

Expression pattern of promoters driving eGFP expression in *Arabidopsis thaliana* hairy roots

Nga T. P. Mai* 

University of Science and Technology of Hanoi (USTH), Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.

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ABSTRACT

Plants are widely used expression system for the production of recombinant proteins. The expression systems mostly rely on promoters. In our study, hairy roots (HRs) of *Arabidopsis thaliana* were chosen to express the model protein enhanced green fluorescent protein (eGFP). To enhance recombinant protein expression, a strongly expressed promoter in the HRs of *A. thaliana* from microarray data – *MT* promoter – was used to control eGFP expression. We obtained different transformation HR lines with equal or lower eGFP production compared with the strong line controlled by the 35S promoter. In rhizocalli, which was induced by growing HRs in the medium supplemented with 2.4-D hormone, much lower eGFP content was quantified. The histological analysis showed that, under the control of *MT* promoter, eGFP was only expressed in the context, but not in the style or calli-like structure of roots which resulted in lower eGFP production. The structural interaction between the *MT* promoter and *eGFP* gene may be responsible for this low eGFP production. Further studies need to be generated to understand the different expression patterns of *MT* promoters toward heterologous protein eGFP. The discussion was raised when choosing promoters for heterologous protein production to get high productivity.

1. INTRODUCTION

Based on important advantages over other prokaryotic or eukaryotic systems, plants have gained great importance for recombinant protein production. Among the plant systems, different hosts have been tested including leafy, seed, or oil crops, plant cell suspensions, hairy roots (HRs), and microalgae, the choice of plant host depends on a broad range of criteria including the nature of the protein, ability for transformation and regeneration, post-translational modifications, scale-up of production and maintenance costs, and the downstream processing requirements [1,2].

HRs has emerged as a promising expression system to produce fully functional recombinant proteins for decades. The use of HR culture technology offers new opportunities for *in vitro* production of plant secondary metabolites as well as recombinant proteins [3]. It can also help to elucidate biosynthesis pathways and physiological processes, assist molecular breeding, and enhance phytoremediation [4]. Their fast growth, genetic stability, low doubling time, ease of maintenance, and ability to synthesize an extensive range of chemicals offer additional advantages over undifferentiated plant cells [5]. Moreover, HRs are highly branched and can be cultured indefinitely on simple hormone-free media containing only minerals, vitamins, and sugar [6].

Furthermore, they are less sensitive to mechanical damage than other tissues, and biomass can be easily separated from the culture medium. These advantages make HRs a very useful expression system for the production of recombinant proteins.

Green fluorescence protein, which originated from jellyfish, has been widely used in living organisms as a fusion tag in gene therapy research to monitor protein localization [7], a reporter of gene expression and protein targeting [8], in studying of pathogen-host interaction [9], and in protein-protein interactions [10], etc. Green fluorescent protein (GFP) has been engineered to become enhanced GFP (eGFP) to increase the fluorescent intensity, making it more applicable in research.

The expression of genes, in particular the *GFP* gene, strongly depends on the promoters. Promoter fusions with reporter genes *in vitro* can help to determine how gene expression is regulated in cells and whole organisms. There are different kinds of promoters, such as constitutive, inducible, and tissue-specific promoters [11]. While the constitutive promoters drive the gene expression at any time, this over-expression approach sometimes causes side effects, for example, phenotypic abnormalities and sterility [12], which could be minimized using tissue-specific or inducible promoters.

In the present study, eGFP has been used as a model protein to evaluate its expression under the control of the highly expressed promoter, which was taken out from microarray results in *Arabidopsis thaliana* HR system and compared with the 35S promoter. Hence, we expected to observe an increase in eGFP expression. Our results demonstrate

*Corresponding Author:

Nga T. P. Mai,

University of Science and Technology of Hanoi (USTH), Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.
E-mail: mai-thi-phuong.nga@usth.edu.vn; maiphuongnga@gmail.com

the different expression patterns of a promoter to control heterologous protein production.

2. MATERIALS AND METHODS

2.1. Plants Material

The HRs of *A. thaliana*, which expressed the *eGFP* gene, were obtained from the previous study [13] by using plant ecotype Columbia and *Rhizobium rhizogenes* ATCC15834 bacteria as materials. The root of wild type *A. thaliana* ecotype Columbia was used as a control.

