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DEVELOPING AUTO-INDUCIBLE P*grac57* PROMOTER IN *BACILLUS SUBTILIS*

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ABSTRACT

Bacillus subtilis has many advantages such as: safe, non-pathogenic, endotoxin-free features. Therefore, B. subtilis has been widely ultilized in therapeutic proteins and important industrial enzymes production. Over the last few years, the potential application of B. subtilis for recombinant proteins synthesis has been significantly enhanced by using pHT - vector system with Pgrac promoter. In this study, we developed inducer-free expression vector for B. subitlis based on P*grac57 promoter and used GFP protein as a reporter. The results showed that we successfully constructed inducer-free vector pHT1686 with* P*grac57 promoters. These vectors are totally able to express GFP protein without any inducer in B. subtilis strain 1012. Moreover, GFP expression level reached 11% of the total cellular proteins. Additionally, GFP activities are in the same range with the inducer-free vector.*

Keywords: Bacillus subtilis, GFP, Pgrac57, auto-inducible expression vector.

TÓM TẮT

Nghiên cứu sử dụng promoter* Pgrac57 *để tạo vectơ tự biểu hiện mang chỉ thị GFP cho Bacillus subtilis

Bacillus subtilis có nhiều ưu điểm như: An toàn, không gây bệnh, không có nội độc tố nên được sử dụng nhiều trong sản xuất protein trị liệu và enzyme công nghiệp quan trọng. Tiềm năng ứng dụng B. subtilis cho biểu hiện protein tái tổ hợp được nâng cao trong những năm gần đây với sự ra đời của vectơ cảm ứng pHT sử dụng

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promoter Pgrac. Trong nghiên cứu này, chúng tôi phát triển hệ thống vectơ biểu hiện không cần chất cảm ứng cho B. subtilis dựa trên promoter P*grac57. Kết quả đã xây dựng được vectơ tự biểu hiện pHT1686 điều hòa bởi promoter* P*grac57, cho phép biểu hiện GFP mà không cần chất cảm ứng trong chủng B. subtilis 1012, lượng biểu hiện protein GFP đạt mức 11% protein tổng số của tế bào và hoạt tính protein GFP ngang bằng với hệ thống có cảm ứng.*

Từ khóa: *Bacillus Subtilis,* GFP, P*grac57,* vectơ tự biểu hiện.

1. Introduction

Recombinant protein production is one of the most powerful techniques which has been widely ultilized in medicine, research and biotechnology. The use of recombinant protein in medicine has recently emerged as a protein therapy for many diseases including cancers, diabetes and anemia. Therefore, development of a host strain with safe and efficient manners for recombinant protein expression in medical use is preferentially needed. *Bacillus subtilis* (*B.subtilis*) has become an orthogonal and potential host strain which has been ultilized for recombinant protein expression with some advantages: i) *B. subtilis* has been evaluated and designated by the U.S. Food and Drug Administration as an safe organism that is Generally Regarded As Safe (GRAS); (ii) its ability to well grow and be highly efficient fermentationin the low cost media; (iii) The culture and purification processes for production are significantly simple and low cost[1],[2].

The vector system of *B. subtilis* has been developed for protein expression with chemical inducer and without chemical inducer. The promoters such as P*spac* and P*xyl* used in expression vector of *B. subtilis* are normally inducible. The level of protein expression which was regulated by P*xyl* promoter increased 150 to 300 folds upon adding 0.5% xylose to the culture medium. The generation of P*spac* promoter by fusing SPO-1 promoter (bacteriophage) to *lacO* promoter (*E. coli*) allows inducing protein expression in *B. subtilis* by IPTG [3]. Using the P*spac* promoter for expression of penicillinase in *B. subtilis* I168 showed that the amount of IPTG-induced protein expression was 100 times higher than that of protein expression without inducer [3]. Over the last few years, the potential application of *B. subtilis* for recombinant proteins expression has been significantly enhanced by using pHT - vector system consisting of P*grac* promoter with IPTG inducer [4],[5]…There was currently a set of over 80 P*grac* promoters have been generated for protein expression in *B. subtilis*. These promoters have different conservation regions such as UP element, -10 and -35 regions that allows to ultilize the orthogonal promoters for expression of interest proteins. Previous studies have shown that the amount of protein expression by using P*grac* promoter was 60 and 30 times higher than that of protein expression by using P*spac* and P*xyl* promoters, respectively[6].

