Matching is the Key Factor to Improve the Production of Patchoulol in the Plant Chassis of *Marchantia paleacea*

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**ABSTRACT:** The valuable terpenoids, such as artemisinin acid, have achieved bioproduction in the chassis of microbes recently. In this study, *Marchantia paleacea* L, a promising plant synthetic biology chassis, was used to explore the possibility of patchoulol production by constructing a synthetic biology pathway composed of FPS and PTS. The experiment results show that the maximum yields based on the cytoplasm and plastid pathway were 621.56 and 1006.45 μg/g, respectively. However, there is no statistically significant difference in the yield of patchoulol between transformant plants with different subcellular compartment-targeting pathways. However, it was found that the highest yield of patchoulol was achieved in transformant plants with similar transcription levels of FPS and PTS. Also, the optimized transcription ratio between PTS and FPS is determined at 1.12 based on statistical analysis and model simulation. Therefore, two kinds of new optimized pathway vectors were constructed. One is based on the fusion protein method, and the other is based on protein expression individually, in which the same promoter and terminator were used to derive the expression of both FPS and PTS. The effect of pathway optimization was tested by transient and stable transformation. The production of patchoulol in transient transformation was the same for the two abovementioned kinds of matching pathway and higher than that for the original pathway. Also, in stable transformation, the yield of patchoulol reached up to 3250.30 μg/g, being three times the maximum content before optimization. It is suggested that *M. paleacea* is a powerful plant chassis for terpenoid synthetic biology and the matching between enzymes may be the key factor in determining the metabolic flux of the pathway in the study of synthetic biology.

**INTRODUCTION**

Plant secondary metabolites, such as terpenoids, phenolics, polyketides, and alkaloids, are structurally diverse with potential biology activity and excellent chemical properties, while these products are usually present in low abundance.1 Many researchers have attempted to efficiently produce plant secondary metabolites in microbial chassis such as *Escherichia coli* and yeast. Martin et al.2 established an *E. coli* strain to produce amorpha-4,11-diene, the precursor of malaria treatment drug artemisinin, in large amounts (25 mg/L) by introducing the MVA pathway and amorpha-4,11-diene synthase (ADS) with other several modifications. This procedure was used in a commercial scale in 2013 by Paddon et al.3

Patchoulol is a sesquiterpene alcohol present in the patchouli oil extracted from the patchouli (*Pogostemon cablin*) leaves.4 Patchoulol is widely used in the cosmetics industry because of its peculiar and long-lasting scent. Besides, patchoulol also has many pharmacological functions such as antibacterial,5 anti-inflammatory,6 and antiviral capabilities.7 PTS (patchoulol synthase), the key enzyme of the patchoulol synthesis pathway, has been characterized.8,9 Because of the finite natural plant resources and the limited yield of the extraction procedure, biotechnological engineering on different chassis plays a more important role in the patchoulol heterologous production.10

Several articles have reported on patchoulol heterologous production using the recombinant microbial platform. Patchoulol (11.5 mg/L) was harvested by suppressing ERG9 expression in engineered *Saccharomyces cerevisiae*, harboring the PTS gene.11 Also, the yield of patchouli can be increased to about five times in *S. cerevisiae* by expressing the patchoulol synthase gene (PTS) and farnesyl pyrophosphate synthase (FPS) as a fusion protein.12 The global metabolic engineering strategy (GMES) also was used to engineer the Mevalonate pathway in *S. cerevisiae*, in which 0.05 mg of patchoulol could be produced for every gram of glucose consumed.13 Furthermore, the combination metabolic engineering strategy,
overexpressing PTS and its precursor gene while suppressing the carotenoid-like byproduct pathway, could efficiently improve the carbon flux and produce patchoulool at 60 mg/L. The highest yield, 466.8 mg/L of patchoulool in engineered yeast was reported by suppressing the squalene pathway and overexpressing PTS and transcription factor UPC2-1 genes.

Plant chassis harbors great potential for synthetic biology, in that photosynthesis can provide sufficient energy and substances, field cultivation costs are low, and plant-specific storage organs can provide storage places for metabolites. However, using the terpenoid synthetic biology approach in plant chassis is challenging because of our limited understanding of plant genetic networks and increased complexity due to multicellularity. The tobacco plant Nicotiana tabacum has been used for patchoulool heterologous production; 0.03 mg/g yield was obtained in the leaves. Targeting the sesquiterpenoid synthase to the plastids is the initial improvement of tobacco chassis, which increases the yield by 4000-fold. HMGR is the key regulatory enzyme in the Mevalonate pathway, and increasing its activity to redirect more carbon flux to target terpenoid precursors is a potential strategy. For example, by overexpressing truncated HMGR in Physcomitrella patens and targeting the PTS and FPS enzymes to plastids, the yield of patchoulool was raised to 1.34 mg/g dry weight. It is the highest yield reported for patchoulool heterologous synthesis based on plants so far. Yet, the production is still limited.