2.2. Plant Roots and HR Growth

HRs obtained from the previous study [13] were grown in Petri dishes containing liquid Gamborg B5 medium [14] supplemented with 5% sucrose. The petri dishes were placed in the dark on a shaker at the speed of 60 rpm at 21°C for 2 weeks. The wild type roots were obtained from wild type *in vitro* 2-week-Col plants.

2.3. RNA Extraction using Trizol

To prepare materials for microarray, RNA from HRs and roots was extracted following the protocol of Azizi *et al.* [15] with some modifications. First, 0.2 g of roots were frozen and ground in a pre-cooled mortar and pestle using liquid nitrogen to obtain a fine powder and then transferred into a 2 mL tube. The sample was then lysed in 1.5 mL of Trizol Reagent (Thermo Scientific, Waltham, MA, USA), and incubated for 5 min at room temperature before being centrifuged at 12000 rpm for 10 min. Chloroform was added to the supernatant in a new tube, and the mixture was vortexed for 15 s before being incubated for 5 min at room temperature. After that, the tubes were centrifuged at 12000 rpm for 5 min, and the supernatant was taken. One-tenth volume of 3 M sodium acetate and 0.6 volume of isopropanol were added, and the mixture was incubated on ice for 10 min before being centrifuged at 12000 rpm for 10 min to pellet RNA. The pellet was washed twice with 70% ethanol and air-dried at room temperature. Finally, the pellet was dissolved in 50 µL of milli-q water. The concentration and quality of RNA were checked using the Nanodrop-2000 Ultraviolet (UV) Spectrophotometer (Thermo Fisher, MA, USA). The RNA concentration needed for the microarray was 166–250 ng/µL. The A 260/280 ratio must be over 1.8 to ensure good RNA quality.

2.4. Microarray Procedure

The microarray procedure followed the Affymetrix for the GeneChip WT PLUS Reagent Kit (Thermo Scientific, Waltham, MA, USA) and the study of Lee *et al.* [16]. In detail, single-strand cDNA was synthesized from mRNA, fragmented, and then labeled by terminal deoxynucleotidyl transferase using the Affymetrix proprietary DNA Labeling Reagent that was covalently linked to biotin. Finally, fragmented and labeled single-strand cDNA was hybridized with WT GeneAtlas array strip on a GeneAtlas® instrument at 48°C. The data were analyzed by the GeneAtlas® software.

2.5. Cloning of the MT Promoter in a Vector

The promoter from *MT1A* (*AT1G07590*) gene (*MT* promoter) was first amplified by polymerase chain reaction (PCR). The temperature program was as follows: 95°C for 13 min, 30 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 1.5 min, followed by a final extension at 72°C for 2 min. The PCR product was then loaded on 1% agarose gel to check the size. After that, the correct PCR product was extracted with

phenol: chloroform: Isoamyl alcohol (25:24:1) (Thermo Scientific, Waltham, MA, USA), precipitated with ethanol 70%, and dissolved in Milli-Q sterile water [17]. The PCR product and the pK18 vector were digested with *Hind*III and *Asp*718 restriction enzymes in 40 µL at 37°C for 2 h. After electrophoresis in a 1% agarose gel, the DNA band containing the *MT* promoter was cut off and purified using a Wizard SV gel and PCR clean-up kit (Promega). The ligation reaction between the *MT* promoter and the pK18 vector was carried out in a total volume of 10 µL at room temperature for 2 h. The ligation mixture was transferred into *Escherichia coli* DH5α competent cells by heat shock. The colony PCR was performed on the colonies grown on kanamycin-containing media in combination with blue-white selection to select the positive colonies [18]. Plasmids from positive *E. coli* DH5α colonies were extracted and digested with either *Eco*RI, *Hind*III, or *Asp*718 and *Hind*III restriction enzymes in a volume of 20 µL at 37°C for 2 h. Digestion products were checked on a 1% agarose gel for 25 min at 135V, and visualized under UV light. A plasmid having the expected digestion profile, namely pRP114, was precipitated with ethanol and sent for sequencing.

