

## Early Detection of the Orchid Flowering Gene *PaFT1* in Tobacco Cells Using a GFP Reporter

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### Abstract

Here we describe a novel method of using *green fluorescence protein* (GFP) as a reporter gene for early detection of an integrated T-DNA containing the orchid flowering gene, *PaFT1* (*Phalaenopsis aphrodite Flowering locus T1*) in the tobacco genome. Functional assays that report the presence of exogenous DNA early in development are especially useful in plants where the desired phenotype is only apparent after long periods of vegetative growth. The objective of this study is to establish a method for detecting an inserted *Phalaenopsis* orchid flowering gene and examining its function in tobacco. The p35S::PaFT1-35S::GFP construct was introduced into *Agrobacterium tumefaciens* strain EHA101. Transformed tobacco leaves were cultured on MS medium with addition of 1 mgL<sup>-1</sup> NAA+3 mgL<sup>-1</sup> BAP+50 mgL<sup>-1</sup> Kanamycin+300 mgL<sup>-1</sup> timentin for selection. Results showed bright green GFP fluorescent signals in 11 out of 15 (73%) tobacco leaf cells at a 2-month time point after transformation. GFP and PaFT1 fragments were amplified in genomic PCR using GFP and PaFT1 specific primers. The accumulated PaFT1 transcripts were observed in 3 month-old transgenic tobacco plants containing p35S::PaFT1-35S::GFP. Green fluorescence was observed only in the transgenic plants at the 5 month-old stage but not in the wild type controls.

**Keywords:** Early flowering, *GFP*, *PaFT1*, reporter gene, tobacco

### Introduction

Biotechnology is widely used in plant breeding to alter the genetic composition of plants to improve crop yields, alter cosmetic features, or facilitate transport. The alteration of genetic traits is performed by inserting genes from other plants, or even from other organisms, into the genome of the plant that is to be improved. In this way, new emerging genetic and physiological traits arise and these characters are passed to successive generations (Yuwono, 2006).

Changes in the properties of the transformant plant can be observed from its phenotype, but these may not be detectable for some time, or even until the plant reaches maturity. Selecting transformant plants could be made more efficient if the transformant plant could be detected earlier in its development. Thus, we have designed a system that relies on the plasmid-based genomic insertion of a fluorescent reporter gene to indicate that transformation has taken place. Our system specifically reports the expression of flowering genes before the flowering phenotype can be observed. A number of systems were considered, including screenable markers such as  $\beta$ -glucuronidase (GUS) assay, and luciferase. However, as enzymatic assays both luciferase and GUS assays require the

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addition of an enzyme substrate to visually detect the result. Therefore we chose an approach based on Green Fluorescent Protein (*GFP*). *GFP* is a 27-kDa protein (Xia *et al.*, 2002), which emits bright light when exposed to blue light or ultraviolet radiation (UV) (Brito *et al.*, 2013). *GFP* is widely used because it does not require substrates or exogenous cofactors for fluorescence (Stewart, 2005; Puchta, 2003) and does not destroy the tissues of transformants (Hraska *et al.* 2006). For these reasons, *GFP* was selected as the reporter gene to improve early detection of flowering genes.

In this research, the construction of a plasmid based on pGAS101 was carried out by inserting *GFP* as a reporter gene. The main gene on pGAS101 plasmid is *PaFT1*. *PaFT1* gene is one of *FT* homologous genes isolated from *P. aphrodite* (Jang *et al.* 2015). Analogous to *Arabidopsis* as the model plant, over-expression of the *PaFT1* gene is expected to accelerate flowering in plants. *GFP* was used as an evaluation tool of transformation parameters. Cells and tissues expressing *GFP* could be distinguished easily from those that do not express *GFP* without destroying the tissue sample, and can facilitate separating transformants and non-transformants (Hraska *et al.*, 2006). *GFP* expression is indicated by green fluorescence, while the non-transformant retains its natural red autofluorescence after being exposed under light with a wavelength of 480 nm (Ghorbel *et al.*, 1999).

Plasmid pGAS101 also includes the selection marker genes *Neomycin Phosphotransferase (NPT)* and *tetracycline resistance (*tet*<sup>R</sup>)* genes. The selection marker genes are important for the development of plant transformation technology, because it allows the identification and isolation of cells expressing the cloned DNA and for monitoring and selecting offspring of the transformants (Miki and McHugh, 2004).

