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Evaluation of Constitutive Promoters for Gene Expression in *Dendrobium* Protocorms and Flowers

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ABSTRACT

Actively dividing protocorms of *Dendrobium* Jaquelyn Thomas orchid were produced on VW medium and transferred to $^1/_2$ MS medium before subjecting to particle bombardment to evaluate the promoter efficiency. Three different constitutive promoters, rice Act-1, CaMV35S, and maize Ubi-1, were determined for the efficiency to control spatial transgene expression in protocorms and sepal tissues of *Dendrobium* orchid. Each promoter was fused to *β-glucuronidase* (*uid A*) gene and *nos* terminator in the plant expression vectors including pActin1-D, pAHC27, and p2K7. The plasmid vectors were individually bombarded into protocorms and fresh sepals of *Dendrobium*. Transient *gus* gene expression was assayed after 3 days of bombardment. The results demonstrated that all tested promoters could drive *gus* gene expression in the orchid protocorms at different levels. Ubi-1 promoter conferred the most efficient promoter in protocorms giving the highest level of *gus* gene expression, whereas GUS activity was distinctly observed under the control of Act-1 and CaMV35S in the *Dendrobium* sepal tissues. Both Act-1 and CaMV35S promoters performed well in the vascular tissue of flower as opposed to the Ubi-1 promoter that did not perform in flower.

Key words: *Dendrobium* orchid, promoter, actin, bombardment, transformation

INTRODUCTION

To date, genetic engineering has been commonly used for introducing specific genes into plants for trait improvement including orchids. Because of the desirability of global marketing, many traits are included in biotechnology programs such as novel color and shape of the flower, long vase life, and pests and disease resistance. Since the success of genetic plant transformation is not only due to gene integration into chromosome of transgenic plant but also to high activity in the target tissue. The suitable promoter is an important factor to drive spatial and temporal gene expression. The broadly available constitutive promoters in plant were CaMV35S promoter from the 5′ region of cauliflower mosaic virus gene (Guilley *et al*., 1982), Act1-D promoter from rice actin gene (McEloy *et al*., 1990), Adh-1 promoter from alcohol dehydrogenase gene (Ellis *et al*., 1987), and Ubi-1 promoter from ubiquitin gene (Toki *et*

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al., 1992). For orchid transformations, the constitutive promoters were Act-1, CaMV35S, and Ubi-1. Although CaMV35S was widely used to produce transgenic orchid, previous reports revealed that CaMV35S promoter was not active in all cell types of tobacco (Benfey and Chua, 1989), rice (Terada and Shimamoto, 1990; Wilkinson *et al*., 1997) and also low level expression in transgenic monocots (Zhang *et al*., 1991; Christensen, 1992; Chowdhury, 1997). On the contrary, the promoter isolated from monocotyledon plants showed higher activity in transgenic monocot plants such as rice (Toki *et al*., 1992; Cornejo *et al*., 1993) and maize (Zhong, 1996; Christensen *et al*., 1992). As noted earlier, orchid transformation showed the high potential of CaMV35S promoter to drive the selectable marker gene expression in protocorm during selection (Anzai *et al*., 1996, Yu *et al*., 1999; Tee *et al*., 2003). Nevertheless, proposing the desirable traits in orchid for the improvement has been focused on the flower to create a novel color and shape. So far, there has been no information on the activity of the potential promoter for orchid flower.

In addition, the media culture producing the suitable target tissue for bombardment is of considerably important to achieve the successful orchid transformation (Nan and Huehnle, 1995; Tee *et al*., 2003). Therefore, this paper was to report the media affecting protocorms induction and the efficient promoters for ubiquitous transient gene expression in protocorm and flowers of *Dendrobium* orchid through particle bombardment technique.

