Insertion of a Flowering gene, *PaFT*, into *Phalaenopsis amabilis* orchid using *Agrobacterium tumefaciens*

Ixora Sartika Mercuriani[1, 2*], Aziz Purwantoro[3], Sukarti Moeljopawiro[1, 4], and Endang Semiarti[1, 4]

[1)] Biotechnology Study Program, The Graduate School, Gadjah Mada University, Jl. Teknika Utara Pogung Sleman, Yogyakarta, 55281, Indonesia
[2)] Biology Education Department, Faculty of Mathematics and Natural Science, Yogyakarta State University, Jl. Colombo No. 1, Yogyakarta, 55281, Indonesia
[3)] Faculty of Agriculture, Gadjah Mada University, Sekip Unit I, Yogyakarta, 55281, Indonesia
[4)] Faculty of Biology, Gadjah Mada University, Jl. Teknika Selatan Sekip Utara, Yogyakarta, 55281, Indonesia

Email: * ixomerc@uny.ac.id

Abstract

Flowering locus T (*FT*) genes family have been reported as the important genes to control the transition from vegetative to reproductive growth stage in several plant species. An *FT*- like gene has been isolated from wild orchid *Phalaenopsis amabilis*, hereafter named *PaFT*. To analyze the function of this gene, in this research, we have introduced the *PaFT* gene under the control of Ubiquitin constitutive promoter (*Ubi-pro::PaFT*) into *P. amabilis* genome through *Agrobacterium*-mediated transformation. Integration of *Ubi-pro::PaFT* into the *P. amabilis* genome was analyzed by PCR using Ubi Forward and TNos Reverse primers. PCR analysis from the transgenic *P. amabilis* yield an 1.11 kb DNA fragmen that indicate insertion of *Ubi-pro::PaFT* fragmen. The frequency of transformations was in the ranged from 2.6 to 6.7 percent. Molecular analysis on the function of *Ubi-pro::PaFT* in transformantt orchid plants is now in progress.

Keywords*: Flowering locus T (FT), Agrobacterium-mediated transformation, Ubiquitin promoter (Ubi-pro), Phalaenopsis amabilis*

1. Introduction

Orchid is a high aesthetic plant with a high economic value. There are more than 5000 species of wild-type orchids in Indonesia (Irawati, 2002). *Phalaenopsis amabilis* (L.) Blume is a very potential orchid that is used as a parental plant to produce some hybrid orchids. Flowering of this orchid is very important, in which transition from vegetative to reproductive phase is important to ensure sexual reproduction. In term that orchid is planted for its flowers, therefore short time waiting for flowering is expected. *P. amabilis* has a long period of vegetative phase (about 2 years since the time of seed sowing). It needs advanced technologies to accelerate the flowering. Up to now the acceleration of orchid flowering is mostly done by manipulating environmental conditions, as well as the use of phytohormones, but the result is still insufficient. Another approach such as genetic engineering is needed to get the appropriate target for flowering. Genetic engineering using flowering gene will give benefit appropriately to induce early flowering.

There are about 80 important genes predicted to be involved in flowering, the *Flowering locus T* (*FT*) is predicted as a key gene in the molecular regulation of flowering in *Arabidopsis* (Kobayashi *et al.*, 1999; Kardailsky *et al.*, 1999; Turck *et al.*, 2008). *FT* encodes the formation of a protein that serves as the primary signal in flowering (Florigen). Several genes that have similarity with *FT* (*FT-like* genes) have been identified and characterized from various plants, e.g: *Heading date 3a* (*Hd3a*) and RFT1 from rice (Kojima *et al.*, 2002; Komiya *et al.* , 2008), *Medicago trucantula FT* (*MtFTa1*) from Medicago (Laurie *et al.*, 2011), *Malus domestica FT* (*MdFT1* and *MdFT2*) from apple (Kotoda *et al.*, 2010), *Picea Abies FT* (*PaFT4*) from spruce (Gyllenstrand *et al.*, 2007), and *Poplar FT* (*FT2*) from Poplar (Hsu *et al.*, 2006). Overexpression of

FT-like genes shorten the periode of juvenile phase of plants and induce early flowering, while mutation of these genes resulted in late flowering.