A number of transformation methods, which could be used to transfer foreign genes include particle bombardment, *Agrobacterium tumefaciens*-mediated, electroporation with protoplasts/protocorm,

seed imbibition and transformation through pollen tube (Chai and Yu, 2007). Transformation using *Agrobacterium* is an indirect transformation technique, which is the most frequently used. This technique has several advantages including transformation efficiency with higher copy numbers of single genes and ease of use with simple laboratory equipment. The *Agrobacterium*-mediated transformation method is commonly used to create transgenic plants, because it has more advantages compared to direct gene transfer methods such as particle bombardment, electroporation and silicon carbide fibers. The advantages include (1) stable gene expression; (2) a low number of transgene copies, (3) large DNA segments are able to be transferred (Ko and Korban, 2004).

Ti plasmid in a cell of *A. tumefaciens* contains a DNA fragment called T-DNA (sized 10–30 kb), which can integrate into the DNA of the plant cell nucleus. The Ti plasmid in *A. tumefaciens* cell is currently developed as a vector to insert foreign DNA into plant cells (Yuwono, 2006). This process requires functional transgenic constructs, including the desired main gene, promoter guiding the expression, marker genes, the transgene insertion into the Ti plasmid and the transformation of T-DNA contained in the plasmid into *Agrobacterium* cells (Mohammed and Abalaka, 2011).

In this study, a binary vector containing p35S::PaFT1-35S::GFP construct was then transferred into *A. tumefaciens* strain EHA101 and tobacco leaves. The detection of *GFP* at the beginning of plant growth indicates that *PaFT1* has integrated into plant genome. The p35S::PaFT1-35S::GFP/*A. tumefaciens* strain EHA101 is an efficient system for early detection and functional analysis of Flowering Gene *PaFT1* in tobacco, and it assumed will also work in orchid and other plant species. In the future, the success of *PaFT1* and *GFP* gene insertion will provide a means to more efficiently select for transformant plants that use exogenous *PaFT1* flowering genes to accelerate flowering in plants.

## Materials and Methods

### *Plant materials and bacterial strains*

Plant materials used as explants in this study were leaf dishes from two months old tobacco (*Nicotiana tabacum* L. var. Kemloko 1). The *in vitro* seedlings were cultivated on Murashige and Skoog (MS) medium, under white continuous light, at (25±2)°C room temperature. The leaf dishes were used as explants for *Agrobacterium*-mediated genetic transformation of p35S::PaFT1-35S::GFP /EHA101 construct. For bacterial strains, *Escherichia coli* strain DH5 $\alpha$ , *A. tumefaciens* strain EHA101, and *E. coli* containing pSK35S::GFP, pGAS101 plasmid were used to construct 35S::PaFT1::GFP. The pGAS101 plasmid (12.2 kb in length) was constructed previously to express *PaFT1* gene using *Cauliflower Mosaic Virus* (CaMV) promoter 35S, it contained *Neomycin Phosphotransferase II* (NPT II) gene, which encodes a resistance character against the antibiotic kanamycin as a selection marker. The pSK35S::GFP (15.8 kb in lengths) plasmid was obtained from Prof. Chiyoko Machida (*Chubu University, College of Bioscience and Biotechnology, Japan*) that was constructed based on the pSK1 plasmid (Kojima *et al.*, 1999). The plasmid carries *GFP* gene between *XbaI* and *NotI* restriction sites, which was used as reporter in this research.

### *Insertion of 35S::GFP fragment into pGAS101 plasmid*

The plasmid pGAS101 and pSK35S::GFP were isolated from *E. coli* using alkaline lysis method following the instruction manual of plasmid isolation kits (Qiagen, UK). The isolated pSK35S::GFP plasmids were cut using *EcoRI* restriction enzymes, and then separated using electrophoreses and visualized with a UV Transilluminator. The obtained p35S::GFP fragment bands were cut from the gel and extracted using JETQUICK Gel Extraction Spin Kit following the manual instruction (Genomed, The Netherlands). The gel extraction product was checked by PCR using 2 pairs of specific primers: the *sGFP* Fw (5'-ACGAGAAGCGCGATCACAT-3') - Nos Rev (5'-GTATAATTGCGGGACTCTAATCA-3')

primer pair. The pGAS101 plasmid containing *PaFT1* gene, which was previously cut using *EcoRI* restriction enzyme, and the p35S::GFP fragment was ligated to pGAS101 using Ligation High-DNA ligase at 16°C for 30 minutes. The presence of ligation products was checked using PCR with *sGFP* Fw and Nos Rev primers as before. The p35S::PaFT1-35S::GFP plasmid (16.2 kb in length) was constructed as shown in Figure 1.

