ENHANCED EXPRESSION OF RECOMBINANT MIRACULIN, A TASTING MODIFYING PROTEIN, IN *NICOTIANA TABACUM* HAIRY ROOTS USING 18.2 HEAT SHOCK PROTEIN PROMOTER AND TERMINATOR

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SUMMARY

Miraculin, a taste modifier, is a protein that was first isolated from miracle fruit (*Richadella dulcifica*). It can change a sour taste into a sweet taste when sour acids are consumed, although it does not elicit a sweet response. Miraculin may have the potential in industry as a substitute for sugars and as artificial sweeteners. Since the miracle plant has low fruit productivity, mass production of miraculin is limited. Transgenic hairy root culture is a potential alternative system for the mass production of miraculin. In this study, we investigated the expression of recombinant miraculin in tobacco (*Nicotiana tabacum*) hairy roots. To increase miraculin expression, the heat shock protein 18.2 promoter and terminator were used to drive the expression of miraculin gene in a potential host system. Synthetic miraculin gene was transformed into *Nicotiana tobacum* leaf explants via *Agrobacterium rhizogenes*. The transgenic hairy root clones that contained synthetic miraculin gene showed rapid growth and reached maximum growth after 35-day culture. When the expression of miraculin gene was regulated by heat shock protein 18.2 promoter and heat shock protein terminator, the expression of recombinant miraculin increased than the control regulated by CaMV 35S promoter and nopaline synthase terminator. The recombinant miraculin was 19.97 ng per µg of the total soluble protein and equivalently with approximately 2% of the total soluble protein. For the first time, a taste modifying miraculin was successfully expressed in tobacco hairy root. The results in this study have given a promising approach for the application of the transgenic hairy root system to produce recombinant miraculin.

Keywords: *Agrobacterium rhizogenes*, miraculin, hairy root, heat shock protein, promoter, terminator

INTRODUCTION

Miraculin is a taste-modifying protein that was extracted from the fruit of *Richadella dulcifica*, a shrub native to tropical West Africa. It's ability to change a sour taste into a sweet taste, although its self property is not sweet (Kurihara, Beidler, 1968). Miraculin could be used as a natural low-calorie sweetener by people suffering from diseases linked to the consumption of sugars, including obesity, diabetes, and hyperlipidemia, and to control the palatability of foods (Sun *et al*., 2006). Due to its unique properties and potential as a sweetener, the mass production of miraculin has been greatly anticipated (Hiwasa-Tanase *et al*., 2012). Miraculin was attempted to be expressed in various hosts such as *E. coli* (Matsuyama *et al*., 2009), *A. oryzae*, *S. cerevisiae* (Ito *et al*., 2007; 2010). However, the recombinant miraculin in

such expression systems had no taste-modifying activity or were only one-sixth to one-fifth compared to the native miraculin. These results suggested that not only is glycosylation important, but the types of sugar chains are also crucial for high and stable activity. In transgenic plants, recombinant miraculin was produced in lettuce (Sun *et al.*, 2006), strawberries (Sugaya *et al.*, 2008), tomatoes (Hiwasa-Tanase *et al.*, 2011; Sun *et al.*, 2007). To enhance the level of expression of miraculin in tomatoes, various approaches were performed using promoter and terminator of expression cassette (Nagaya *et al.*, 2010; Matsui *et al.*, 2011) and optimization of codons (Hiwasa-Tanase *et al.*, 2011). Thanks to the ease of transformation and cultivation, plants are suitable for expressing many recombinant proteins, including functional antibodies and vaccines (Daniell *et al.*, 2001). However, low yields and the cost of extraction and purification have limited the large-scale manufacturing of recombinant proteins in plants.

