of floral dip, all the newly developed inflorescences/floral buds/branches (if developed any) were cut down from all treated plants. Through this approach, the transformed inflorescences met the opportunity to attain maturity rapidly in 4 weeks of time while all the newly developed inflorescences got terminated incompletely, thus saved the transformation time, and avoided the wrong collection of untransformed seeds.

2.5. Seed collection and dryness

When the siliques became completely dried and matured (green color of the siliques began to turn yellowish), the seeds were collected on a piece of a paper from all treated plants (Figure 1E). About ten thousand seeds were collected from plants transformed

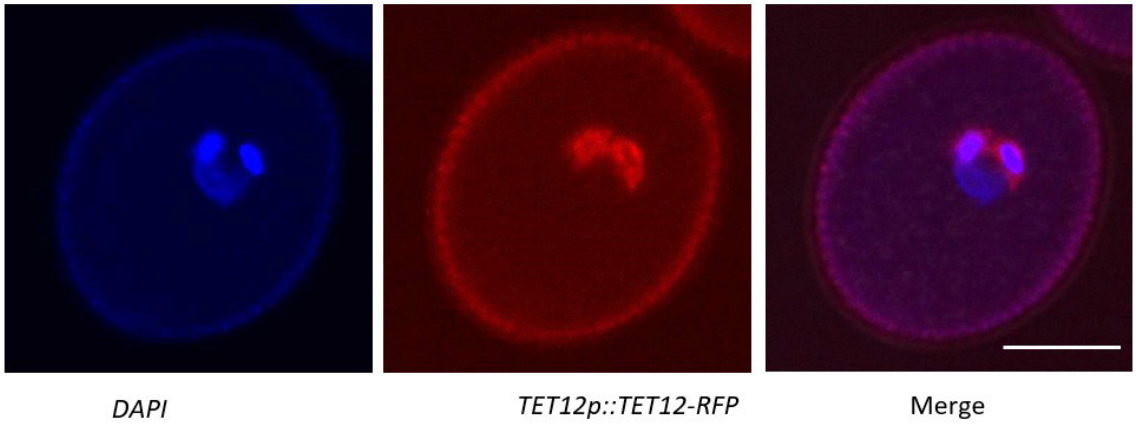

Figure 2. Confirmation of positive transformants of *Arabidopsis* transgenic line *TET12p::TET12-RFP* through confocal microscope. *Arabidopsis* pollen showing *DAPI* fluorescence (left section) for sperm cells and vegetative nucleus, red fluorescence (*TET12p::TET12-RFP*; middle section) for sperm cells and Merge (right section) in wild type (Col-0) plants. Bar scale 10 µm.

with normal procedure while a total of three thousand seeds were collected from plants transformed with modified procedure. All the seeds were kept on 37 ºC incubator for two days for complete dryness, followed by one week at room temperature. At this stage, 1500 harvested seeds from both experiments were regerminated for positive transformants screening while remaining half seeds were stored for a long time at 4 ºC.

2.6. Transformants selection

The seeds were sterilized with 75% ethanol for 5 minutes, followed by 20% bleach for 8 minutes and then rinsed four times with sterile water. The surface sterilized seeds were spread on Murashige and Skoog (MS) medium (10 g sucrose, 0.5 g 2-(N-morpholino) ethanesulfonic acid (MES), 4.3 g Murashige & Skoog salts, 8 g agar per liter; pH 5.7) plates with hygromycin antibiotics (60 µg/mL) for transformants selection. Plates were kept at 4 ºC for three days for vernalization and then transferred to 28 ºC and kept until the seedling appeared. Positive transformants were selected based on true leaves and roots or tall green seedling as compared to small yellowish seedling in case of non-transformants (Figure 1F). Sixteen positive transformants were found in 1500 screened seeds through classical procedure (1% transformation rate), while 154 positive transformants were found in 1500 screened seeds through modified procedure (10% transformation rate) confirmed that modified floral dip method enhanced the transformation efficiency significantly. The positive transformants were transplanted to pots of well-watered soil in greenhouse or growth chamber and grown in healthy conditions till flowering/seed collection. The positive transformants were further confirmed through DNA amplification by polymerase chain reaction (PCR) or through the availability of red fluorescence while observing the pollen of the transgenic lines (*TET12p::TET12-RFP*) by using a confocal laser scanning microscope (Figure 2).

3. Conclusion

Using modified floral dip transformation method, *TET12p::TET12-RFP* and three other constructs were transformed into wild type *Arabidopsis* plants and in all cases the transformation efficiency was found much higher than the other classical transformation methods. Therefore, this study concludes that drought stress enhances the efficiency of floral dip method of Agrobacterium-mediated transformation in the model plant *Arabidopsis thaliana* and significantly reduces the workload, cost, and time of the transformation procedure. Therefore, the application of this method will benefit the scientific society to easily introduce novel, valuable genes into economically important crops and to produce new genetically modified plants within a short period of time.

Acknowledgements

This work was supported by grants from Higher Education Commission (HEC), Pakistan (Grant no. SRGP/NAHE/HEC/2020/326) to Ali. I.

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