### *Transformation of p35S::PaFT1-35S::GFP plasmid into E. coli strain DH5 $\alpha$*

Before the transformation, competent cells of *E. coli* strain DH5 $\alpha$  were prepared according to the methods described in Sambrook and Russell (2001). Competent cells were subjected to transformation through the heat shock method. A volume of 800  $\mu$ L of competent cells was divided into 8 microtubes with 100  $\mu$ L competent cells each. A mixture of competent cells and p35S::PaFT1-35S::GFP plasmid were soaked in liquid SOC medium in a microtube and the obtained mixture was incubated for 45 minutes in water bath at 37°C. A 100  $\mu$ L culture was taken from SOC medium, then inoculated it onto SOB solid medium plate containing 20 mM MgSO<sub>4</sub> and 50 mgL<sup>-1</sup> of kanamycin antibiotic using the spread method, and then it was incubated at 37°C. Colony PCR was performed to ensure that bacterial plasmids contained pGAS101 using a pair of degenerate primers DegPaFT1 (F1: 5'-GAKATGAATAGAGAGASRGAC-3') and DegPaFT1R1 (5'-TCAATCYTGCATYCT-TCTTCC-3'), and pSK35S::GFP was checked using the primers of *sGFP* Fw (5'-ACGAGAAGCGCGATCACAT-3') and Nos Rev primer (5'-GTATAATTGCGGGACTCTAATCA-3'), using PCR reactions with the following parameters: 94°C for 40 sec (initial denaturation) ; 94°C for 30 sec (denaturation); 60°C for 40 sec (annealing) for *sGFP* Fw and Nos Rev primer pair, 54°C for 40 sec for DegPaFT1 F1 and R1 primer pair; 72°C for 50 seconds (extension) and 72°C for 8 min (final extension), repeated for 40 cycles. The amplification products were checked using electrophoresis and visualized by a UV Transilluminator.

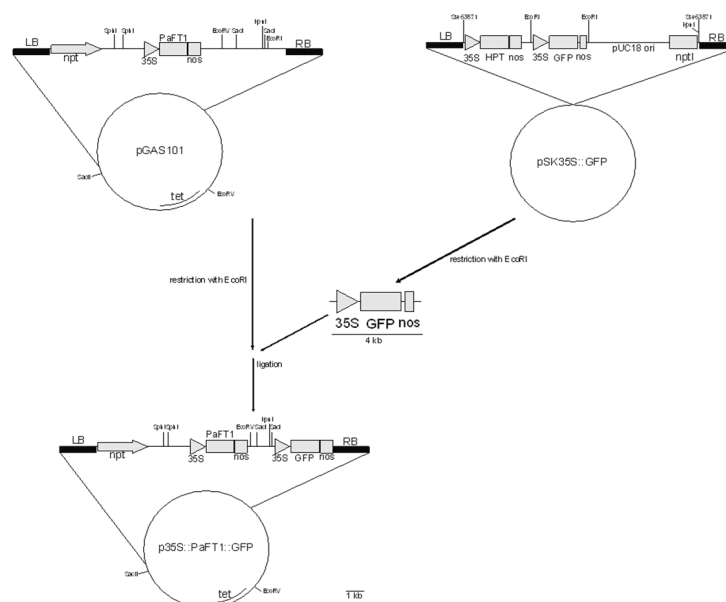
### Bacterial transformation by insertion of p35S::PaFT1::GFP plasmid into *A. tumefaciens* strain EHA101

The p35S::PaFT1-35S::GFP plasmids were transferred into *A. tumefaciens* competent cells, following procedures described by Widyasari and Suhandono (2007) with modification. *A. tumefaciens* competent cells in a volume of 100  $\mu$ L in a 1.5 ml Eppendorf tube were mixed with 1  $\mu$ L plasmid in cold condition with liquid nitrogen for 5 minutes. The mixture was then incubated at 37°C for 25 min. A volume of 1 mL liquid LB medium was added into the tube, and then the bacteria were grown at room temperature (28°C) for 3 hours with shaking at 50 rpm. Bacterial cells were later harvested by centrifugation at 12,000 rpm for 30 sec. Pellets were suspended with 1 mL LB. After that, 200  $\mu$ L cell suspension was spread into the selection media (LB + 5 mgL<sup>-1</sup> tetracycline antibiotics + 50 mgL<sup>-1</sup> kanamycin), and then incubated at 28°C for two days to obtain transformed colonies. The transformation results were checked by PCR with primer pair of Deg PaFT F2 and R1 to

detect the presence of *PaFT1*, and sGFP Fw and Nos Rev to detect *GFP*, in the same way of that used in *E.coli*. The recombinant p35S::PaFT1-35S::GFP / *A. tumefaciens* strain EHA101 was then ready to be used for genetic transformation in tobacco leaf explants.