Hairy roots, are established at wound explants infecting with *Agrobacterium rhizogenes*. Hairy roots cultures were applied in biotechnology because they have many advantages, e.g. fast-growing, genetically and biochemically stable, easy to maintain, and can grow in phytohormone free media. So, they served as a promising transgenic system for compound production, including foreign proteins (Huang, 2012). Plant-derived hairy roots culture has many advantages for commercial production, such as producing recombinant proteins (Wongsamuth, Doran, 1997). Cocultivation of explants with *A. rhizogenes* results in the production of hairy roots that are easily distinguished by their rapid, highly branching growth on hormone-free medium and plagiotropic root development. *A. rhizogenes*-mediated transformation has been used to introduce a range of foreign genes (Christey, 2001). Similar to the expression plant system, hair roots provide a suitable environment for protein folding and disulfide bond formation, correct assembly of multimeric proteins, post-translational modifications, processing, and secretion. It is necessary to produce recombinant

miraculin protein that has taste-modifying activity. The objective of this study was to investigate to enhance the level of expression of miraculin and produce commercial recombinant miraculin using modified heat shock protein 18.2 promoter and terminator in a potential host system, which was hairy roots culture.

MATERIALS AND METHODS

Materials

The miraculin gene (*Mir*), which contains 762 bp, was optimized base on the coding sequences on GenBank (accession number: D38598.1 và AB512278.1) and commercially synthesized by Epoch Life Science Inc. *Nicotiana tabacum* L. cv K326, *E. coli* DH5α, *Agrobacterium rhizogenes* ATCC 15834 were provided by the Laboratory of Plant Cell Biotechnology, Institute of Biotechnology, Vietnam Academy of Science and Technology (VAST). Primers used to amplify *Mir* gene were Mir_opt_F: 5' ATGAAGGAACTTACTATGTTGAG 3' and Mir_opt_R: 5' AGGATCTGAATGGTTCC 3'. Two sets of primer specific for *rol*C and *vir*C genes were used for the characterization of transformants. Primers used to amplify *rol*C were rolC_F: 5' ATGGCTGAAGACGACCTGTGTT 3' and rolC_R 5' TTAGCCGATTGCAAACTTGCAC 3'. Primers used to amplify *vir*C were virC_F: 5' ATCATTTGTAGCGACT 3' and virC_R: 5' AGCTCAAACCTGCTTC 3'.

Construction of the expression vector pBI121/Mir

Two expression vectors, pBI121/HSP-pro/Mir/HSP-ter and pBI121/35S-pro/Mir/Nos-ter, were constructed and consist of kanamycin-resistance genes. Synthetic miraculin gene was regulated by CaMV 35S (35S-pro) promoter and nopaline synthase terminator (Nos-ter) or Heat shock protein 18.2 promoter (HSP-pro) and Heat shock protein 18.2 terminator (HSP-ter). In addition, a c-myc gene was fused in-frame of the miraculin CDS. Heat shock protein 18.2 promoter and heat shock protein 18.2 terminator were isolated from *Arabidopsis thaliana* results

from our previous studies (La Viet Hong *et al*., 2015). These recombinant vectors were transformed into *A. rhizogenes* ATCC 15834 by electroporation method.

Induction of tobacco hairy roots

Total 90 *in vitro* tobacco leaf explants of 0.5–1.0 cm^{2}size were prepared in 1/2 Murashige and Skoog liquid medium (Murashige, Skoog, 1962). They were inoculated for 30 mins with a *A. rhizogenes* harboring recombinant vector (OD$_{600}$ nm = 0.5). After that, co-cultivation explants were transferred on solid MS medium at 25°C free antibiotics in the dark for two days. Then, infected explants were transferred to fresh MS medium supplemented with 400 mg/L of cefotaxime and 150 mg/L of kanamycin to remove bacteria and maintained at 25°C under dark conditions for hairy root induction. About 7–10 days, the hairy roots formed at the incision sites of the leaf fragments were subsequently transferred at two-week intervals to fresh MS agar containing cefotaxime and kanamycin and were incubated at 25°C in the dark.