2.6. Fusion of MT Promoter to the eGFP Gene

The pRP36 vector, which contains 35S promoter – signal peptide – eGFP – polyA, was the receiving vector. Both pRP36 and pRP114 were digested with *Asp*718 and *Hind* III in a volume of 40 µL at 37°C for 2 h. Phenol/chloroform extractions and ethanol precipitations were carried out to collect the products. The ligation reaction between the *MT* promoter and the plasmid containing signal peptide – eGFP – polyA was performed in a volume of 10 µL at room temperature for 2 h. The ligation mixture was transformed into *E. coli* DH5α competent cells by heat shock at 42°C. A plasmid showing the correct profile was selected and named pRP115. It contains the fusion *MT* promoter – SP – eGFP – polyA construct. The fusion *MT* promoter – signal peptide – eGFP – polyA was then transferred into the pRD400 binary vector [19] using *Asp*718 and *Bgl*II enzymes. Plasmids were analyzed by digestion with either *Eco*RI or *Hind*III in 20 µL at 37°C for 1 h. Digestion products were checked by electrophoresis on a 1% agarose gel for 25 min at 135V. A plasmid showing the expected profiles was selected and named pRP116. Finally, the plasmid was transferred into *R. rhizogenes* 15834 competent cells by electroporation. Bacteria containing pRP116 vector were grown in 5 mL liquid MGL medium (5 g/L Tryptone, 2.5 g/L Yeast extract, 100 mg/L NaCl, 5 g/L Mannitol, 1 g/L glutamic acid, 250 mg/L KH₂PO₄, 100 mg/L MgSO₄, pH7) [20] containing kanamycin at 50 µg/mL at 28°C, 200 rpm for 24 h for being used for *A. thaliana* transformation. The transformation protocol followed the protocol from Mai *et al.* (2016).

2.7. eGFP Assay

eGFP was quantified on a BioRad Versafluor fluorimeter fitted with 490/10 nm excitation and 510/10 nm emission filters, following the protocol from Mai *et al.* [13]. The machine was calibrated using a 10 mg eGFP/L standard bought from Sigma Company. Before measuring, the culture media were diluted at suitable dilution factors in 50 mM Tris/HCl pH 7.5 buffer.

2.8. Statistical Analysis

Data were analyzed by analysis of variance single factor and Turkey *post hoc* test at a 5% probability level ($P \leq 0.05$). Experiments were done at least in triplicates. All data were expressed as mean ± confidence interval ($P = 0.95$).

3. RESULTS

Microarray data have shown the difference in gene expression between wild-type roots and HRs in *A. thaliana* plants. There are 28295 genes that are expressed higher in the HRs than in the wild-type roots. These genes encoded for structural proteins, proteases, transporters, transcription factors, etc. Among these genes, the *Atlg07590* gene, which encodes the metallothionein 1A, the so-called *mt1A* gene, was the most highly expressed and annotated in HRs than in wild-type roots [Supplemented Table 1].

3.1. Cloning *MT* Promoter from *MT1A* Gene to Drive the *eGFP* Gene

The result from the microarray has given a hypothesis that the promoter of the *mt1A* gene (*Atlg07590*) linked to the *eGFP* gene would likely result in a high level of eGFP production by *A. thaliana* HRs. Therefore, we cloned the *MT* promoter from this gene to direct the expression of the *eGFP* gene.

The *MT* promoter from the *mt1A* gene was amplified by PCR and digested with *HindIII* and *Asp718* for cloning into the pK18 vector. The PCR products were visualized after electrophoresis in an agarose gel [Figure 1]. The result showed only one band corresponding to the size of the *MT* promoter, which is about 1.6 kb, demonstrating the correct amplification.

Then, the *MT* promoter was inserted into the pK18 vector (2661 bp) and transferred into *E. coli* DH5 α bacteria cell. Colony PCR was carried out on four white colonies. The plasmids from positive PCR results were extracted and digested with either *Asp718* and *HindIII* or *EcoRI* restriction enzymes [Figure 2]. All four plasmids showed the correct profiles after being digested by two enzymes. The plasmid from clone 2, which indicated the brightest bands after digestion, was kept and named pRP114.

The *MT* promoter from the pRP114 plasmid was inserted into vector pRP36, using *Asp718* and *HindIII* enzymes, replacing the 35S promoter. Then, the new vector was transformed into *E. coli* DH5 α to select the good transformant.