### Transfer of T-DNA containing p35S::PaFT1-35S::GFP fragment into tobacco leaves

A single colony of *A. tumefaciens* strain EHA101 containing p35S::PaFT1-35S::GFP was cultured in a 15 mL corning tube containing 2 mL LB liquid medium with 50 mgL<sup>-1</sup> kanamycin and 5 mgL<sup>-1</sup> tetracycline antibiotics, incubated at 28°C for 48 h on a rotary shaker. A volume of 100  $\mu$ L *Agrobacterium* culture was added to 10 mL LB medium with 50 mgL<sup>-1</sup> kanamycin and 5 mgL<sup>-1</sup> tetracycline antibiotics, and incubated at 28°C for 48 h on a rotary shaker. Bacteria were then harvested by centrifugation at 6,000 rpm for 3 min. Bacterial cells pellet obtained was suspended in 10 mL liquid MS medium without sugar. After that, 2 mL bacterial culture was poured into 8 mL liquid MS



**Figure 1.** Construction of p35S::PaFT1-35S::GFP. The fragment of 35S::GFP (4,000 bp in size) in pSK35S::GFP plasmid was located between 2 *EcoRI* restriction sites, from which we cut the 35S::GFP fragment from the plasmid with *EcoRI* restriction enzyme. The pGAS101 plasmid has a single *EcoRI* restriction site, which facilitated linearization with *EcoRI* and subsequent insertion into 35S::GFP. The 35S::GFP DNA band with a size of 4,000 bp containing fragment were cut by *EcoRI*, and then gel extracted using Jet quick gel extraction kit (Genomed, Netherlands). The fragment of 35S::GFP and opened pGAS101 were mixed in a ratio 3:1, then ligated with T4 DNA ligase. The results of DNA ligation were checked using PCR with a pair of primers, the sGFP Fw and the Nos Rev. PCR products were visualized using UV Transilluminator. The final recombinant plasmid contains p35S::PaFT1-35S::GFP cassette.



medium without sugar, and then homogenized. Next, the *in vitro* leaf explants (1 cm<sup>2</sup> in size) were soaked into bacterial suspension for 30 min. Leaf explants were later drained on sterile filter papers, then planted on MS medium with addition of 1 mgL<sup>-1</sup> NAA, 3 mgL<sup>-1</sup> BAP, and 100 mgL<sup>-1</sup> acetosyringone, then it were incubated in the dark room for 3 days. The transformed explants were then washed using 1,000 mgL<sup>-1</sup> timentin for 5 minutes, then rinsed its with liquid MS medium without sugar twice for 3 min each. Finally, the explants were dried up with sterile filter papers and transferred onto MS solid medium with addition of 1 mgL<sup>-1</sup> NAA, 3 mgL<sup>-1</sup> BAP, 50 mgL<sup>-1</sup> kanamycin, and 300 mgL<sup>-1</sup> timentin for maintenance to form buds. About 2 months after *Agrobacterium* infection, some leaf pieces were analyzed for *GFP* expression, leaf pieces were placed on object glass, dropped with glycerin, covered with a cover glass, and then was observed with a fluorescent microscope under UV light, and photographed using Canon IXUS digital camera (Japan).

#### ***Analysis of PaFT1 expression in transgenic tobacco plants***

Detection of gene expression was performed by isolating mRNA from young leaves of 3 months old of p35S::PaFT1-35S::GFP containing tobacco transformant plants, followed with cDNA synthesis from the mRNA. Total RNA was isolated using *Total RNA Mini Kit Plant* (Geneaid) and cDNA was synthesized from mRNA using *Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit* (Thermo Fisher Scientific Inc.). To detect the expression of *PaFT1* gene in putative transgenic, RT-PCR was performed. The cDNA was used as template for RT-PCR analysis using the primers specific for the *PaFT1* gene (deg PaFT F2 and R1) in putative *PaFT1* transgenic plants. RT-PCR was also conducted for *GFP* gene using a pair of sGFP Fw and Nos Rev primers.

#### ***Phenotypic analysis of transgenic tobacco plants containing p35S::PaFT1-35S::GFP***

Two month-old regenerated plants from leaf explants cultivated on MS medium with

addition of 3 mg.L<sup>-1</sup> BAP + 1 mg.L<sup>-1</sup> NAA and 50 mg.L<sup>-1</sup> Kanamycin+300 mg.L<sup>-1</sup> timentin were transferred into MS medium for rooting. The plants were transferred into soil in pots and maintained in a greenhouse with natural environmental conditions and light photoperiods. The number of leaves, length of stem and time until detection of fluorescence were measured.