Development of auto-inducible or constitutive promoters has also been reported which is able to solve the limitation of chemical inducers about cost and safety. Therefore, some auto-inducible promoters such as *gsi*B, *pst*, mannose, *cry*3Aa, *apr*E, *srf*A… have been generated which allows these vector systems to express interest proteins without using any inducers in *B. subtilis*. However, these promoters have not currently been revealed the efficiency of protein expression yet.

Mutation of P*grac* promoter system by deletion of a part or full *lacI* gene in the vector system is able to replace inducible system to auto-inducible system [7]. Previously, we were successful to generate vectors consisting of P*grac01* and P*grac*100 for protein expression without using inducer. The GFP expression under these P*grac*01 and P*grac*100 promoters showed high yield which accounted for 9-13% of cellular protein total. In this study, the P*grac*57 promoter has been developed by deletetion of *lacI* which allows the system to express interest proteins without using any inducers. This novel promoter opens a promising approach to synthesize interest recombinant proteins at large-scale industry without using inducer.

2. Materials

E. coli OmniMAX strain was used for cloning and *B. subtilis* 1012 [5]was used as a host strain for expression of GFP reporter. *Pfu* DNA polymerase, *Taq* DNA polymerase and restriction enzymes including *Bam*HI and*Kpn*I were supported by Thermo Scientific. PCR kit and cloning kit and basic materials for molecular biology were supported by Qiagen, Thermo Scientific, Sigma-Aldrich, Merck-Millipore and BioBasic. Plasmid pHT01 as negative control, pHT1198 as a template for targeted gene and pHT1652 as vector for cloning[8]. All of plasmids were supported by center for bioscience and biotechnology, University of Sciences, National University-HCMC. All primers for PCR were described in **table 1**.

Table. *Primers were used for PCR*

Primers	Primer sequences	Target
ON2063	GGCCATGAGCTCAATTGCGTTGCGCTCACTGCCGGTACC AAAGGAGGTAAGG	PCR from pHT1198 vector
ON2064	GGCCATGGATCCTTCCTCCTTTATATGG	
ON925	GAATTAGCTTGGTACCAAAGGAGGTAAGGATCACTAG	Screening *E. coli*consisting of pHT1686 vector
ON1278	GGCCATGACGTCTTTGTAAAGCTCATCCATGCCATGTGT	
ON886	TCACCCTCTCCTCTGACAGAAAATTTGTGCCCATTAAC	Sequencing of pHT1686 vector

3. Methods

3.1. Cloning pHT1686 consisting of Pgrac57 promoter.

Cloning was carried out as figure 1. Firstly, *Pgrac*57 gene was amplified from PCR with a pair of primer ON2063/ON2064 and template. Gene and vector was treated with 2 restriction enzymes: *Kpn*I và *Bam*HI. Products were then ligated by T4 DNA ligase, resulting in pHT1686 vector consisting of P*grac*57 promoter. The ligated product was transformed into *E. coli* OmniMAX strain by chemical transformation approach. The transformed products were then cultured in LB-agar plates with 100 µg/mL ampicilline. Colony PCR was subsequently screened by using a pair of primer ON925/ON1278. The selected colonies were then inoculated in liquid media. Plasmids consisting of pHT1686 were isolated by using QIAprep Miniprep kit (Qiagen) and these plasmids were finally confirmed by sequencing with a primer ON886.