Colony PCR was undertaken on *E. coli* DH5 α transformants. Plasmids from the four positive colonies were digested either with *Asp718* or *HindIII*. All four plasmids showed the correct profile which contained the *MT* promoter [Figure 3]. The plasmid from clone 2 was kept and named pRP115.

The *MT* promoter – Signal Peptide – *GFP* gene – polyA fusion in pRP115 was transferred into the pRD400 binary vector using *Asp718* and *BglII* restriction enzymes. The plasmids from four white colonies were digested with either *EcoRI* or *HindIII*. Three of four plasmids showed the correct profile which contained the inserted *MT* promoter – Signal Peptide – *GFP* gene – polyA structure [Figure 4]. The plasmid from clone 2 was kept as pRP116. Then, the pRP116 was electroporated into *R. rhizogenes*.

A. thaliana hypocotyls were transformed with *R. rhizogenes* containing pRP116 vector. Putative HRs were first visualized under UV light to confirm their transformation status. The results showed the green HRs in both 35S-*egfp* line 26 [Figure 5a] and *MT-egfp* line 41 [Figure 5b] which indicated the expression of the *eGFP* gene in HRs, and confirmed the successful transformation.

However, the results also showed that eGFP was expressed in the whole root, even in root hairs in HR 35S-*egfp* line 26, whereas it is only expressed in the cortex in HR *MT-egfp* line 141 [Figure 5a and b].

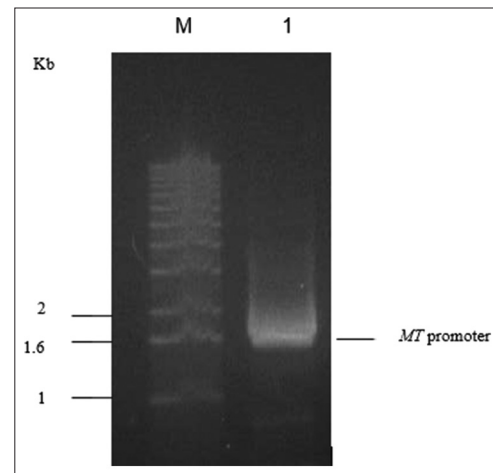


Figure 1: The *MT* promoter in an agarose gel after polymerase chain reaction (PCR) amplification. M: Marker (Invitrogen 1 Kb Plus DNA Ladder); 1: PCR product (1.6 Kb).

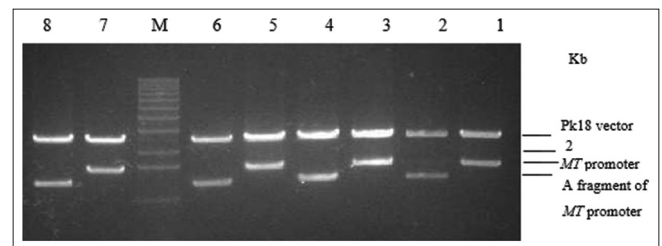


Figure 2: Analysis of the digestion of the pK18 vector containing the *MT* promoter. M: Marker (Invitrogen 1 Kb Plus DNA Ladder); 1, 3, 5, 7: Plasmids of colonies 1, 2, 3, 4 digested with *Asp718* and *HindIII*; 2, 4, 6, 8: Plasmids of colonies 1, 2, 3, 4 digested with *EcoRI* and *HindIII*.

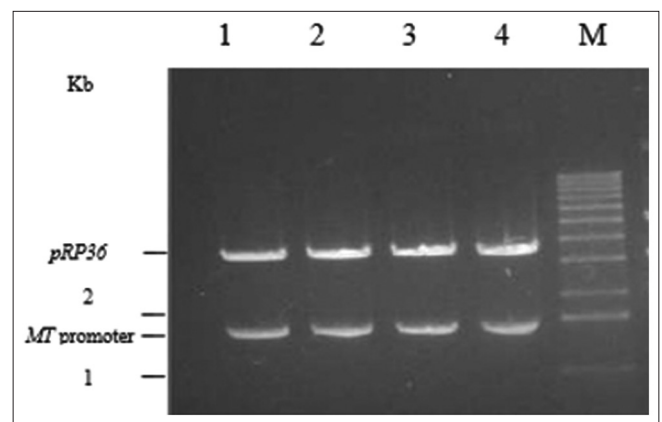


Figure 3: Analysis of the digestion of the pRP36 vector containing *MT* promoter. M: Marker (Invitrogen 1 Kb Plus DNA Ladder); 1, 2, 3, 4: Plasmids 1, 2, 3, 4 digested with *Asp718* and *HindIII*.