Recently a *FT-like* gene has also been isolated from *P. amabilis* namely *PaFT* by Dr. Seonghoe Jang (Academia Sinica, Biotechnology Center in Southern Taiwan, 2010, personal communication). This gene has been cloned in pGA plasmid under the control of constitutive promoter Ubiquitin (Ubi). Overexpression of this *FT* gene (*Ubi-pro::PaFT*) in *P. amabilis* was carried out using *Agrobacterium tumefaciens*. Compare to other method, the use of *Agrobacterium* relatively easy and cheap, therefore it has been used in genetic transformation of orchid (Zhang *et al*., 2010; Semiarti *et al*., 2007; Shrawat and Lorz, 2006; Shrestha *et al.*, 2007; Sjahril *et al*., 2006; Mishiba *et al.*, 2005; Liau *et al.*, 2003; Yu *et al*., 2001; Belarmino and Mii, 2000). The objective of this research is to insert *Ubi-pro::PaFT* fragmen to *P. amabilis* genome through *Agrobacterium*-mediated transformation to get a new orchid cultivar with acceleration of flowering.

2. Methods

Materials

The plant materials were 3-6 weeks old *P. amabilis* protocorms. Protocorms are produced by sowing seeds from mature fruit (4 months after self pollination) on New Phalaenopsis (NP) medium that have been modified by addition of 150 $ml.L^{-1}$ coconut water and 100 $mg.L^{-1}$ tomato extract (Semiarti *et al*., 2010).

Plasmid Vectors and Bacterial Strains

Agrobacterium tumefaciens strain LBA4404 that contains *Ubi-pro::PaFT* carrying plasmid (pGAS102) and empty plasmid (pGA3426) is used as the source of gene and control experiment, respectively. These clones were obtained from Dr. Soenghoe Jang (Academia Sinica, Biotechnology Center in Southern Taiwan).

Co-cultivation and Selection

Agrobacterium-mediated transformation is done according to Semiarti *et al.* (2007) with several modifications. A colony of both *Agrobacterium* that carry pGA3426 and pGAS102 inoculated in 5 ml of liquid LB medium containing 5 $mg.L^{-1}$ Tetracycline and incubated at 28^{o}C for 2 days. After that, 1 ml of this culture was subcultured in 9 ml of new medium containing 50 $mg.L^{-1}$ Acetosyringone and incubated overnight. 10ml of *Agrobacterium* culture (OD600 = 0.8-1) were centrifuged at 5000 rpm for 10 minutes. Bacterial pellets then resuspensed with NP medium. Suspension of bacterial culture was then diluted with liquid NP medium at ratio 1: 4 (v / v) and used for cocultivation.

Prior to cocultivation, orchid protocorms were subcultured for 4 days in solidified NP medium containing 1 mgl^{-1} 2,4-D and 50 mgl^{-1} acetosyringone. In cocultivation, precultured protocorms were collected and immersed in diluted *Agrobacterium* suspension that is supplemented with 2 drops of Tween20 and 50 mgl^{-1} acetosyringone in a new sterile petridish for 30 min. These protocorms were then transferred onto sterile filter paper to dry and cultured onto solidified NP medium containing 1 mgl^{-1} 2,4-D and 50 mgl^{-1} acetosyringone. Elimination of *Agrobacterium* was done by washing protocorms with sterile destilated water for 3 times and continues with washing medium (no sugar added liquid NP medium containing 25 $mg.L^{-1}$ Meropenem). The last washing was done 2 days (the medium was changed every day) with agitation (100 rpm) . Protocorms were then transferred onto sterile filter paper to dry and cultured onto solidified NP medium containing 5mM 2-IP, 0.15mM NAA, and 100 mgl^{-1} carbenicillin for a week to inhibit *Agrobacterium* overgrowth. Growing were rinsed thoroughly with liquid NP medium containing 25 $mg.L^{-1}$ Meropenem, then transferred onto medium containing 5mM 2-IP, 0.15mM NAA, 100 mgl^{-1} carbenicillin and 10 $mg.L^{-1}$ Hygromycin as selection agent for 6 weeks. Subculturing on the same selection medium was done every 2 weeks. After selection, protocorm were regenerated on solidified NP medium containing 5mM 2-IP, 0.15mM NAA.