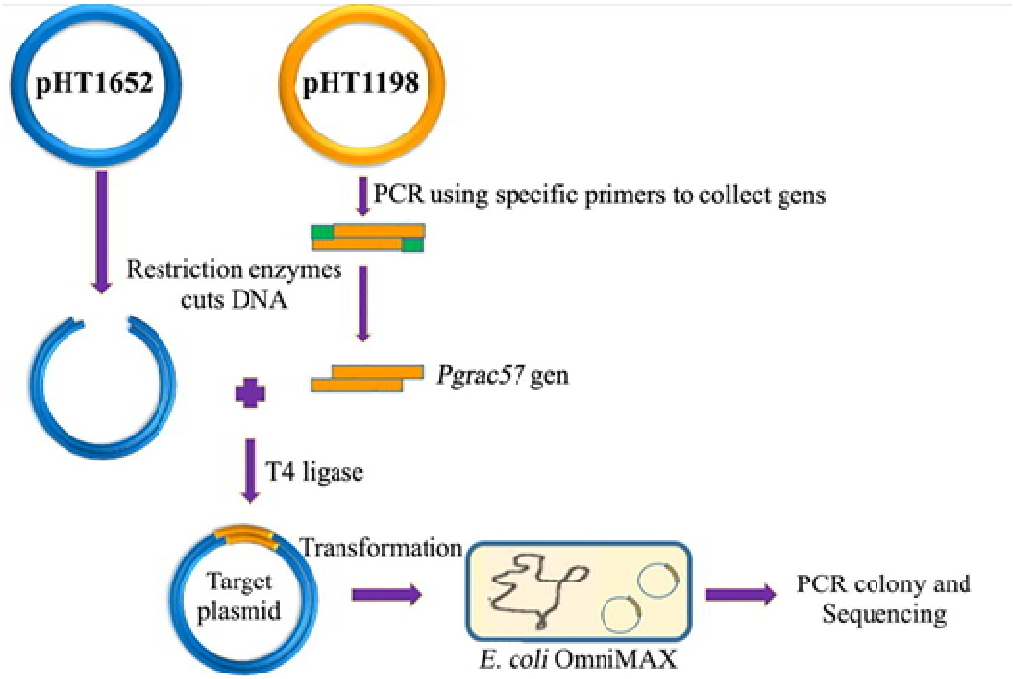

Figure 1. *Cloning of pHT1686*

3.2. Evaluation of expression of GFP reporter protein in B. subtilis 1012 strain

For protein expression, pHT1686 vector was transformed into *B.subtilis* 1012 competent cells. The transformed cells were cultured in LB-agar plate with 10 µg/mL chloramphenicol. A single colony was then inoculated in 10 ml liquid LB medium overnight, at 25°C and 220 rpm. A small cultured medium was stranfered into 40 ml liquid

LB medium (10 µg/mL chloramphenicol) which was adjusted to reach 0.1 of OD_{600} value. This cell culture meidum was continuously incubated at the same condition above. When OD_{600} reached 0.8-1, the sample was harvested at 0h, 2h and 4h.

The expression of GFP protein was evaluated by gel electrophoresis assay. The cellular solution samples at OD_{600}=2,4 wereadded in 100 µL lysis buffer (25 mM Tris-HCl pH 8,0; 0,25 M Sucrose) with addition of 0,2 µL lysozyme (50 mg/mL) and this mixture was then incubated at ở 37ºC in 5 minutes. After that, add 25 µL loading buffer (5X) into the sample and heat at 95˚C in 5 minutes. The sample was then analyzed by electrophoresis gel with 12.5% polyacrylamide.

The cellular solution samples at OD_{600} = 1,2 were added in 480 µl lysis buffer (140 mM NaCl, 2,7 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 1,8 mM KH_2PO_4, 400 µg/ml lysozyme) and mixed well. This solution sample was incubated at 37°C in 30 minutes. After that, the samples were centrifuged at 13000 rmp in 5 minutes. The suspended solution was harvested and added (50 µl) to the plate (384 wells). The GFP protein in the samples were measured by plate reader, CLARIOstar (BMG LabTech) with parameters: excitation at 470 ± 8 nm; emission at 515 ± 8 nm; focal height: 6.9 nm; Gain: 1400. Intensity of fluorescent protein is calculated by the measured value divided by OD_{600} value. The experiments were carried out in triplicates.

4. Results and discussion

4.1. Cloning of pHT1686 vector consisting of Pgrac57 promoter for expression of GFP reporter protein

The targeted DNA from PCR was ligated to the plasmid vector. The ligated product was transformed into *E. coli* OmniMAX^{TM} strain (Invitrogen) and then spreaded the transformed solution on LB-agar plate with antibotic. Four colonies were selected for colony PCR with a pair of designed primer. The colony consisting of targeted DNA was then isolated and sequenced. The results of DNA sequencing which was analyzed by Clone Manager 9.0 showed 100% homologous sequence between analyzed and designed DNA sequence of pHT1686 plasmid.