The HRs were then grown in liquid B5 medium in parallel with a positive line 26 HRs, which expressed very strong eGFP protein under the control of 35S promoter, to compare the capacity of eGFP production under the control of two different promoters (35S and *MT*).

Out of ten tested lines driven by *MT* promoter, HR lines 7 and 141 gave the highest eGFP production after 1 month of culture, at around 63–68 mg eGFP/L. Most lines produced between 10 and 20 mg/L

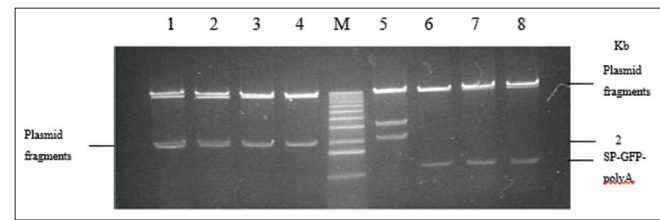


Figure 4: Analysis of new pRD400 binary vector containing *MT-SP-egfp-polyA*. M: Marker (Invitrogen 1 Kb Plus DNA Ladder): 1, 2, 3, 4: Plasmids digested with *Hind*III. 5, 6, 7, 8: Plasmids digested with *Eco*RI.

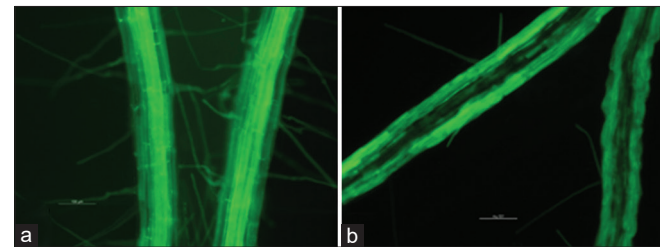


Figure 5: Hairy roots expressing the enhanced green fluorescent protein (*eGFP*) gene of *35S-egfp* line 26 (a) and *MT-egfp* line 141 (b).

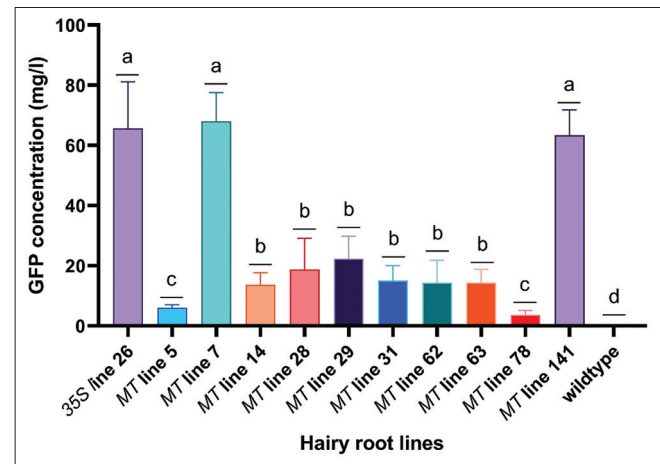


Figure 6: Green fluorescent protein (GFP) production by *MT-enhance GFP (egfp)* and *35S-egfp* hairy root lines after 1 month of culture in liquid B5 medium. Values are presented as means $\pm$ standard deviation calculated from ten replicates. Different letters indicate the significant differences ($P < 0.05$).

[Figure 6]. There was no significant difference between GFP produced by *35S* promoter line 26 and *MT* promoter line 7 and 141 ($P > 0.05$).

In the previous study, we showed that when treated with the 2,4-D hormone, the HRs developed into thick organs called rhizocalli that produced more protein than untreated roots [21]. In the present study, HRs from these three strong lines (HR lines 7 and 141 from *MT* promoter and line 26 from *35S* promoter) were treated by 2,4-D at a concentration of 0.5 mg/L to induce the development of rhizocalli. The GFP production was recorded after 15 days of culture in a liquid B5 medium.

