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Transient Transformation of Potato Plant (*Solanum tuberosum* L.) Granola Cultivar Using Syringe Agroinfiltration

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ABSTRACT

Genetic transformation has been used as an alternative approach to improve the quality and the productivity of potato plant. In this study, different conditions have been set up to optimize transient GFP (Green Fluorescence Protein) expression in potato cv. Granola. Leaves of potato were infiltrated with *Agrobacterium tumefaciens* strain C58C1 harboring pK7FWGF2 vector with a nuclear-targeted GFP by simple pressure. GFP signals allowed simple evaluation of transformation efficiency which were indicated by GFP expression in nucleus of leaf cells in infiltrated areas. The results showed that leaf position, co-cultivation time, optical density and the presence of acetosyringone significantly affected the transformation efficiency. The fourth terminal leaves from four-week old plants were the optimum age for transformation. Furthermore, the highest transient transformation efficiency was obtained upon 48 h post-infiltration at an $OD_{600} = 0.8$, and the presence of 200 μ M acetosyringone. In conclusion, the developed protocol will be useful to study gene function as well as to generate stable transformation of this potato cultivar.

INTRODUCTION

Potato belongs to Solanaceae family and is considered as one of the most important food crops in the world. Potato tuber contains high carbohydrates, proteins and vitamins, minerals and antioxidant (Abelenda, Navarro, & Prat, 2011). This crop is not only used as an alternative food, but also used as a plant model to study the physiological processes in plants (Dobnik et al., 2016). Potato has been predicted as the most important crop for the next twenty years in developing countries in term of food security (Birch et al., 2012). Therefore, several attempts have been performed to improve the quality and the productivity of potato.

The production of potato in many countries faces some problems because it is prone to different kind of pests, diseases, weeds and abiotic stresses such as drought and high temperature (Birch et al., 2012; Park, Vleeshouwers, Jacobsen, Van Der Vossen, & Visser, 2009). These stresses have greatly influenced yield and tuber quality. On the

other hand, conventional potato breeding could not be the only solution to overcome these problems since it is hampered by potato characteristics like autotetraploidy, self-incompatibility and high heterozygosity (Bhaskar, Venkateshwaran, Wu, Ané, & Jiang, 2009).

Agrobacterium-mediated transformation is a gene transfer system that has been successfully used on many important crops (Ziemienowicz, 2014). This system offers a potential tool that could support potato conventional breeding program. One of the *Agrobacterium*-mediated transformation methods that has been applied to several plants was syringe agroinfiltration. Syringe agroinfiltration is a simple, yet powerful tool to analyze gene expression transiently. This method has been successfully applied in crop plants such as grapevine, tomato and tobacco (Ben-Amar, Cobanov, Buchholz, Mliki, & Reustle, 2013; Matsuo, Fukuzawa, & Matsumura, 2016; Shah, Ali, Jan, Jalal-Ud-Din, & Ali, 2015), as well as in medicinal plants (Faizal & Geelen,

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2012; Mirzaee, Sharafi, & Hashemi Sohi, 2016). In potato, this method was used for discovery and analysis of resistant genes candidate which can be expressed transiently as well as to generate stable transformation (Bhaskar, Venkateshwaran, Wu, Ané, & Jiang, 2009).

Potato transformation highly depends on genotype, hence there are so many protocols available for its transformation. Most potato cultivars have been successfully transformed including cultivar of Désirée, Bintje, Russet Burbank and Kennebec (Vinterhalter, Nevena, & Ivana, 2008). However, there is no report about transformation of Granola cultivar using this method. Therefore, this study was performed to develop transient transformation system of granola cultivar using syringe agroinfiltration. The condition described in this study can be used for functional gene analysis and/or to generate stable transformation of granola cultivar.

MATERIALS AND METHODS

The research was performed from January 2016 to June 2017 at Micropropagation and Transformation Laboratory of School of Life Sciences and Technology, Institut Teknologi Bandung.