4.2. Evaluation of expression of GFP reporter protein in B. subtilis 1012 strain

The recombinant *B. subtilis* 1012 was successfully generated. This strain was ultilized to be as a host strain for protein expression in liquid LB medium. The plasmids of pHT1686 consisting of P*grac*57, pHT01 (negative control) and pHT1652 consisting of P*grac*01 were transformed into *B. subtilis* 1012 for protein expression. The amount of protein expression of these plasmid vectors were analyzed by SDS-PAGE (Figure 2)

Figure 2 showed that auto-inducible protein expression of vectors in *B. subtilis* increased in time-dependent. The gel showed the band of protein is approximately 27 kDa which are in line with the molecular weight of GFP. The pHT1686 and pHT1652 vectors in *B. subtilis* are able to express GFP reporter protein without any inducers. The level of this GFP protein increased at the following times 0h, 2h and 4h. In contrast, negative control pHT01 vector showed slightly protein expression of GFP. Both pHT1652 and pHT1686 vectors express the same level of GFP protein after 4h. These results showed the level of protein expression under P*grac01* promoter and P*grac*57 promoter systems are similar each other.

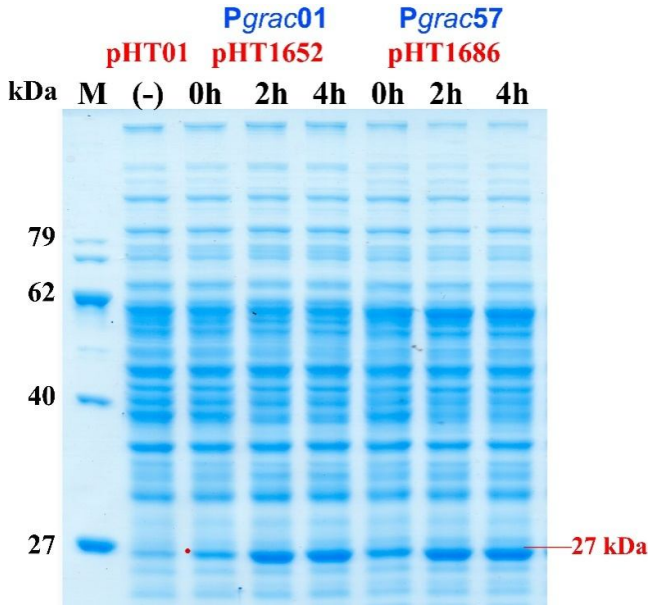

Figure 2. *Analysis of auto-inducible protein expression in B. subtilis at the following times*

The level of protein expression was also analyzed by the software of AlphaEaseFC 4.0 (Figure 3). The results showed that the protein expression level under P*grac*01 promoter system was high yield which accounted for 11.4 % of celllular protein total. This result are in line with previous study about auto-inducible protein expression of P*grac*01 promoter which was reported in 2017 [7]. In auto-inducible system, the GFP protein expression in *B. subtilis* under P*grac*57 promoter control was also high which accounted for 11% of cellular protein total. This protein level is similar to protein level under P*grac*01 (11.4%) and P*grac*100 (9-13%) [7]. Previous study, the amount of β-galactosidase mRNA expression under inducible P*grac*57 promoter system was 40 times higher than that of β-galactosidase mRNA expression under inducible P*grac*01 promoter system in *B. subtilis* [3]. The generation of auto-inducible promoter system by deletion a part or full

lacI gene showed that the amount of GFP expression under auto-inducible P*grac*57 promoter system is similar to that of GFP expression under auto-inducible P*grac*01 promoter system in *B. subtilis*. This molecular mechanism can be explained that the structure of P*grac*57 promoter might be changed because of deletion of *lacI* gene. In addition, expression of different targeted proteins shows the different amount of proteins.