## **Results and Discussion**

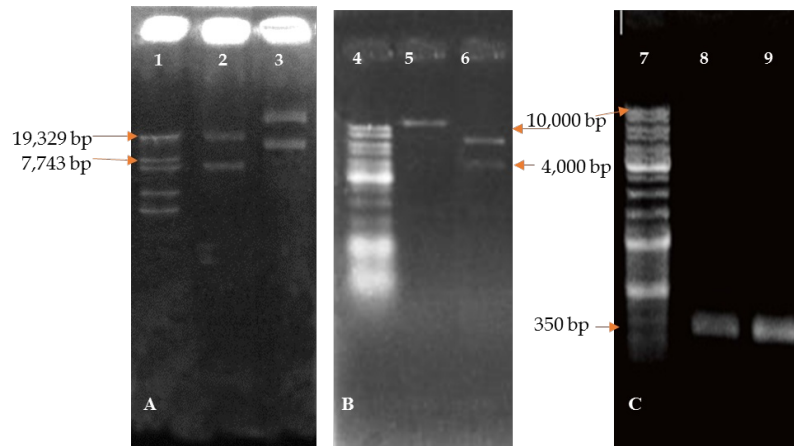
### ***Construction of p35S::PaFT1-35S::GFP***

The 35S::GFP that originally came from pSK35S::GFP plasmid was successfully inserted into pGAS101 (Figure 2). Figure 2A shows isolated 12.2 kb circular plasmid DNA of pGAS101 DNA and pSK35S::GFP (12 kb). *EcoRI*-digested plasmid showed a single linear plasmid of pGAS101. On the other hand, pSK35S::GFP was split into two bands: 8 kb and 4 kb, indicating that pGAS101 has only one *EcoRI* restriction site, and pSK35S::GFP has 2 *EcoRI* restriction sites (Figure 2 B). The presence of *GFP* in these two plasmids was confirmed by PCR analysis using sGFP Forward primers and Nos-ter primers, that resulted in 350 bp amplified DNA fragments.

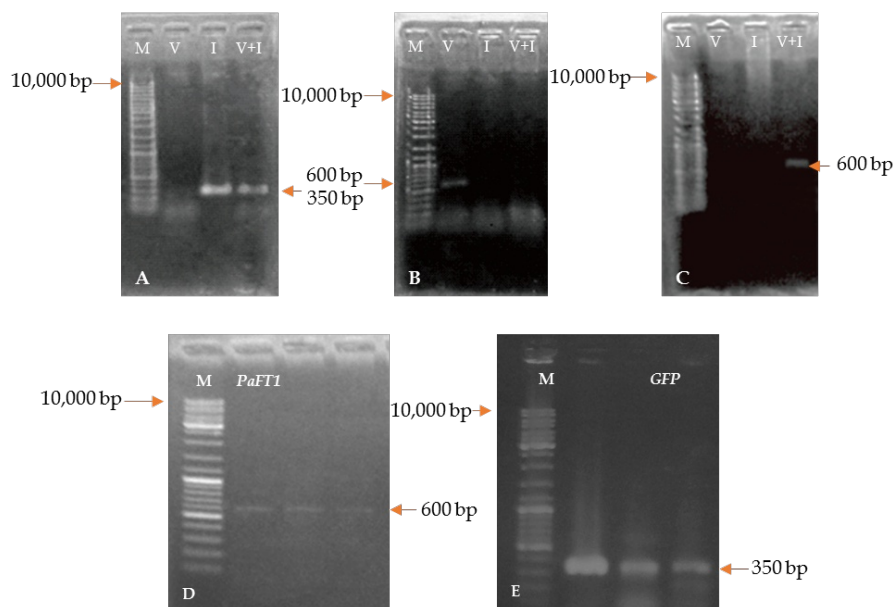
The linear plasmid DNA pGAS101 was ready to be inserted with *GFP* gene. A 35S::GFP DNA fragment of about 4 kb in length was gel extracted with the *Jet quick gel extraction kit* (Genomed).

DNA bands resulting from amplified DNA pSK 35S::GFP using PCR with primers of sGFP Fw and Nos Rev were evaluated by gel extraction for the presence *GFP* gene. As a negative control experiment, the detection of *HPT* gene was checked using a pair of *HPT* primers (*HPT* Fw and *HPT* Rev). PCR products were then checked using electrophoresis and visualized using a UV Transilluminator (Figure 3). The presence of *GFP* was detected. This data confirmed that the *GFP* had been amplified.