MATERIALS AND METHODS

Cultured medium

Young shoots of *Dendrobium* Jaquelyn Thomas orchid (3-4cm in length) were soaked in 70% ethanol for 10 min and in 2.5% sodium hypochlorite for 20 min followed by three thorough washings in sterilized distilled water. Shoot tips and auxiliary buds (2 mm) were excised from the shoots, after removal of the leaf sheaths. The tissues were cultured in a modified VW medium (Vacin and Went, 1949) with shaking at 100 rpm under artificial light of 35$\mu molm^{-2}s^{-1}$ with a light/dark cycle of 16/8 h at 25°C. Protocorms were induced and sub-cultured to fresh medium at 14-day intervals. After 90 days of subculture, the second protocorms were used for the growth and regeneration tests in VW, $\frac{1}{2}$MS (Murashige and Skooge, 1962), MS+VW, and KC (Knudson, 1946) medium supplemented with 2% sucrose, 0.2% active charcoal, and 0.8% agar.

Plant materials

The protocorms of *Dendrobium* orchid obtained from young apical buds were sub-cultured to produce new active dividing tissues in liquid VW for 21 days. The protocorms were pretreated to decrease cell osmotic pressure by culturing on MS medium supplemented with 0.8% manitol, 0.2% sucrose, and 0.8% agar for 4 h before subjecting to particle bombardment. The sepals at the opening stage of flower development of *Dendrobium* flower collected from Rapee Sagarik Orchid Garden, Kasetsart University, Thailand, were used for determining gene expression. They were surface-sterilized by immersing for 15 min in 2.5% sodium hypochlorite and rinsed three times with sterile distilled water. The sterilized sepals were placed on a wet 3MM paper in Petri- dish.

Plasmids

Three plant vectors, pAct1-D, pAHC27, and p2K7, containing rice Act-1, maize Ubi-1 and CaMV35S promoters, respectively, having *uid A* gene and *nos* terminator (Figure1) were used. The plasmid DNA preparations were carried out according to the supplier instruction (QIAprep® spin miniprep kit, QIAGEN).