Plant Materials and *Agrobacterium* Strain

Potato tuber was submerged in 3 % fungicide for 10 minutes and incubated in the dark at 28 °C for sprout induction. Approximately 1-2 cm sprout were planted for 3, 4 and 5 weeks on the combination of compost and husk medium. *A. tumefaciens* strain C58C1 harboring a binary vector pK7FWGF2 was used in this research. This vector also contained a nuclear localization signal::green fluorescent protein (NLS::GFP) construct that is targeted to the nucleus and driven by CAMV35S promoter. A pre-culture was initiated by incubating a single colony of *A. tumefaciens* in 5 mL YEB medium (1 g L⁻¹ yeast extract, 5 g L⁻¹ beef extract, 5 g L⁻¹ peptone, 5 g L⁻¹ sucrose, and 1 M MgSO₄·7H₂O) supplemented with appropriate antibiotic for 48 h at 28 °C on a rotary shaker at 135 rpm. Small aliquot of pre-culture was subsequently transferred to fresh YEB medium and incubated overnight at the same culture condition (Fig. 1a). The bacterial culture was pelleted by spinning it at 3000 g for 15 minutes at room temperature. This step was repeated 3 times. The pellet was resuspended with infiltration medium (10 mM MgCl₂·7H₂O, 10 mM 2-[N-morpholino] ethanesulfonic acid (MES) pH 5.6) and diluted to different OD₆₀₀ (Fig. 1b). The bacterial

suspension was incubated for 2-3 h in the dark at room temperature. Induction of *Agrobacterium* virulence was modulated by supplementing infiltration medium with various concentrations of acetosyringone (0, 50, 100, 150 and 200 µM).

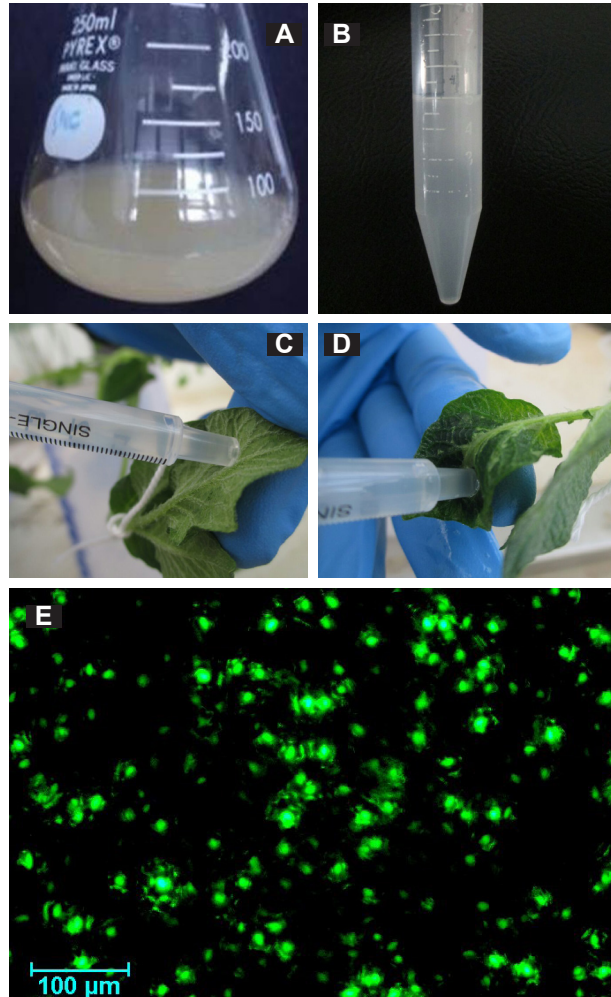


Fig. 1. Illustration of experimental procedure of transient transformation in potato leaves via syringe agroinfiltration. A. The single colony of *A. tumefaciens* C58C1 NLS::GFP were cultured in YEB medium in the dark at 28 °C; B. The suspension was centrifuged and the obtained pellet was subsequently resuspended in infiltration medium to a final optical density; C. *Agrobacterium* suspension were injected to the abaxial part of potato leaf; D. The suspension was injected to the whole potato leaf; E. Visualization of GFP expression in nucleus by fluorescence microscope.