Products of gel extraction process targeting 35S::GFP fragment were mixed with pGAS101 plasmids, which were already digested with *EcoRI* and ligated with T4 DNA ligase. The ligation mixture was then incubated



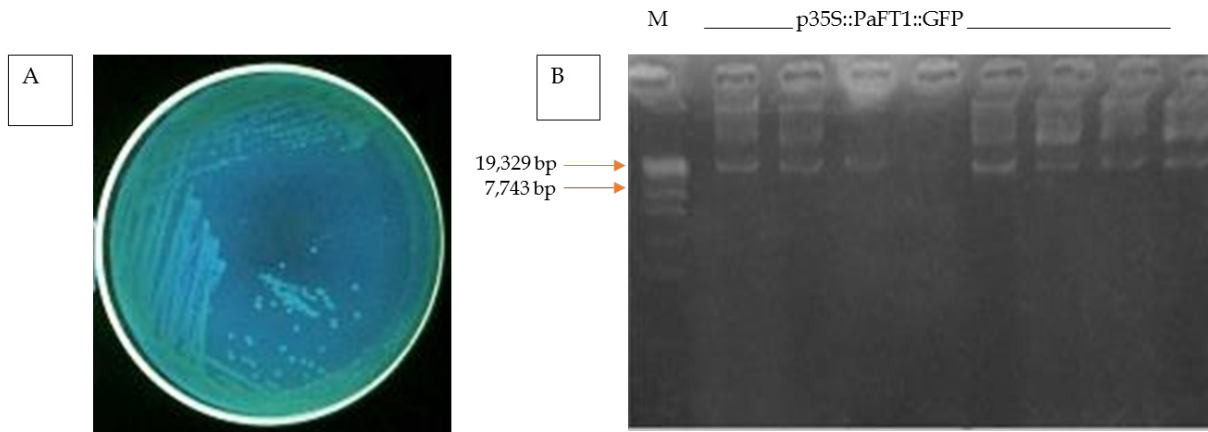
**Figure 2.** Confirmation of pGAS101 and pSK35S::GFP plasmids. (A) Isolated plasmid DNA of pGAS101 and pSK35S::GFP plasmids; (B) *EcoRI* restriction enzyme-digested pGAS101 and pSK35S::GFP plasmids; (C) Amplified DNA from pSK35S::GFP [Lane 1. Marker  $\lambda$ /styI DNA, lane 2. pGAS101 plasmid DNA, lane 3. pSK35S::GFP plasmid DNA, lane 4. Marker of 1kb DNA ladder, lanes (1, 4 and 7). *EcoRI* restriction enzyme digested pGAS101 (5) and pSK35S::GFP plasmid (6); GFP amplified DNA from pSK35S::GFP (8 and 9)].



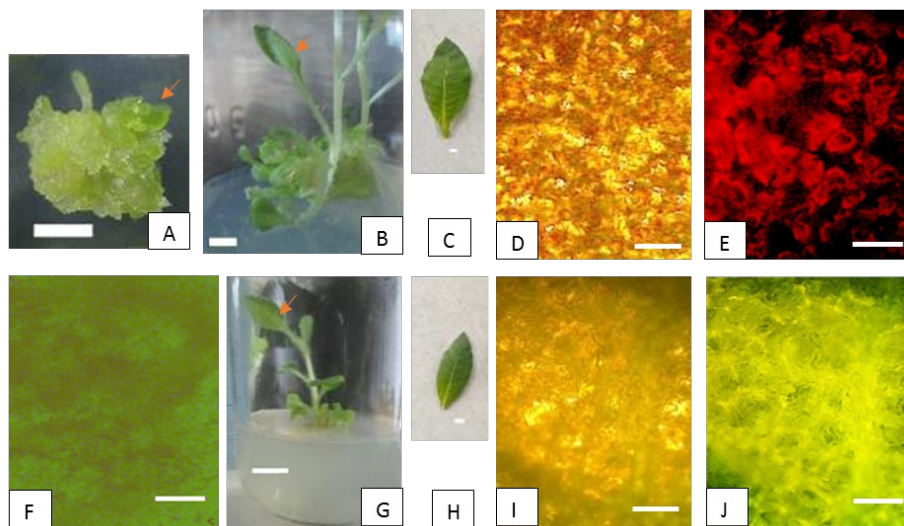
**Figure 3.** Detection of *PaFT1* and *GFP* in bacterial plasmid. (A, B, C) Amplified of *PaFT1* and *GFP* from *E. coli* DH5 $\alpha$  and (D, E) *A. tumefaciens* EHA101 using *GFP* and *PaFT* primers. (A) s*GFP*, (B) Deg*PaFT* (54°C), and (C) Deg*PaFT* (57°C). PCR products matched the size of *GFP* (350 bp) from a colony containing pSK35S::*GFP* (I) and a colony of *p35S::PaFT1::GFP* (V+I), while products matching with size of *PaFT* 1 (600bp) were obtained from a colony of pGAS101 (V) and a colony of *p35S::PaFT1::GFP* (V+I). (D) *PaFT1* and (E) *GFP* from *p35S::PaFT1::GFP/A. tumefaciens* EHA 101, (M) 1kb DNA ladder.