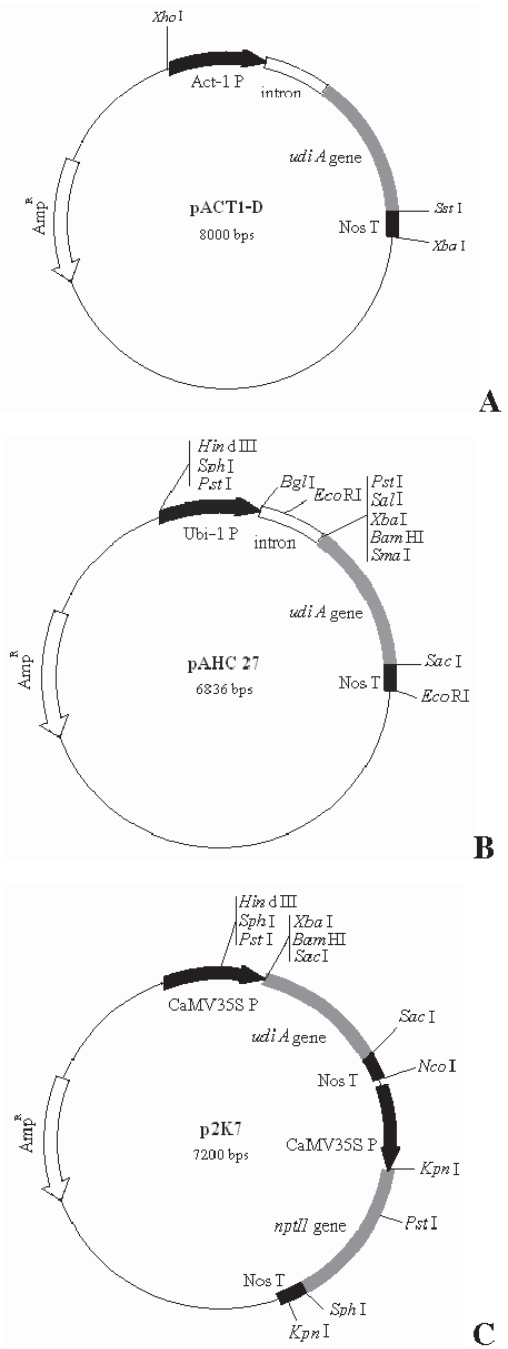

Figure 1 Schematic draw of the cassette plasmid utilized in transient *gus* expressions of the protocorm and flower of *Dendrobium* Jaquelyn Thomas. All plasmids were consisted of a different promoter fused to *β-glucuronidase* (*uid A*) gene and *nos* terminator. A) the pAct1-D plasmid contained rice Act-1 promoter; B) the pAHC27 plasmid contained maize Ubi-1 promoter; C) the p2K7 plasmid contained CaMV35S promoter.

Microprojectile bombardment

The 3.5 μg (1.0 μm in diameter) of gold microcarriers (Bio-Rad) were coated with 40 mg plasmid DNA as described by Christou *et al*. (1991). The protocorms were bombarded with coated microparticles at the helium gas pressure of 600 Psi, the target distance of 10 cm, and different vacuum chambers (-20, -25, or -40 inch Hg) by particle inflow gun and subsequently cultured under a 16 h photoperiod of cool white light at 25°C.

The coated microcarriers were accelerated into the orchid sepals under the following condition: the helium gas pressure of 600 Psi, the target distance of 12 cm, and vacuum chamber at -25 inch Hg using PHD 1000/Helium biolistic device (Bio-Rad Laboratories, Hercules, California).

Gus histochemical assay

After 3 days of bombardment, both treated protocorms and sepals of flowers were examined for GUS activity by histochemical process (Jefferson *et al*., 1987). The tissues were immersed in 5-bromo-4-chloro-3-indonyl-β-D-glucuronic acid (X-gluc) buffer containing 1mM X-gluc, 100mM sodium phosphate, pH 8.0, 0.5mM potassium ferricyanide, and 0.5mM potassium ferrocyanide and incubated overnight at 37°C. After staining, the chlorophyll of bombarded tissues were removed using 70% ethanol. The blue spots on the protocorms and the sepals were counted and recorded.

RESULTS

Plant culture

The actively dividing protocorms derived from the seeds of *Dendrobium* responded well to all four solid media of VW, $\frac{1}{2}$MS, MS+VW and KC. Within the first 14 days of sub-cultured protocorms, the development of protocorms in each medium revealed the same growth rate. After 21 days, however, the protocorms in $\frac{1}{2}$MS, MS+VW began to regenerate into small multiple shoots unlike those in VW and KC (Table1). The latter were continuously multiplied as protocorms forming a big lump shape. Interestingly, the protocorm type in KC medium was rather unique having dark green color, succulent, clear and shiny lump. From these results, the tissues culturing in VW media showed the most abundance of actively dividing protocorms (Figure2A), while those in the $\frac{1}{2}$MS medium could regenerate the plantlets within 60 days. Therefore, VW medium was selected and subjected to the next step of transient gene expression test (Figure2B).

Effect of promoters on protocorm expression

To compare the efficiency of each constitutive promoter, Act-1 (pAct1-D), CaMV35S (p2K7) and Ubi-1 (pAHC27) upon controlling *uid A* gene expression in orchid protocorms, the bombarded protocorms were

Table 1 Growth and development of *Dendrobium* protocorm-like bodies (Plb) on four different media.