GFP production by rhizocalli was much higher in *35S-egfp* line 26 than in *MT-egfp* lines 7 and 141. Only approximately 22.5 and 11.5 mg/L GFP were produced by *MT-egfp* rhizocalli lines 7 and 141, which were even lower than by HRs, while approximately 120 mg/L eGFP was produced by rhizocalli line 26 [Figure 7].

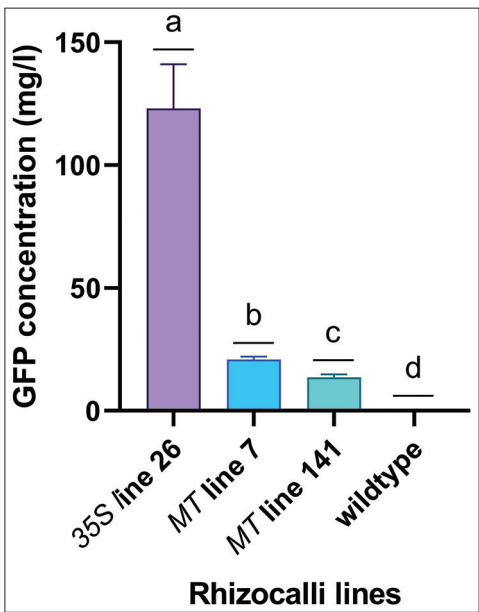


Figure 7: Green fluorescent protein (GFP) production by *MT-enhance GFP (egfp)* and *35S-egfp* rhizocalli lines after 15 days of culture in liquid B5 medium supplemented with 0.5 mg/L 2,4-D. Values are presented as means $\pm$ standard deviation calculated from ten replicates. Different letters indicate the significant differences ($P < 0.05$).

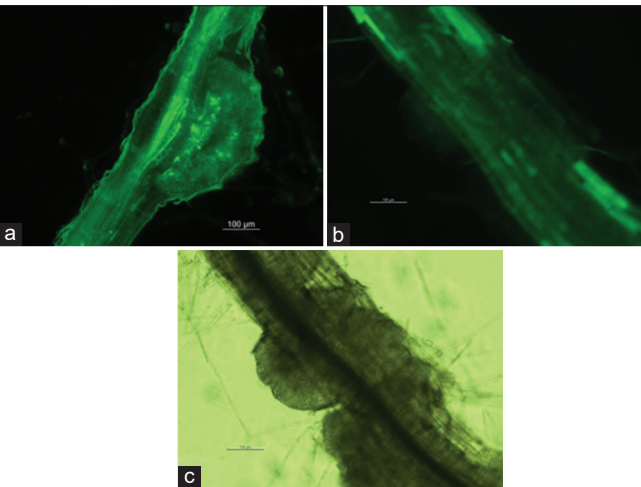


Figure 8: Rhizocalli expressing the enhanced green fluorescent protein (*egfp*) gene of *35S-egfp* line 26 (a) and *MT-egfp* line 141 (b) under UV light and *MT-egfp* line 141 under white light (c).

The results of rhizocalli visualized under the UV light showed that eGFP was strongly expressed in rhizocalli of *35S-egfp* line 26 [Figure 8a] whereas rhizocalli of *MT-egfp* line 141 [Figure 8c] seems to not express, only the cortex in HR part expressed a little eGFP [Figure 8b].

The dry weight of rhizocalli driven by *35S* and *MT* promoters showed that there was no significant difference in the dry weight of all three tested lines ($P > 0.05$) [Figure 9], indicating that the promoter does not affect the growth of rhizocalli but strongly affect the eGFP expression.

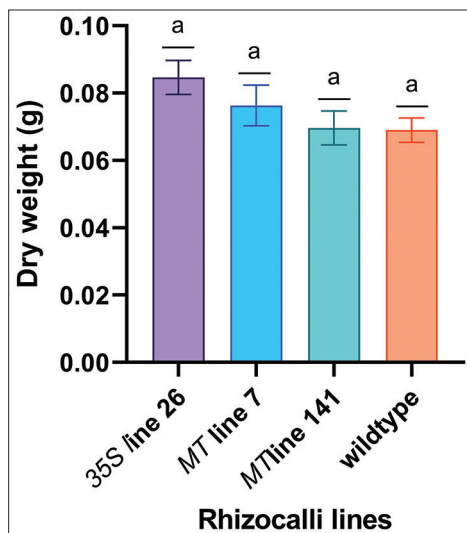


Figure 9: The dry weight of rhizocalli driven by 35S and MT promoters after 2 weeks of culture in liquid B5 medium supplemented with 0.5 mg/L 2,4-D. Values are presented as means $\pm$ standard deviation calculated from ten replicates. Different letters indicate the significant differences ($P < 0.05$).