at temperature of 37°C for 30 mins. Products of the ligation process were used to transform the *E. coli* strain DH5 $\alpha$  competent cells using the heat shock method. The heat shock method takes advantage of the membrane permeability created when cells undergo sudden temperature changes in a short period. Competent cells were diluted in CaCl<sub>2</sub> (0.1M)

cold solution (Sambrook and Russell, 2001) to induce competence so the cells could accept foreign DNA molecules and to undergo transformation (Madigan *et al.*, 2012). The *E. coli* competent cells were obtained by inducing the bacterial cells with CaCl<sub>2</sub> treatment. The treatment resulted in cells that could be transformed by allowing DNA to



**Figure 4.** p35S::PaFT1-35S::GFP plasmid/*A. tumefaciens* EHA101. (A) Colonies of *A. tumefaciens* bacterial culture; (B) Isolated plasmid DNA of p35S::PaFT1-35S::GFP /*A. tumefaciens* EHA101, M=Marker ( $\lambda$ /styI).



**Figure 5.** Expression of *GFP* in leaves of transgenic tobacco containing p35S::PaFT1-35S::GFP. (A) The 1.5 months old leaf culture of transgenic tobacco containing p35S::PaFT1-35S::GFP on MS medium + 1 mgL<sup>-1</sup> NAA, 3 mgL<sup>-1</sup> BAP, 50 mgL<sup>-1</sup> kanamycin, and 300 mgL<sup>-1</sup> timentin; (F) The callus explants exposed under UV light illumination showed green fluorescence; (B) A 2 months old plantlet of Wild type/non transformed on MS medium + 1 mgL<sup>-1</sup> NAA, 3 mgL<sup>-1</sup> BAP; (C) Leaf of (B); (D) 2 months old Wild type/non transformed leaves examined under bright light, and under UV light (E); (G) A 2 month-old plantlet of transgenic tobacco containing p35S::PaFT1-35S::GFP on MS medium + 1 mgL<sup>-1</sup> NAA, 3 mgL<sup>-1</sup> BAP, 50 mgL<sup>-1</sup> kanamycin, and 300 mgL<sup>-1</sup> timentin; (H) Leaf of (G); (I) the leaf of 2 month-old plantlet of transgenic tobacco containing p35S::PaFT1-35S::GFP under white light and under UV light (J). The bright green fluorescence was detected in the leaf of transformant (J), but not in wild type (E) that showed red autofluorescence. Bars (A, B, C, G, H) 1 cm; Bars (D, E, F, I, J) 100  $\mu$ m.

**Table 1.** The initiation of flowering in transgenic tobacco containing p35S::PaFT1-35S::GFP.

Treatment	Number of dish	Kanamycin positive	PCR positive	GFP positive	Number of leaves*		Plant height (cm)*		Flowering time (month)	
					NT	T	NT	T	NT	T
1	25	10/25 (0.4)	4/6 (0.67)	3/5 (0.6)	18	17	125	120	6	5
2	25	11/25 (0.44)	5/8 (0.625)	4/5 (0.8)	17	14	120	115	6	5
3	25	15/25 (0.6)	3/6 (0.5)	4/5 (0.8)	17	17	130	120	6	5
Total		36/75 (0.48)								

\*5 months old; NT: non-transformant, T: transformant



pass through the *E. coli* cell wall and membrane, with  $\text{Ca}^{2+}$  ions neutralizing the negative charges in DNA molecules so the DNA could more readily enter the cells following *heat shock* treatment (Casali, 2003). At the same time multiplication of pGAS101::GFP recombinant plasmids was occurred.

Checking whether bacteria carry pGAS101, pSK35S::GFP, and p35S::PaFT1-35S::GFP plasmids was performed by colony PCR to amplify DNA fragments using two pairs of primers, the DegPaFT F1 - R1 and sGFP Fw - Nos Rev. Amplified DNA fragments matching the sizes of *GFP* and *PaFT1* genes, respectively were detected (Figure 3D and E).