Week[1/]	Size and development of protocorms			
	VW	$\frac{1}{2}$MS	MS+VW	KC
1	0.2-0.5 cm Plbs	0.2-0.5 cm Plbs	0.2-0.5 cm Plbs	0.2-0.5 cm Plbs
2	0.5-2 cm Plbs	0.5-2 cm Plbs	0.5-2 cm Plbs	0.5-2 cm Plbs
3	1.5-3 cm Plbs	Tiny shoots	Lots of tiny shoot	2 cm shiny Plbs
4	Tiny shoots	4-6 leaf	3.5 cm Plbs+shoot	Smooth shiny Plbs
6	Plbs+4-6 leaves	Small plantlets	Plbs+shoot+leaves	Shiny Plb+2-4 leaves
8	Plbs+plantlets	Rooting plantlets	Plantlets	Shiny Plb+2-4 leaves

[1/] The number of weeks after transferring to the tested media

determined for the transient GUS activity using histochemical assay. In this experiment, the promoter expressions were evaluated from the total area of staining on the protocorm surface due to the dispersion of GUS solution in the vacuoles contained in the cell expressing *gus* gene. The results showed that all the promoters tested could drive transgenes in the protocorms. The GUS activity under the control of Ubi-1 promoter with vacuum chamber of -25 inch Hg was as high as 81-100%, while those driven by CaMV35S and rice Act-1 promoters were only 61-80 % and 41-60 %, respectively (Table2). However, at the level of -40 inch Hg, CaMV35S did not positively control the expression of this gene. As for the intensity of stain, the protocorms bombarded with Ubi-1 promoter (Figure3B) and Act-1 (Figure3C) gave intense blue staining on the surface, but less on the protocorms under the control of CaMV35S (Figure3D) suggesting that maize Ubi-1 and rice Act-1 promoters were more efficient in driving gene expression of orchid protocorms.

Effect of promoters on floral expression

Each of the three plasmids consisting of constitutive promoters, Act-1, Ubi-1, and

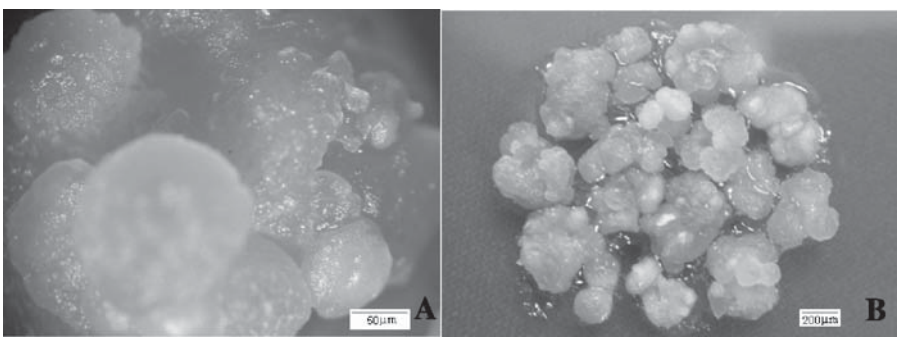

Figure 2 The protocorms culturing in VW medium and used as a target tissue for particle bombardment. A) characterization of the protocorms containing actively dividing cells; B) the suitable protocorms placed on the osmotic media prior to genetic transformation.

Table 2 Effect of constitutive promoters on *gus* expression in bombarded protocorms of *Dendrobium* Jaquelyne Thomas at different levels of vacuum chamber.

Plasmid	Promoter	Vacuum chamber (inch Hg)	GUS activity [a]
pAHC27	Ubi-1	-20	+++
		-25	++++
		-40	++
pAct1-D	Act-1	-20	+++
		-25	++
		-40	+
p2K7	CaMV35S	-20	++
		-25	+++
		-40	-

a: Determining transient GUS activity by area and intense of blue spots cells of *Dendrobium* protocorms; ++++: 81-100%, +++: 61-80%, ++: 41-60%, +: less than 40%, and -: no blue spots.