Selection and screening transgenic hairy roots

Genomic DNA of hairy root clones was extracted by the CTAB method (Xin, Chen, 2012). The quantity and concentration of the DNA were measured using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were used as templates in PCR reactions for *Mir, rolC* and *virC* genes to confirm the transgenic status of hairy roots.

Evaluation of hairy root growth

An amount of 0.2 g of selected transgenic hairy roots (H.WT, H.HSP1, H.HSP3, H.H35S1) was transferred into 250 mL Erlenmeyer flask containing 20 mL liquid MS medium added 100 mg/L kanamycin antibiotics and grown 28°C in the dark by shaker (110 rpm) for 42 days. The fresh weight of transgenic hairy roots was determined at intervals of 7 days. In the same way, single root tips of selected transgenic hairy roots were excised in 1.0–1.5 cm length and cultured on solid MS supplemented antibiotics

for 21 days. The length of root tips was measured at intervals of 7 days.

Western blot analysis and enzyme-linked immunosorbent assay (ELISA)

Extraction of the total soluble protein

All transgenic hairy roots at 35 old days were sampled for extraction of the total soluble protein. Three hairy roots (H.HSP1, H.HSP2, and H.HSP 3) with HSP-pro/Mir/HSP-ter construct were pretreated with heat shock (Lee *et al*., 2002), heat shock at 37±1°C in 2 hours, after recovery at 25°C in 4 hours. After 1.0 gram of each hairy root clone (H.HSP1, H.HSP2, H.HSP, and H.35S1) was ground under liquid nitrogen, and the powder was suspended in 1:1 (w:v) phosphate buffer (100 mM, pH 7). Then, the supernatant was prepared by centrifugation at 10000 rpm for 10 min at 4°C. The amount of extracted total protein was determined using the Bradford method (Bradford, 1976). The resulting supernatant was used for Western blot analysis and ELISA.

Analysis of expression of recombinant miraculin by Western blotting

The crude proteins (20 μL per lane) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gel and transferred onto nitrocellulose membrane (Amersham Biosciences) on Pierce Fast blotter (Thermo Scientific). Then, plates were blocked with skim nonfat-milk powder 10% in PBS 1X. After that, the blot was reacted with anti-c myc antibody, followed by incubation with Goat anti-mouse IgG conjugated horseradish peroxidase. Immunoreactive signals were detected by staining with TMB (3,3′,5,5′-Tetramethylbenzidine).

Indirect quantification of recombinant miraculin by ELISA

The total soluble protein of transgenic hairy root clones and a wild-type hairy root clone included in the assay for reference were diluted to 100 μg/mL with coating buffer (50 mM Na$_2$CO$_3$ and 0.02% (w/v) NaN$_3$, pH 9.6). ScFv-

c-myc protein supplied by Laboratory of Plant Cell Biotechnology, Institute of Biotechnology, VAST) diluted to different contents (25 ng/µL, 50 ng/µL, 100ng/µL, 200ng/µL, and 400ng/µL) were used to construct a standard equation. 100 µL of those aliquots were applied to a microtiter plate (ImmunoPlate Maxisorp, Nalgen Nunc International, Roskilde, Denmark) and incubated overnight at 4°C. The plate was blocked with 5% fat free milk in TBS-T (10 mM Tris-Cl, 150 mM NaCl, pH 7.5, and 0.05% Tween-20). For immunological reactions, the plate was initially incubated with 100 µL of anti c-myc antibody dilution (10000 times), followed by incubation with Goat anti-mouse IgG conjugated to horseradish peroxidase dilution (5000 times in 5% fat-free milk in TBS-T). The enzymatic substrate, 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher), was added. After 20 min incubation, the reaction was stopped by HCl 1M. The absorbance signal was measured at 450 nm on the ELx808 Biotek plate reader device using Genedoc5 software. Data were analyzed using Excel software.

Statistical analysis

Statistical analyses of fresh weight, length, and recombinant miraculin data were carried out by ANOVA tests. Significance was assigned at $p < 0.05$ with the Least Significant Difference test. All analyses were performed using the SPSS statistical package (SPSS, Chicago).