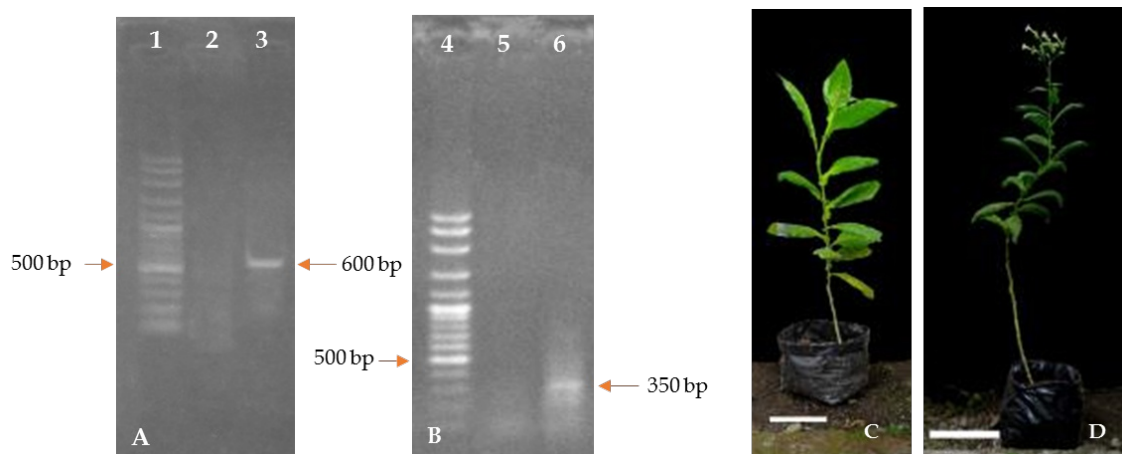
The p35S::PaFT1-35S::GFP recombinant plasmid was isolated from *E. coli* strain DH5 $\alpha$ . The plasmid was then transformed into competent cells of *A. tumefaciens* strain EHA101. Procedures of plasmid transformation into competent cells of *A. tumefaciens* was adopted from a method described by Widyasari and Suhandono (2007). The p35S::PaFT1-35S::GFP recombinant plasmid was isolated from *A. tumefaciens* strain EHA101, and then it was checked by electrophoresis and visualized with UV Transilluminator (Figure 4).

The p35S::PaFT1-35S::GFP/*A. tumefaciens* EHA101 plasmid obtained as transformation products were checked using PCR with 2 pairs of primers, the degPaFT F2 - deg PaFT

R1 and sGFP Fw - Nos Rev. The PCR products showed the presence of *PaFT1* and *GFP* genes (Figure 3).

Thus, the recombinant p35S::PaFT1-35S::GFP/*A. tumefaciens* strain EHA101 was ready to be used for genetic tobacco transformation to accelerate flowering. Examination of the tobacco leaves under UV light showed the bright green fluorescence of *GFP* in the leaves of 2.5 months old transformants. (Figure 5, Table 1).

To examine gene expression in transformant plants, we used RT-PCR using gene-specific primers. This method was also used by Semiarti *et al.* (2010) to detect *GFP* gene insertion in *P. amabilis* orchid protocorms, to evaluate transgenic plants carrying a copper-inducible reporter gene expression system in *Arabidopsis thaliana* by Saijo and Nagasawa (2015), to evaluate the efficiency of T-DNA delivery in transformants of *Phalaenopsis violacea* by Zulkifle *et al.* (2010) and to analyze FT expression in plant by Xu *et al.* (2012). In this study, *PaFT1* and *GFP* transcripts were detected in putative p35S::PaFT1-35S::GFP transgenic tobacco plants (Figure 6) indicating that the T-DNA of p35S::PaFT1-35S::GFP was integrated into the tobacco genome and functionally active. Transgenic tobacco plants with p35S::PaFT1-35S::GFP construct showed accelerated flowering with 14–17 leaves and 115–120 cm height at 5 months whereas wild



**Figure 6.** Early flowering of *N. tabacum* induced by p35S::PaFT1-35S::GFP. Detection of *PaFT* (A) and *GFP* (B) expression in the putative transgenic tobacco containing p35S::PaFT1-35S::GFP by RT-PCR. (Lane 1 and 4, 100 bp DNA Ladder; lane 2 and 5, non-transformant plants; lane 3 and 6, transformant plants). (C) 5 month-old wild type/non-transformed plant. (D) 5 month-old transgenic plant. Bars 25 cm.



type (non-transformant) plants flowered around one month later than transgenic tobacco plants with more leaves (Table 1, Figure 6C and 6D).

These results indicate that *PaFT1* can mildly reduce the vegetative growth period and accelerate flowering in tobacco. The use of *GFP* for efficient and early detection of transgenes was very helpful for early judgement of positive transformants.

### Conclusions

The use of *GFP* for p35S::*PaFT1*-35S::*GFP* construct creates an efficient system for *Agrobacterium*-mediated plant transformation in plant. The recombinant plasmid, p35S::*PaFT1*-35S::*GFP* is a potential tool for genetic transformation in plants to accelerate flowering.

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