CaMV35S, was delivered into fresh sepal of *Dendrobium* Jaquelyn Thomas to evaluate the GUS activity. Bombarded sepal tissues were determined for *gus* gene transient expression by histochemical assay one day after bombardment. The results revealed that the promoter activity for driving *gus* expression was different in floral tissues. The gene expression under the control of Act-1 promoter (Figure4A) was more active than under the control of CaMV35S (Figure4B), whereas Ubi-1 gave the least effect. The high level of expression with Act-1 promoter appeared throughout the sepal base and vascular tissue, while CaMV35S promoter gave no expression in the sepal base but worked well particularly within the vascular tissue (Figure4C). Interestingly, no blue spot was observed in orchid sepal under the control of Ubi-1 promoter (Figure4D). As the results, the Act-1 and CaMV35S were considered highly efficient in expressing the *gus* gene of the sepal base and the vascular tissues of orchid flower.

DISCUSSION

For stable transformation in orchid, not only a suitable type of tissue is needed as a target tissue for bombardment but the developmental stage also affects the production of transgenic orchid (Nan and Kuehnle, 1995). Due to integration of transgene in dividing cells at metaphase and G_2-phases is more stable than in the cells at stationary and G_1-phases (Iida *et al.*, 1991), the type of tissue culture medium used would be considered helpful in inducing the protocorm production. To select a suitable medium for orchid transformation, protocorms of *Dendrobium* Jaquelyn Thomas were cultured in four different formulae of medium, VW, $\frac{1}{2}$MS, MS+VW, and KC. The results showed that the earliest development of protocorm to plantlet was obtained in $\frac{1}{2}$MS medium within 6 weeks and these plantlets could further produce root within 2 weeks. The protocorms cultured in other media

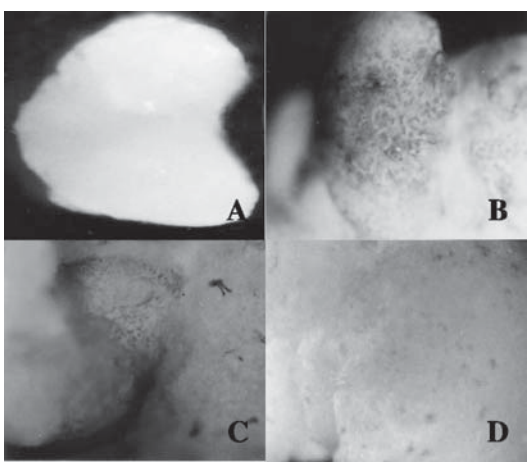

Figure 3 Transient GUS activity in protocorm-like bodies of *Dendrobium* orchid with different promoters at the vacuum chamber level of -25 inch Hg; A) negative control; B) Ubi-1 promoter; C) Act1-D promoter; D) CaMV35S promoter.

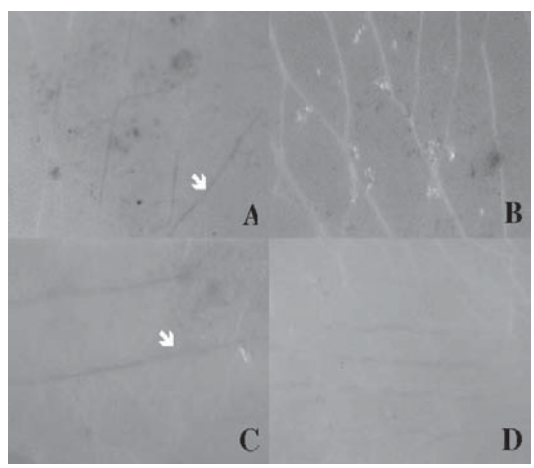

Figure 4 Transient GUS expression in sepal of *Dendrobium* orchid with different promoters. A) Act1-D promoter; B) CaMV35S promoter expressed in the sepal base; C) CaMV35S promoter activity in the vascular tissue; D) Ubi-1 promoter. White arrow indicated blue color of GUS activity in vascular tissue.

took as long as 8 weeks to produce plantlet but not root, while the protocorms culturing in VW medium gave abundance of actively dividing cells which would be efficient for transformation. The results suggested that the protocorm to be used for further bombardment should be cultured in VW medium prior to transformation and then regenerated transgenic plantlets by transferring into $\frac{1}{2}$MS medium during selection process.