Potato Leaf Agroinfiltration

Infiltration protocol was conducted referring to Sparkes, Runions, Kearns, & Hawes (2006). The third, fourth and fifth leaves counted from the apex of 3, 4 and 5-week old potato plants were used for transformation. The abaxial of potato leaves were infiltrated with *Agrobacterium* suspension using 1 mL needleless syringe. It took about 0.6 mL of bacterial suspension to infiltrate the whole potato leaf. Three days after infiltration, the transformed leaves were collected to observe GFP expression using Nikon Eclipse E800 fluorescence microscope equipped with NIS-Element BR version 4.40.00 (64-bit) software (Fig. 1c-e). A number of fluorescence nuclei from the images were calculated using ImageJ software. After monitoring GFP expression, leaf discs were fixated with ethanol: acetic acid buffer (3:1) for 2 h and were washed twice. Afterwards, 2 ng μL^{-1} 4', 6' diamino-2 phenylindole (DAPI) were used to stain the leaf nuclei for 1 h and the stained leaves were washed twice. The leaves were mounted on slide for microscopic observation. Frequency of transient transformation was obtained by comparing the number of GFP fluorescence nuclei to the number of DAPI stained-nuclei and multiplied by 100 %.

Statistical Analysis

The experiment was replicated three times. The frequency of transient transformation from each parameter was analyzed with one way ANOVA with Duncan multiple range test using SPSS ver. 21.0. All significant differences between means were adjusted at $P < 0.05$.

RESULTS AND DISCUSSION

GFP Transient Expression of Potato Leaf

The leaf of Granola cultivar was infiltrated

with *A. tumefaciens* C58C1 NLS::GFP by a syringe agroinfiltration. After 3 days, the leaves were collected and observed under fluorescence microscope. The cells of potato leaves expressed GFP. GFP signal was detected from each nucleus because GFP gene was fused with NLS (Fig. 2C). NLS was a sorting signal for GFP, which guided this protein to enter and accumulated in nucleus. The strong expression of GFP exhibited that CAMV35S as a constitutive promoter actively transcribed genes in plant tissues so that the gene product can be increased (Hernandez-Garcia, Martinelli, Bouchard, & Finer, 2009).

GFP expressions were observed in all infiltrated areas. Previous study reported that the high frequency of transgene in leaves depended on the region of infiltrated leaf which were mostly detected on basal part than on tip part (Wroblewski, Tomczak, & Michelmore, 2005). On the contrary, GFP was almost expressed equally from the tip until basal part of the leaves in granola cultivar (data not shown).

Furthermore, by using syringe agroinfiltration, *A. tumefaciens* transformed more cells from mesophyll tissues than the epidermis. The suspension of *A. tumefaciens* were infiltrated through stomata and distributed into mesophyll tissues which was located between upper and lower epidermis (Ben-Amar, Cobanov, Buchholz, Mliki, & Reustle, 2013). These intercellular spaces provided extra space for *A. tumefaciens* to infect cells on mesophyll tissues. Therefore, the number of fluorescence cell was detected mostly in mesophyll than those in the epidermis. In addition, potato leaf structure may affect infiltration process due to the thricomes on abaxial part which limited bacterial penetration through stomata.

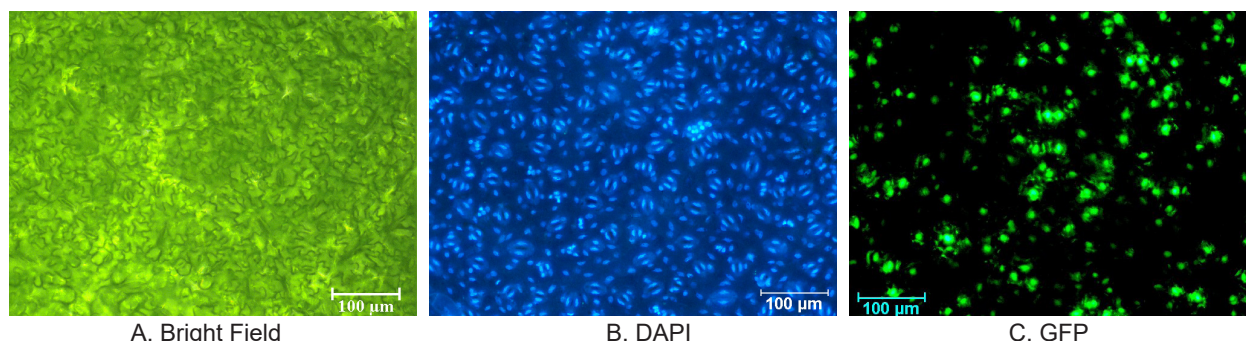


Fig. 2. Microscopic analysis of GFP transient expression in transformed potato leaf. (A). Potato leaf cells under normal light; (B). Nuclei of potato leaf cells exhibited fluorescence of DAPI; (C). GFP expressed in the nuclei of potato leaf.