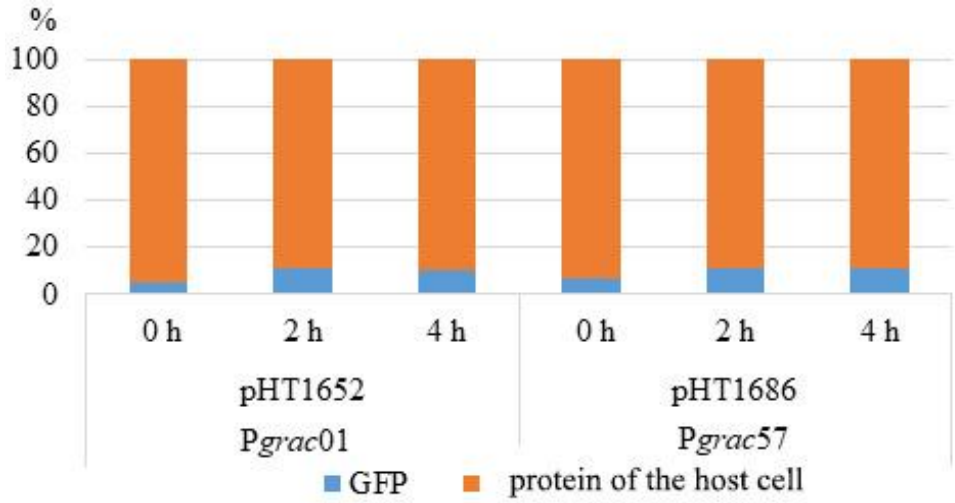

Figure 3. *Analysis of the GFP protein expression level in B. subtilis via the software of AlphaEaseFC 4.0.*

4.3. Evaluation of the activity of GFP reporter protein by flourescent spectrometry

The auto-inducible system that targetd protein is continueously expressed in the *B. subtilis* host strain without controlling. Therefore, over protein expression under these strong promoter systems might cause the change of protein structure, resulting in inactivation of protein function. Beside analysis of protein level, the evaluation of targeted protein activation is more important and needs to be established. The activation of GFP was evaluated by flourescent spectrometry (plate reader) (Figure 4).

Negative control pHT01 vector which was considered as a blank point showed no fluorescent signal. Previous study, auto-inducible protein expression of pHT1652 consisting of P*grac*01 showed the activity of GFP which was expressed in this system was 0.84 time compared to that of inducible pHT10 vector system in *B. subtilis* [8]. In this study, the activity of GFP under auto-inducible P*grac*57 promoter was higher than that of GFP under autio-inducible P*grac*01 promoter in *B. subtilis*. From results above, we conclude the activity of GFP under auto-inducible P*grac*57 promoter (pHT1686) is more less the same with the activity of GFP under inducible promoter system (pHT10-*gfp*+) in *B. subtilis.*