Although, establishing transgenic plant depends largely on the optimization of culture condition, having stronger promoters to ensure the successful production of transgenic plants is also necessary. The use of an effective promoter to drive transgene is crucial to achieve higher levels of expression. To determine the constitutive promoter for specific control of *gus* gene expression, protocorm and sepal of *Dendrobium* orchid tissue were bombarded with pAct1-D, pAHC27 and p2K7 plant vectors containing rice Act-1, CaMV35S and maize Ubi-1 promoter, respectively. Even though previous reports revealed that CaMV35S promoter was an efficient promoter for callus of *Phalenopsis* (Anzai *et al*., 1996) and *Dendrobium* (Tee *et al*., 2003) while Act-1 and CaMV35S promoters were highly active in the protocorms of *Dendrobium* (Yu *et al*., 1999), these results showed that rice Act-1 and maize Ubi-1 promoters gave higher level of *gus* expression in orchid protocorms comparing with using CaMV35S promoter. Similar to these results, several research groups demonstrated that the rice Actin-1 and maize Ubi-1 promoter had higher potential than CaMV35S promoter when used in monocotyledons, including maize and barley (Schledzewshi and Medel, 1994), oil palm (Chowdhury *et al*., 1997) and rice (Zhang, 1991**;** Zhongyi *et al*., 1997). These could imply that the transcription factor presenting in orchid protocorms might be more effective in recombining with *cis*-element of the promoter isolated from monocotyledons, rice Act-1 and maize Ubi-1, than the CaMV35S promoter isolated from dicotyledons.

On the contrary, these results revealed that maize Ubi-1 promoter was unable to drive *gus* transgene in the mature *Dendrobium* sepals. This suggested that the recognition of *cis*-element of the promoter and *trans*-acting factor in the orchid flower might be limited as also reported by Benfey and Chua (1989). Although, Ubi-1 promoter had high potential to express in actively dividing and growing cells (Cornejo *et al*., 1993; Takimoto, 1994; Plesse, 2001) but it might not be active in the mature flower. Gurbarino and Belknap (1994) showed that a potato ubiquitin promoter showed the highest expression in meristematic tissue but declined during leaf expansion in transgenic potato and rose. These explained why the transformed protocorms of orchids containing actively dividing cells showed the highest *gus* expression driving under maize Ubi-1 promoter but the lowest expression in mature flower compared with using rice Act-1 and CaMV35S promoter.

Rice Act-1 promoter, on the other hand, was a more efficient promoter for gene expression in orchid sepal and protocorm than CaMV35S promoter. The high level of expression with rice Act-1 has been reported in shoot and floral meristematic tissues in transgenic maize (Zhong *et al*. 1996) and in most cell types of transgenic rice (Zhang *et al*., 1991). Strong expression under the control of CaMV35S was particularly active in anther and pollen tissues of transgenic tobacco and most cell types of transgenic cotton (Sunilkumar *et al*., 2002), implying that CaMV35S promoter was a more efficient promoter in dicotyledon tissue than in monocotyledon tissue, including orchid. This was confirmed by the less *gus* gene expression in *Dendrobium* when CaMV35S promoter was used.

CONCLUSION

There are many factors affecting plant

genetic transformation. Of these, the target tissue for bombardment and the promoter controlling transgene expression are the most critical to establish transgenic orchid. Based on these results, to obtain high efficiency of bombardment transformation in *Dendrobium* orchid, the protocorms tissue should be sub-cultured in VW medium prior to bombardment and transferred into $\frac{1}{2}$MS medium to further generate transgenic plantlet. Although maize Ubi-1 promoter showed the highest expression in the protocorms, its expression was not detected in the flower. Consequently, rice Act-1 promoter would be a high potential promoter for use in for orchid flower improvement.

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