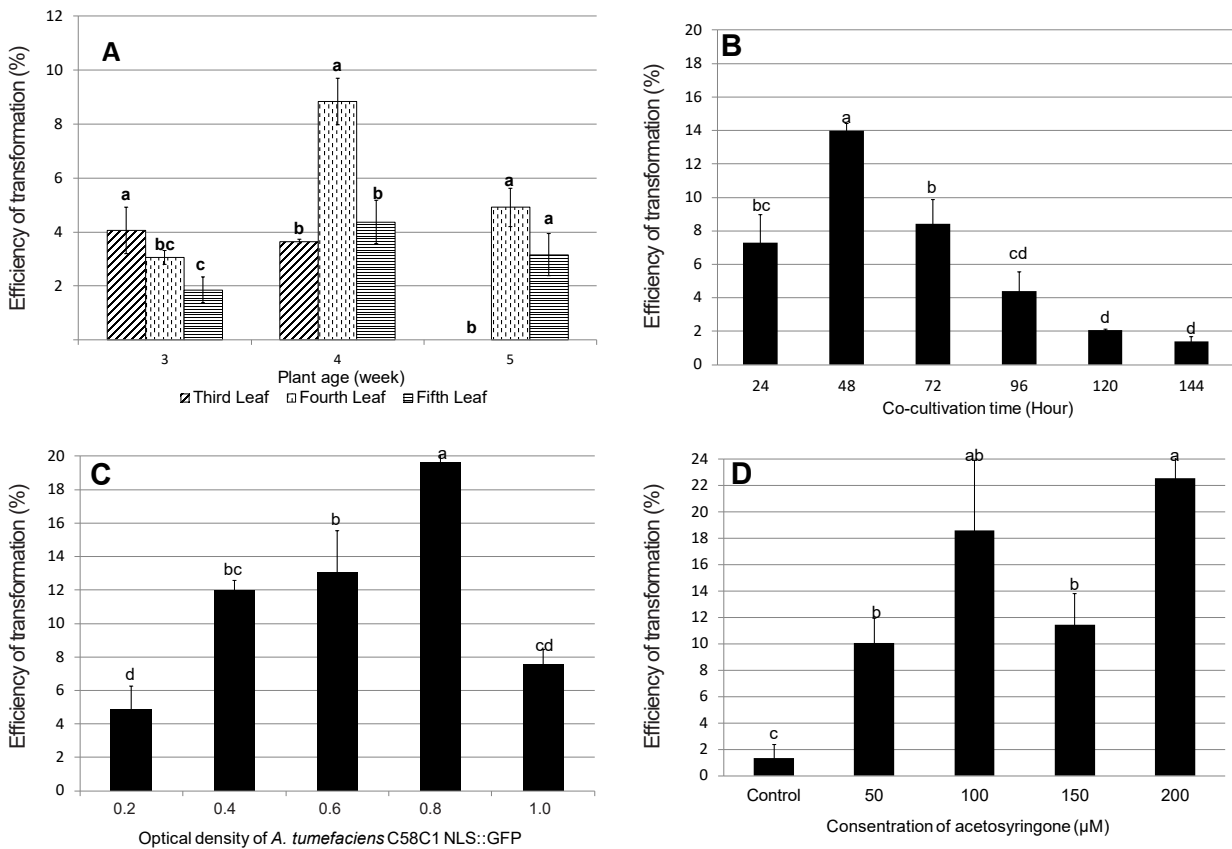


Fig. 3. Frequency of transient GFP expression of potato cv. Granola for the effect of plant ages and leaf position (a), co-cultivation time (b), optical density (c), and the presence of acetosyringone (d). Different letters in each column indicate significant differences between the mean values at P < 0.05.

The Effect of Plant Age and Leaves Position

In this study, various ages and leaf positions of potato plant were also tested for transformation. Fig. 3a showed the frequency of GFP expression on 3, 4 and 5-week old potato plant with the third, fourth and fifth leaves counted from the apex. We found that frequency of transformation was not affected by plant age. However, leaf position significantly affected the frequency of transformation. The highest frequency of GFP expression was 8.84 ± 0.85 % and obtained from the fourth leaf of four-week old potato plant.