Analysis of the transgene in *P. amabilis* genome

Analysis of gene insertions in *P. amabilis* genome was performed by PCR. Genomic DNA was isolated from small cut of protocorms. Amplification of *Ubi-pro::PaFT* DNA fragment from *P. amabilis* genome performed using Ubiquitin (forward: 5'-TTGTCGATGCTCACCCTG-3 ') and TNos (reverse: 5'-GATCTAGTAACATAGAT GACACCGCG-3') primers. The DNA amplification was carried out by PCR using the following conditions: 5 min at 94^{o}C for the first denaturation of DNA; 35 cycles of 1 min at 94^{o}C for denaturation, 30 sec at 59^{o}C for annealing, and 1.5 min at 72^{o}C for elongation; and 5 min at 72^{o}C for Post PCR. Amplified DNA was then checked on 1% gel electrophoresis, stained with green fluorescence staining (Good View), and visualized with UV-transilluminator.

3. Results and Discussion

In this research we use hygromycin containing medium to select putative transformant. This selection agent is due to *HPT* gene that is used as selectable marker. Almost all of the protocorms are died in 6 weeks after sub-cultured in selection medium (Table 1 and Figure 1). All of the non-cocultivated protocorms were able to regenerate on medium without hygromycin, while only 0.3 % protocorms retain green on hygromycin containing medium. We got several putative transformantt from cocultivated protocorm with both pGA3426 and pGA102 (Table 1 and Figure 1). At first (4 weeks after selection), most cells of the protocorms were turned into brown and finally died on selection medium, but then (in 2 weeks later) small creamy white cell colonies with hygromycin-resistance initiated to grow from the browned or dead cell population. These cells then grow to be a small greenish yellow protocorm-like bodies (PLB) (Figure 2). This result indicates there is only several cells of protocorm that their genome were successfully inserted with the *HPT* and *PaFT* transgene. It is similar to the previous result of *Agrobacterium*-mediated transformation in *Phalaenopsis* (Sjahril *et al*., 2006). There are 6.7 % putative transformant resulted from pGA3426 containing *Agrobacterium* cocultivated protocorms and 2.7 % putative transformant resulted from pGAS102 containing *Agrobacterium* cocultivated protocorms. The difference of transformation frequency is assumed because of vector (pGA3426) has smaller size of the T-DNA. This makes it easier to be inserted in the target genome.

Table 1. Transformation Frequency of pGA3426 and pGAS102 containing *Agrobacterium* into protocorms

Treatment		Hygromycin (10 mg.L^{-1})	Replication	Total Protocorm	Retaining Green Protocorm	Transformation Frequency (%)	Average of Transformation Frequency (%)
[a] NT	1	-	1	335	335	-	-
			2	327	327	-	
	2	+	1	357	1	-	-
			2	329	1	-	
[b]T#1		+	1	382	25	6.5	6.7±0.2
			2	411	28	6.8	
[c]T#2		+	1	546	12	2.2	2.6±0.6
			2	565	17	3.0	

[a]non cocultivated (Wild Type/Non Transformant) protocorms
[b]pGA3426 containing Agrobacterium cocultivated protocorms
[c]pGAS102 containing Agrobacterium cocultivated protocorms