RESULTS

Establishment of trangenic tobacco hairy roots

Total 90 leaf explants were inoculated with *A. rhizogenes* carrying gene transferred vector, and 30 explants were cultured as the negative control using live and killed *A. rhizogenes,* respectively, for infection. After 7–10 old days co-cultivation, the hairy root was only induced at the wound site of leaf explants and total hairy root clones were obtained 297 and 265 respectively explants infected 35S/Mir/NOS and HSP-pro/Mir/HSP-ter constructions (Figure1 and Table 1). All hairy root clones were transferred on selected medium, after 4–5 subcultures, survival hairy root clones were 297 and 265.

Table1. Induction of miraculin transgenic tobacco hairy root.

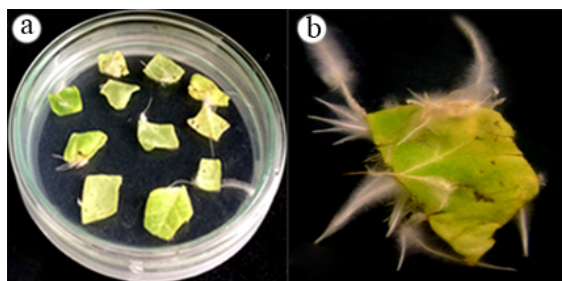| Cassettes expression	Total explants	Induction and selection of hairy root			
		Responded explants	Hairy root number mean per explant*	Total hairy root clones	Survival clones
35S-pro/Mir/NOS-ter	90	31	11.18±1.21[a]	297	297
HSP-pro/Mir/HSP-ter	90	30	10.73±1.19[a]	265	265
wt	30	22	19.14±2.16[b]	276	-

* After 15 old days co-cultivation.

Figure 1. Induction, selection and morphological characterization of hairy root transferred miraculin gene. (a, b). Hairy root clone after co-cultivation 7-10 and 15 old days with *A. rhizogenes* carrying desired vectors.

Screening of transgenic hairy root

DNA of five hairy root clones of each transformant that shown a high growth on medium containing antibiotics was extracted and used as a template in this amplified reaction. The results showed that miraculin fragment was detected in all selected clones (Fig. 2) but absent in wild type clone. All these transgenic hairy root clones were carrying *rolC* gene but not virC (data not shown). This result revealed that the transformation of the synthetic gene into tobacco leaf explant for inducing hairy roots was simple

and easy.

Evaluation the growth of selected transgenic hairy root clones

Hairy roots are fast-growing and laterally highly branched and can grow in a hormone-free medium.

a. Fresh weight of some selected hairy roots

Two HSP-pro/Mir/HSP-ter clones and one 35S-pro/Mir/Nos-ter in miraculin positive were investigated in a flask containing liquid medium.

As shown in Figures 3 and 4, the growth of transgenic hairy roots comprised four distinct phases: lag phase (from 0 to 7 days), exponential phase (from 7 to 21 days), stationary phase (from 28–35 days), and the death phase (from 42 days, the growth of hairy root clones was slow, browning and necrosis). The growth curves of four transgenic clones were similar. Fresh weights of these reached the highest on 35-old day cultures (approximately 0.82–0.87mg/flask) and were not a significant difference between clones.

b. Length and morphological characterization of the single tip of transgenic hairy root

The length of selected transgenic hairy root clones was determined to assess their growth (Figs 3 and 5).
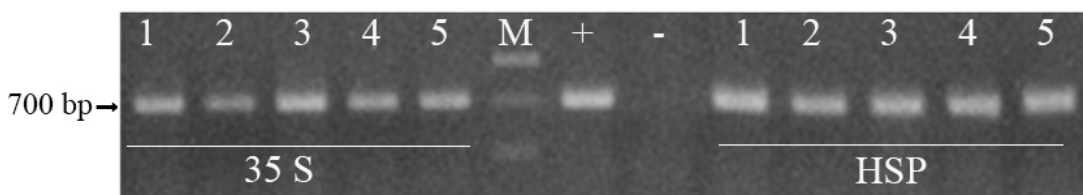