4. DISCUSSION

To date, extensive efforts have been undertaken to enhance the production of secreted recombinant proteins. The precise regulation of recombinant protein expression is very important to balance the intricate metabolic pathways in the host cells and to ensure the high productivity of the desired recombinant proteins. Promoters are the key regulatory elements controlling the level of recombinant protein expression in hosts and have been extensively studied to maximize the expression of genes in different host systems including plants [22]. Our previous studies have demonstrated the high production capacity of heterologous proteins using *A. thaliana* HRs as an expression system [13,21]. The present study shows the different expression patterns of a promoter toward heterologous genes in *A. thaliana*. We obtained a very high expression level of the *mt1A* gene (*At1g07590*) in HRs compared to normal roots of *A. thaliana* from microarray data; however, this promoter showed quite low expression when it was used to drive the expression of a heterologous eGFP protein in HRs of *A. thaliana*. The histology analysis showed that in HRs receiving the pRP116 plasmid, the MT promoter was only expressed in the cortex but not in the style of roots [Figure 5]. That's why we obtained a quite low eGFP concentration in most HR lines with MT promoter than the one with 35S promoter [Figure 6]. An impact of the reporter gene on the promoter activity which is represented by the amount of recombinant protein production was also observed in the study of Stadlmayr *et al.* where the relative productivities of *eGFP*, *HSA*, and *lacZ* genes varied broadly under the control of *ePET9* promoter in *Pichia pastoris* even the identical cloning strategy [23,24]. Therefore, additional investigations focusing on promoter architecture and the interaction with the heterologous gene structure would be necessary to understand this bottleneck. Promoter engineering could also be performed to create promoters with better properties [25].

In our previous study, for the 1st time, the rhizocalli structure, which was formed by treated HRs with a suitable concentration of 2,4-D hormone, showed outstanding heterologous protein expression over HRs [21]. However, we obtained much lower eGFP production in rhizocalli with MT promoter than the one with 35S promoter [Figure 7]. Histological analysis showed that the calli-like structure in rhizocalli

did not express eGFP protein [Figure 8b]. Further studies need to be carried out to explain for this phenomenon. This result also suggests a specific interaction between MT promoter and *eGFP* gene structure leading to low eGFP production.

Surprisingly, the MT promoter did not affect the growth of HRs which was indicated by no significant difference in the root weight between lines under the control of the MT promoter and 35S promoter indicating that the MT promoter only affected heterologous protein production but not the host system growth. A similar observation was reported when the seed-specific promoter was utilized, as it promoted the accumulation of foreign proteins while minimizing potential effects on plant health and embryo development [26].

Among ten studied HR lines controlled by MT promoter, we obtained diverse expression profiles of eGFP even though they have the same promoter and GFP coding sequence. These differences can be explained by the position where the gene was inserted in the *A. thaliana* genome. The difference in the expression activity may also be correlated with the copy number of the foreign gene [27]. Furthermore, after gene insertion, there were also some other variations in the genomic sequences of the host, including small deletions at the integration sites, superfluous DNA inserted between T-DNA and genome, and translocation of genomic DNA in the flanking regions which could affect the level of transgene expression [28].

5. CONCLUSION

The production of model GFP proteins under the MT promoter did not exceed that with the standard promoter 35S while we detected the different expression locations of the MT promoter in HRs and rhizocalli in *A. thaliana*. Importantly, the MT promoter specifically drives GFP expression within the context, while remaining inactive in the style or calli-like structure of the HRs. This finding presents a novel strategy for utilizing this promoter for spatially controlled gene expression. The discussion emerged during the selection of promoters for heterologous protein production to achieve high productivity. Additional promoters from various genes identified through microarray analysis could also be investigated for their potential in heterologous protein production.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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