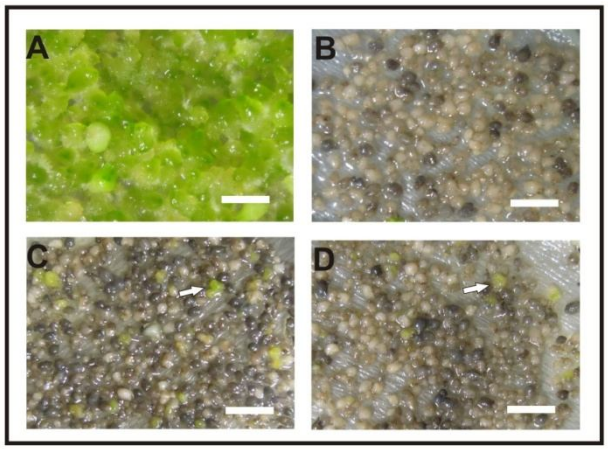

Figure 1. The growth of putative transformantt on NP medium with and without hygromycin, in 6 weeks after subculture. A, NT protocorms on NP medium without hygromycin as positive control. **B**, NT growth on hygromycin containing medium as negative control. **C**, T#1 growth on hygromycin containing medium. **D**, T#2 growth on hygromycin containing medium. Arrows point to the green protocorm that survived growth on Hygromycin containing NP medium. Bar: 6 mm

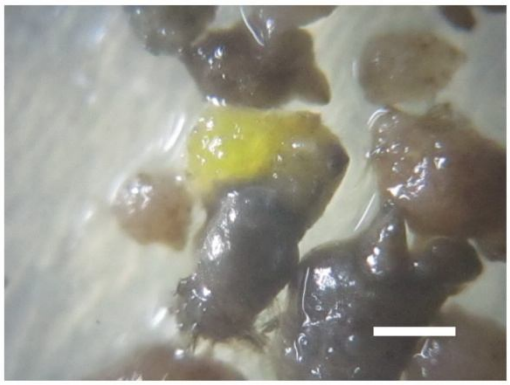

Figure 2. Regenerated small cell colonies forming greenish PLB. Bar: 1 mm

We have analyze *Ubi-pro::PaFT* fragment insertion in a putative transformantt genome from both leaves and roots. There is a 1.11 kb fragment that indicates *Ubi-pro::PaFT* inserted in *P. amabilis* orchid genome (Figure 3). This Molecular analysis is still in progress. We still analyze transgene integration with other putative transformantts and will be continued with trangene expression analysis in RNA level.

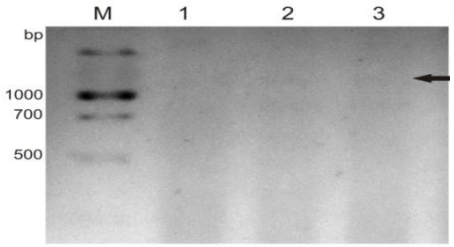

Figure 3. Detection of *Ubi-pro::PaFT*/transgene insertion in orchid transformantt. M, molecular marker. **1**, *Ubi-pro::PaFT* insertion analysis from T0. **2**, *Ubi-pro::PaFT* insertion analysis from leaf of T2. **3**, *Ubi-pro::PaFT* insertion analysis from root of T2. Arrow points to 1.11 kb fragment.

4. Conclusion

Several putative tranformants have been regenerated from both T#1 and T#2 with transformation frequency about 2.6 and 6.7 %. *Ubi-pro::PaFT* insertion analysis from both leaf and root of T#2 by PCR yielded 1.11 kb fragment.

Acknowledgement

We wish to thank Dr. Seonghoe Jang, Academia Sinica, Biotechnology Center in Southern Taiwan for providing pGA3426 and pGAS102 containing Agrobacterium. This work was supported by National Strategy Grant 2011 from Directorate General of Higher Education, Indonesia.

Reference

Journal article

[1] Belarmino, MM., and Mii, M. 2000. *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. *Plant Cell Rep* 19: 435–442

[2] Gyllenstrand, N., Clapham, D., Ka¨llman ,T., and Lagercrantz, U. 2007. A Norway Spruce FLOWERING LOCUS T Homolog IsImplicated in Control of Growth Rhythm in Conifers. *Plant Physiol.* 144: 248–257

[3] Kardailsky, L., Shukla, V.K., Ahn, J.H, Dagenais, N., Christensen S.K., Nguyen J.T., Chory J., Harrison M.J., and Weigel, D. 1999. Activation tagging of the floral inducer FT. *Science* 286: 1962-1965.

[4] Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. 1999. A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286: 1960-1962.