Figure 2. PCR product electrophoresis in agarose gel (0,.8 w/v). (1-5 left) lanes were transgenic hairy root clones with35S-pro/Mir/Nos-ter; M: 1 kb DNA marker (Fermentas, Germany); (+) positive control (using pBI121 containing miraculin gen); (-) negative control; (1-5 right) lanes were transgenic hairy root clones with HSP-pro/Mir/HSP-ter.
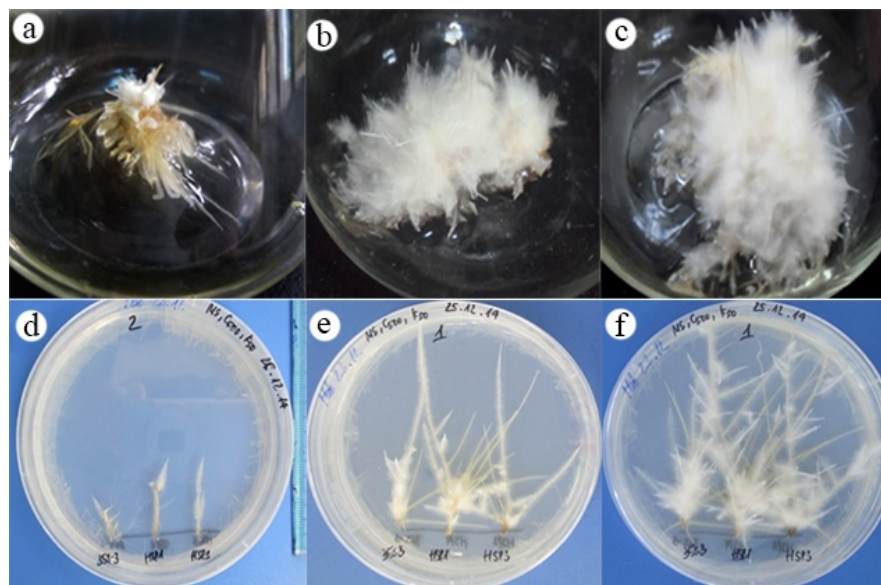

Figure 3. The growth of transgenic hair roots on liquid and solid medium. (a, b, c) transgenic hairy root on liquid culture: (a), (b), (c): 7, 28 and 35 days. (d, e, f) single tip of transgenic hairy root on solid culture: (d). 7 day; (e). 14 day and (f) 21 day.

Some selected hairy root clones showed rapid growth and branching after 14 days of culture. At 21-old day culture, the H.HSP1 and H.HSP3 had elongated rapidly than H.35S1 and reached 7.23 and 6.90, respectively. The growth rate of hairy roots may vary significantly between species, but differences are also observed between different root clones of the same species (Sevón *et al.*, 1998).
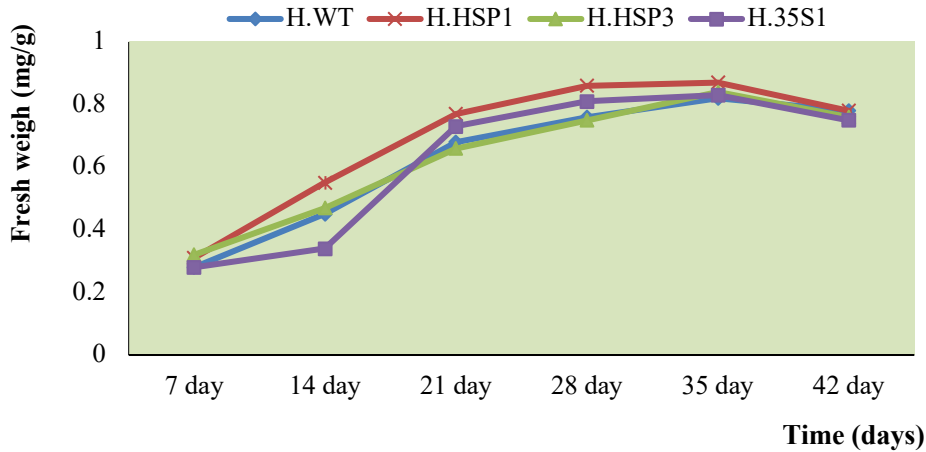