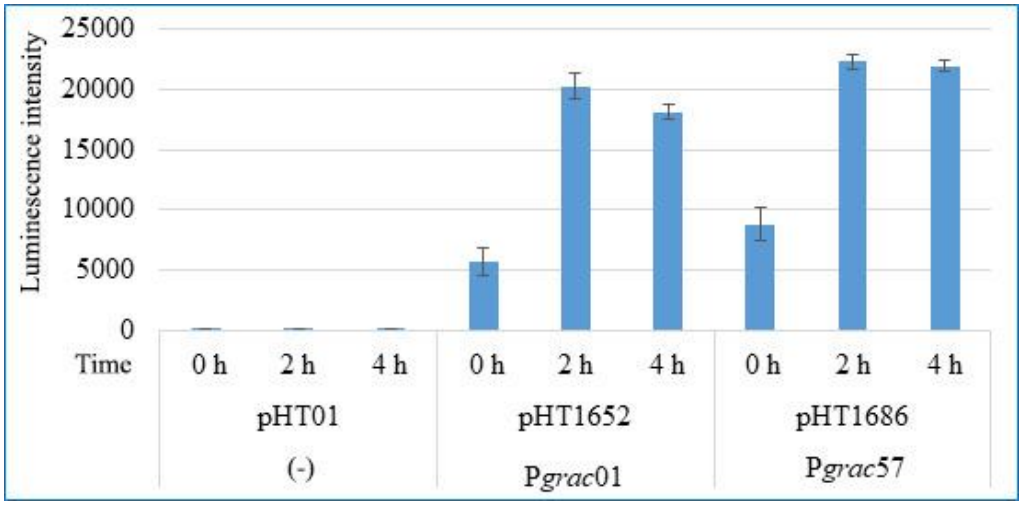

Figure 4. *Activities of gfp expression in B. subtilis*

Previous study, generation of P*grac*57 promoter by mutation of -35 (TTGAAA => TTGACA) and -15 regions (TCT => ATG) and deletion of UP element showed that expression of β-galactosidase under this inducible P*grac*57 promoter was 10 times higher that that of β-galactosidase expression under the inducible P*grac*01 promoter. However, these promoter systems were regenerated (inducible systems into auto-inducible systems) by deletion of *lacI* gene (upstream of promoter sequence) that the level of protein expression under both P*grac*57 and P*grac*01 promoters (auto-inducible systems) was similar each other. Molecular mechanism can be explained that deletion of *lacI* gene might effect on the structure of promoter. These results are homogeneous with the analysis of protein expresison on SDS-PAGE by AlphaEaseFC 4.0 software (Figure 3).

B. subtilis is a microorganism that can respond to the changes of culture medium, so it is possible that interest protein is continuously expressed at the begining of the log phase. That also reduces the expression of side proteins which are necessary for cell growth. In addition, our researches showed that the length of *lacI* gene effects on expression of protein under P*grac* promoter controlling. Therefore, deletion of *lacI* also effects on this auto-inducible promote, resulting in decrease of amount of GFP expression under P*grac*57 promoter controlling (pHT1686) in *B. subtilis*. Expression of protein under the P*grac*57 promoter controlling shows a high yield, accounting for 11% of the total protein of *B. subtilis* host. This promoter will provide a potential auto-inducible protein expression system in *B. subtilis* for large-scale industrial applications.

5. Conclusion

The auto-inducible pHT1686 vector consisiting of P*grac57* has successfully been generated. The GFP protein which has been expressed in *B. subtilis* under P*grac57* promoter accounted for 11% of cellular protein total. Interestingly, the amount of targeted protein which was expressed by using this auto-inducible promoter system is similar to the amount of chemical-inducible protein expression. This novel P*grac57* promoter opens a promising approach to synthesize interest recombinant proteins at large-scale industry without using inducer.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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REFERENCES

[1] W. Schumann, "Production of Recombinant Proteins in *Bacillus subtilis*," in *Advances in Applied Microbiology*, vol. 62, Supplement C vols., Academic Press, 2007, pp. 137–189.

[2] T. T. P. Phan, H. D. Nguyen, and W. Schumann, "Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in *Bacillus subtilis*," *Protein Expr. Purif.*, vol. 46, no. 2, pp. 189–195, Apr. 2006.

[3] D. G. Yansura and D. J. Henner, "Use of the Escherichia Coli Lac Repressor and Operator to Control Gene Expression in Bacillus Subtilis," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 81, pp. 439–43, Feb. 1984.

[4] H. D. Nguyen, Q. A. Nguyen, R. C. Ferreira, L. C. S. Ferreira, L. T. Tran, and W. Schumann, "Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability," *Plasmid*, vol. 54, no. 3, pp. 241–248, Nov. 2005.

[5] T. T. P. Phan, H. D. Nguyen, and W. Schumann, "Development of a strong intracellular expression system for *Bacillus subtilis* by optimizing promoter elements," *J. Biotechnol.*, vol. 157, no. 1, pp. 167–172, Jan. 2012.

[6] D. T. M. Tran *et al.*, "Development of inducer-free expression plasmids based on IPTG-inducible promoters for *Bacillus subtilis*," *Microb. Cell Factories*, vol. 16, p. 130, Jul. 2017.

[7] H. Saito, T. Shibata, and T. Ando, "Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg," *Mol. Gen. Genet. MGG*, vol. 170, no. 2, pp. 117–122, Feb. 1979.

[8] Do Thanh Hung, "Development of inducer-free expression vectors based on P*grac*01 promoter using GFP reporter protein," *University of Sciences*, National University-HCMC, Vietnam, 2016.