[5] Kojima S, Takahashi, S., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., Yano, M. 2002. Hd3a, a Rice Ortholog of The Arabidopsis FT Gene, Promote Transition to Flowering Downstream of Hd1 under Short-Day Conditions. *Plant Cel Physiol.* 43(10): 1096-1105.

[6] Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S., Shimamoto, K. 2008. *Hd3a* and *RFT1* are essential for flowering in rice. *Development* 135: 767-774.

[7] Kotoda, N., Hayasi, H., Suzuki, M., Igarashi, M., Hatsuyama, Y., Kidou, S., Igasaki, T., Nishiguchi, M., Yano, K., Shimizu, T., Takahashi, S., Iwanami, H., Moriya, S., Abe, K. 2010. Molecular characterization of Flowering Locus T-Like genes of apple (Malusxdomestica Borkh.). Plant Cell Physiol. 51(4): 561-575

[8] Laurie, R.E., Diwadkar, P., Jaudal, M., Zhang, L., Hecht, V., Wen, J., Tadege, M., Mysore, K.S., Putterll, J., Weller, J.L., Macknight, R.C. 2011. The Medicago *Flowering Locus T* homolog, *MtFTa1*, is a key regulator of Flowering Time. *Plant Physiol.* 156: 2207-2224.

[9] Liau C.H., You, S.J., Prssad, V., Hsiou, H.H., Lu, J.C., Yang, N.S., Chan, M.T. 2003. *Agrobacterium tumefaciens*-mediated transformation of an Onchidium orchid. Plant Cell Rep. 21: 993-998.

[10] Mishiba K.I., Chin, D.P., Mii, M. 2005. Agrobacterium–mediated transformation of *Phalaenopsis* by targeting protocorm at an early stage after germination. Plant Cell Rep. 24: 297-303.

[11] Semiarti E, Indrianto, A., Purwantoro, A., Isminingsih, S., Suseno, N., Ishikawa, T., Yoshioka, Y., Machida, Y., Machida,C. 2007. *Agrobacterium*-mediated transformation of the wild orchid species *Phalaenopsis amabilis*. *Plant Biotechnol*. 24: 265-272.

[12] Semiarti, E., Indrianto, A., Purwantoro, A., Martiwi, I.N.A., Feroniasanti, Y.M.L, Nadifah, F., Mercuriani, I.S, Dwiyani, R., Iwakawa, H., Yoshioka, Y., Machida, Y., and Machida, C. 2010. High-frequency genetic transformation of *Phalaenopsis amabilis* orchid using tomato extract-enriched medium for the pre-culture of protocorms. *Journal of Horticultural Science & Biotechnology* 85 (3) 205–210

[13] Shrestha, B.R, Chin, D.P, Tokuhara, K., and Mii, M. 2007. Efficient production of transgenic plantls of *Vanda* through sonication-assisted *Agrobacterium*-mediated transformation of protocorm-like bodies. Plant Biotechnol. 24: 429–434.

[14] Sjahril, R., Chin, DP., Khan, RS., Yamamura, S., Nakamura, I., Amemiya,Y., Masahiro Mii, M. 2006. Transgenic *Phalaenopsis* plants with resistance to *Erwinia carotovora* produced by introducing wasabi defensin gene using *Agrobacterium* method. Plant Biotech. 23: 191-194

[15] Turck, F., Fornara, F., Coupland, G. 2008. Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annu Rev Plant Biol* 59: 573–594

[16] Yu, H., Yang, S.H., Goh, C.J. 2001. Agrobacterium-mediated transformation of a Dendrobium orchid with the class 1 knox gene DOH1. *Plant Cell Reports* 20: 301–305

[17] Zhang, L., Chin, D.P., Fukami, M., Ichikawa, H., Nakamura, I., Mii, M. 2010. Agrobacterium–mediated transformation of Cattleya with an Odontoglossum ringspot virus replicase gene sequence. Plant Biotechnol. 27: 421–426

Monograph, edited book, book
[1] Irawati, 2002. Pelestarian jenis anggrek Indonesia. Buku panduan Seminar Anggrek Indonesia 2002. Hal: 34-45