Figure 4. The growth of transgenic hairy root clones were determined by fresh weigh of hairy roots (mg/g) at intervals of 7 days during 42 days of growth period. H. WT: wilt type clone; H. HSP1 and H.HSP3: transgenic hairy root clones with HSP-pro/Mir/HSP-ter; H. 35S1: transgenic hairy root clones with35S-pro/Mir/Nos-ter.
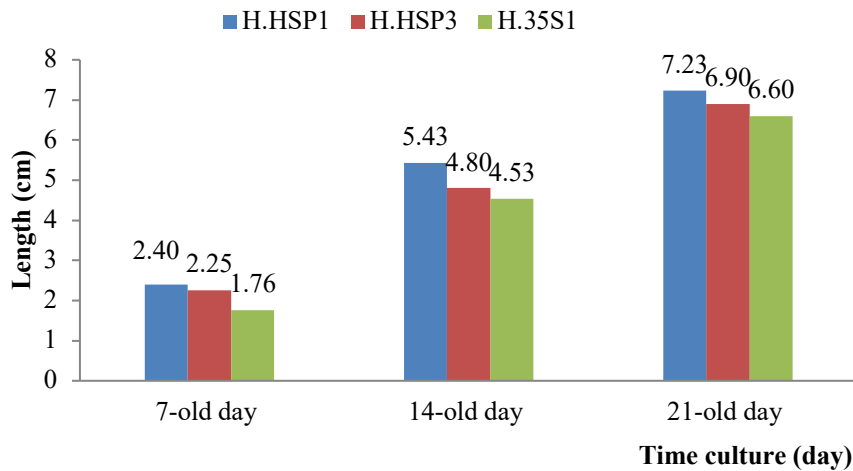

Figure 5. The length of selected transgenic hairy root clones (in cm).

Expression of miraculin protein in transgenic tobacco hairy root

Total protein was used for Western blot analysis (Fig. 6). The 50–54 kDa band was found in some hairy root clones but not in the non-transgenic hairy root clone. The size of this band correlated with expected recombinant miraculin protein in dimer form. This indicates recombinant miraculin having sweet-tasting activity.

According to Moriwaki *et al.* (1999) the optimum temperature for the sHSP18.2 promoter

in transgenic *Nicotiana plumbaginifolia* explants is 42°C. We also performed heat-induction at 42°C in 2 hrs and recovery at 25°C in 4 hrs, but recombinant miraculin was not detected in transgenic hairy root with HSP-pro/Mir/HSP-ter (data not shown).
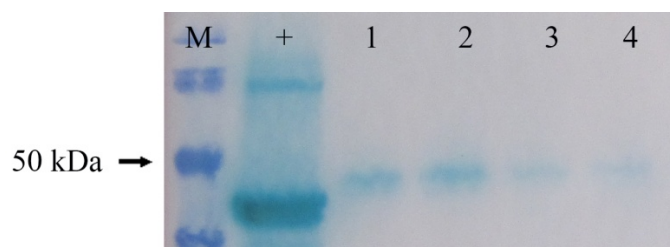

Figure 6. Expression of recombinant miraculin protein in transgenic hairy root. Lane 1, protein ladder (Thermo Scientific); (+) positive control; (1, 2, 3, 4) were H.HSP1, H.HSP2, H.HSP 3 and H.35S1, respectively; (-) non-transgenic hairy root. Immunge using anti-body of c-myc protein.