Plant ages and leaf positions were among the critical factors for successful transformation (Bhaskar, Venkateshwaran, Wu, Ané, & Jiang, 2009). Previous study reported that gene expression in transformed leaves was varied depending on developmental stages (Hosein, Lennon, & Umaharan, 2012). The near fully expanded leaves exhibited higher gene expression than fully expanded or younger ones.

Faizal & Geelen (2012) pointed out that tissues of near fully expanded leaves have undergone cell expansion so that the cells actively replicated and were highly responsive to transformation.

The Effect of Co-cultivation Time

Co-cultivation time is one of the important factors in genetic transformation (Mo, Huang, Yang, Zhang, & Luo, 2015). In granola cultivar, the frequency of transformation was significantly influenced by the co-cultivation time. The highest frequency of GFP expression was obtained from leaves at 48 h post-infiltration (Fig. 3b). Low transformation frequency was exhibited from leaves when the co-cultivation time was set too short or too long. GFP signals were firstly detected at 24 h post-infiltration, and increased to optimum at 48 h. Subsequently, the expression of GFP decreased until 144 h post-infiltration.

During co-cultivation time, *A. tumefaciens* was recognized and attached to the plant cells. When

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the recognition process was done, *A. tumefaciens* transferred its T-DNA to plant cell which was supported by interaction between *Agrobacterium* virulence protein and plant cell protein. Optimum co-cultivation time of many plants commonly varied from one to another, however, the optimum co-cultivation time for potato plant was about 48-72 h post-infiltration (Veale, Slabbert, & Van Emmenes, 2012). The decrease of GFP expression after 48 h cultivation was related to an important regulation process in plant cell known as gene silencing mechanism (Bashandy, Jalkanen, & Teeri, 2015; Ben-Amar, Cobanov, Buchholz, Mliki, & Reustle, 2013). Consequently, the gene expression will be reduced afterwards.

The Effect of Optical Density

Previous studies showed that the various optical densities have been adjusted to transform potato plant depending on cultivars background (Bhaskar, Venkateshwaran, Wu, Ané, & Jiang, 2009; Chakravarty & Wang-Pruski, 2010; Du, Rietman, & Vleeshouwers, 2014; Han, Goo, Lee, & Lee, 2015). Here the optical density was adjusted from 0.2 to 1.0. In this study, the frequency of transformation was significantly influenced by the variation of optical density. The frequency of transformation increased gradually and reached the optimum GFP expression when $OD_{600} = 0.8$ (7.72×10^8 cfu mL⁻¹) was used (Fig. 3). The frequency of expression, then, decreased after OD_{600} was adjusted to 1.0. In line with Banerjee, Prat, & Hannapel (2006) and Wang (2006), they transformed other potato cultivars using optical density approximately 0.8. On the other hand, Bhaskar, Venkateshwaran, Wu, Ané, & Jiang (2009); Chakravarty & Wang-Pruski (2010); Du, Rietman, & Vleeshouwers (2014) and Han, Goo, Lee, & Lee (2015) stated that optimum optical density for transformation of several potato cultivars was less than 0.8. It implies that different potato cultivars generated different responses upon *Agrobacterium* transformation.

The Effect of Acetosyringone

The transient expression was intensified by seventeen fold after the inclusion of 200 μ M acetosyringone compared to those in control (Fig. 3d). Acetosyringone is one of the phenolic compounds which is produced by plant cells. Acetosyringone is essential for *A. tumefaciens*-mediated transformation due to its chemoattractant activity (Gnasekaran & Subramaniam, 2015).

Acetosyringone has been known to activate virulence genes of *A. tumefaciens*, especially *VirA* protein will activate *VirG* protein, subsequently this protein acts transcription factor for transcription process of other virulence genes (Krenek *et al.*, 2015).

CONCLUSION AND SUGGESTION

A transient transformation system for potato cv. Granola has been developed via syringe agroinfiltration. The optimum conditions obtained in this study will be useful to study gene function, promoter activity and gene-gene interaction using transient expression system, and also to generate stable transformation in potato cv. Granola.

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