Quantification of recombinant miraculin in transgenic hairy root

To establish the level of expression of the miraculin gene in transgenic hairy roots, the recombinant miraculin was measured indirectly through quantification of c-myc protein using ELISA. In particular, scFv c-myc protein was calculated from the standard equation that expressed the correlation of content protein and $OD_{630\ nm}$. $y = 26.31x - 1.645$ ($R^2 = 0.98$), where y is content of c-myc protein (ng/µL), x is absorption of crude total protein at 630 nm in wavelength.

As shown in Fig. 7, the concentrations of miraculin from the three H.HSP1, H.HSP2, H.HSP3 hairy root, and one H.35S1 were 14.13; 19.97; 13.70 and 14.17 ng per µg total protein, respectively. The H.HSP3 hairy root proved the most expression in all hairy root clones in this experiment. The content of recombinant miraculin in these clones was 1.4-fold higher than that in other clones.
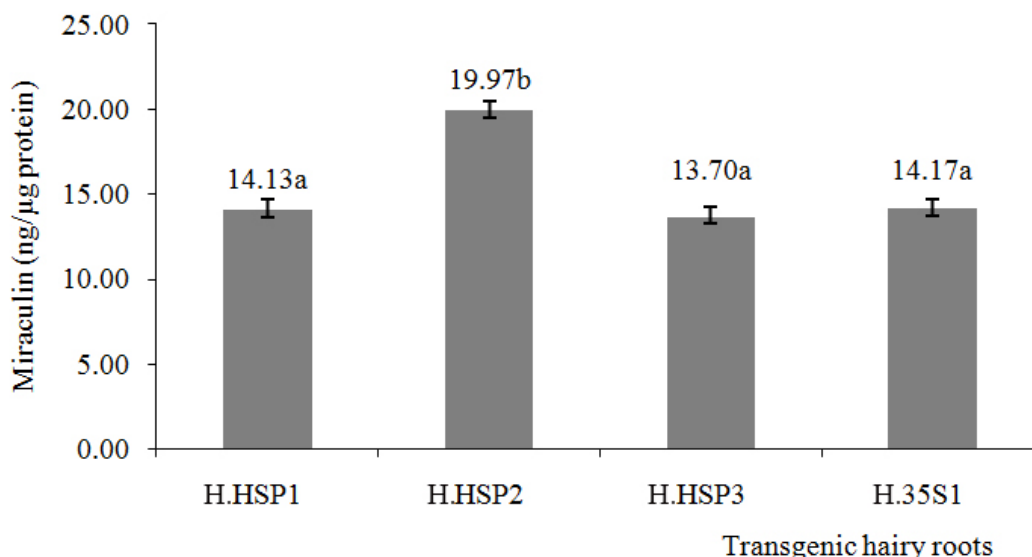

Figure 7. Recombinant miraculin in transgenic hairy root clones**.** The represented data show the average value and bars show standard deviation of three repeats

DISCUSSION

Herein, we report, for the first time, the expression of recombinant miraculin protein in hairy root culture. In this study, two expression cassettes were 35S-promoter/Mir/Nos-terminator and HSP-promoter/Mir/HSP-terminator harboring synthetic miraculin, of which codons were optimized and synthesized. Transgenic hairy root clones were stable and characterized by profuse branching and high-density growth. It is suggested to produce recombinant miraculin for commercial use through hairy root cultivation in suitable bioreactors.

The promoter is an essential element for inducing target gene expression, determining the target tissue, and achieving the desired expression time (Desai *et al.*, 2010). According to Takahashi *et al.* (1992), the high levels of *GUS* (β-glucuronidase) activity were induced in all organs of transformants except for seeds during heat shock. The optimum temperature for expression of *GUS* in *Arabidopsis* was 35°C regardless of the plant growth temperature. The terminator sequence also plays a crucial role in transcript termination, mRNA stability, and mRNA modifications such as capping, splicing, and polyadenylation (Desai *et al.*, 2010). Consequently, it also regulates by these functions the level of mRNA expression and protein accumulation. In particular, polyadenylation is important for regulating mRNA stability, transportation, and translation (Zarudnaya *et al.*, 2003). Different types of terminators influence gene expression levels (Nagaya *et al.*, 2010). A terminator derived from the heat shock protein 18.2 gene increased mRNA expression levels 2.5-fold and 1.5-fold over that with the NOS terminator in transient assays using protoplasts of *Arabidopsis* and rice, respectively (Nagaya *et al.*, 2010).

In this study, the miraculin gene was expressed in *N. tabacum* hairy roots. The recombinant miraculin protein in transgenic hairy roots with HSP-pro/Mir/HSP-ter cassette was 1.4 times higher than that in transgenic hairy root with 35-pro/Mir/Nos-ter, the concentration of recombinant miraculin in this study was approximately 2% of the total soluble protein and was higher than recombinant miraculin in transgenic lettuce, strawberries and tomatoes (approximately 1.0-1.5 %) which miraculin was driven by 35S promoter and NOS terminator (Hiwasa-Tanase *et al.*, 2011; Sun *et al.*, 2007). However, the content of miraculin in this study was lower than the content of miraculin in transgenic tomatoes, driven by 35S promoter and HSP terminator (reached 17.1% of total soluble protein) (Hirai *et al.*, 2011).

These results have provided the way for increasing the mass of recombinant miraculin protein by using a cassette with HSP promoter and HSP terminator and culture transgenic hairy roots in the bioreactor.

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GIA TĂNG BIỂU HIỆN MIRACULIN TÁI TỔ HỢP TRONG RỄ TƠ THUỐC LÁ (*NICOTIANA TABACUM*) SỬ DỤNG PROMOTER VÀ TERMINATOR CỦA PROTEIN SỐC NHIỆT 18.2

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[2]*Đại học Sư phạm Hà Nội 2*

TÓM TẮT

Miraculin là một chất điều chỉnh vị giác, một loại protein được phân lập từ quả thần kỳ (*Richadella dulcifica*). Nó có thể thay đổi vị chua thành vị ngọt khi tiêu thụ axit chua, mặc dù nó không tạo ra phản ứng ngọt. Miraculin có thể có tiềm năng trong ngành công nghiệp như một chất thay thế cho đường và làm chất tạo ngọt nhân tạo. Vì cây thần kỳ có năng suất quả thấp, nên việc sản xuất miraculin lượng lớn bị hạn chế. Nuôi cấy rễ tơ chuyển gen là hệ thống thay thế tiềm năng để sản xuất miraculin. Trong nghiên cứu này, chúng tôi đã khảo sát sự biểu hiện của miraculin tái tổ hợp trong rễ cây thuốc lá (*Nicotiana tabacum*). Để tăng sự biểu hiện của miraculin, promoter và terminator của protein sốc nhiệt 18.2 đã được sử dụng để thúc đẩy sự biểu hiện của gen miraculin trong một hệ thống vật chủ tiềm năng. Các dòng rễ tơ mang gen miraculin chuyển nạp cho thấy sự tăng trưởng nhanh và đạt mức tăng trưởng tối đa sau 35 ngày nuôi cấy. Gen miraculin được điều hòa bởi promoter và terminator của protein sốc nhiệt 18.2, mức độ biểu hiện của miraculin tái tổ hợp đã tăng so với đối chứng sử dụng promoter CaMV 35S và terminator synthase nopaline. Miraculin tái tổ hợp thu được là 19,97 ng/ µg trên tổng protein hòa tan và tương đương với khoảng 2% tổng số protein hòa tan. Lần đầu tiên, miraculin điều chỉnh hương vị đã được biểu hiện thành công trong rễ tơ cây thuốc lá. Kết quả này đưa ra một cách tiếp cận đầy hứa hẹn cho việc ứng dụng sản xuất với miraculin tái tổ hợp.

Từ khóa: *Agrobacterium rhizogenes*, heat shock protein, promoter, miraculin, rễ tơ, terminator