Though plants are a potential chassis for terpenoid metabolism engineering, working with plants is beset with problems because of slow generation times, genetic redundancy, and difficulties in transformation, compared to microbes. To circumvent these difficulties, we adopted Marchantia as a test bed to study terpenoid synthetic biology in plants. This liverwort plant system is fast and easy to culture and transform. Marchantia paleacea was modulated to have higher sesquiterpene content, oil bodies rich in sesquiterpene, and the carotenoid-like byproduct pathway, could be vegetatively propagated by germs; each germ originates from a single cell and can develop into a complete plant. Through germ reproduction, many plants with the same genetic background as the parent can be quickly obtained, effectively reducing the interference of genetic factors. The plant is a haploid and has a genome of about 220 Mb, which shows low genetic redundancy in the regulation of most pathways. Meanwhile, a simplified Agrobacterium-mediated transformation method for sporophytes of Marchantia polymorpha (Agar-Trap) has been explored. It is reported to have a high transformation ratio of up to 100%.

M. paleacea contains oil bodies rich in sesquiterpene, diterpenoid, and aromatic compounds, which suggested that M. paleacea could provide a potential store site for terpenoids in plant chassis.

Here, we reported the construction and optimization of patchoulool pathways in M. paleacea to explore the heterologous production of patchoulool in plants and studied the factors determining the metabolic flux of the patchoulool pathway, such as cellular compartment targeting, the expression level of each enzyme, and the matching between enzymes in the pathway in M. paleacea.

**RESULTS**

Transformants in *M. paleacea*. We modified the 35s and CVM promoter and selected 35s-3 and cvm-6 as the strong promoters in this experiment through the Dual-Luciferase Reporter Assay System (related data were not shown). GV3101, harboring the plasmids PDGB3::tpPTS-T35s:CVM-6-FPS-T35s::cvm-6-HYG-T35s and PDGB3::tpPTS-T35s:CVM-6-tFPS-T35s::cvm-6-HYG-T35s, was used to introduce PTS and FPS into the germ via Agrobacterium-mediated transformation method. After Agrobacterium transformation and Hygromycin selection, the survival T₀ generation germs were placed in the selection medium. Then, we placed T₁ generation and wild-type gemmae in the selection medium again, and about 1 week later, the wild-type germs turned white and lost their vitality (Figure 1). Among them, 17 independent transgenic plants (10 in the tpFPS + tpPTS group and 7 in the FPS + PTS group) were obtained and confirmed to have PTS and FPS using the PCR test. They were named as 1–1, 1–2, 1–3, 1–7, 1–9, 1–10, 1–12, 1–13, 1–14, 1–15, 2–1, 2–2, 2–3, 2–5, 2–8, 2–10, and 2–11, respectively. It demonstrated that FPS and PTS have been successfully integrated into the *M. paleacea* genome, and the Agrobacterium-mediated *M. paleacea* was modulated to have higher sesquiterpene content, oil bodies rich in sesquiterpene, diterpenoid, and aromatic compounds, which suggested that *M. paleacea* could provide a potential store site for terpenoids in plant chassis.

![Figure 1. Resistant seedlings and wild-type seedlings screened in the medium.](https://baike.baidu.com/pic/%E5%9C%B0%E9%92%B1/4906381/1321988/)

Production of Patchoulool in *M. paleacea* Was Feasible. To test whether the transgenic group could produce patchoulool, gas chromatography–mass spectrometry (GC–MS) analysis was performed. As the chromatogram shows (Figure 3), the same peak appeared in the standard sample and transgenic group at about 7.46 min, except the wild-type sample. Then, the mass spectrum was extracted at the corresponding chromatographic peak, and the feature fragments were identified. Thus, the target product could be synthesized by exogenous gene insertion, transcription, and translation in *M. paleacea* [https://baike.baidu.com/pic/%E5%9C%B0%E9%92%B1/4906381/1321988/](https://baike.baidu.com/pic/%E5%9C%B0%E9%92%B1/4906381/1321988/).
Compartmentalized metabolic engineering has proven to be an effective strategy for improving sesquiterpene production. The plant material was divided into two groups: FPS + PTS group and tpFPS + tpPTS group; the tpFPS + tpPTS group showed a higher patchoulol content on average according to GC−MS quantitative analysis. Also, 1−9 in the tpFPS + tpPTS group and 2−3 in the FPS + PTS group yielded the maximum production of 1006.45 and 621.56 μg/g dry weight of patchoulol, respectively. Though the tpFPS + tpPTS group showed higher average yield, there was no significant statistical difference between the two groups via student’s test, defining the significant level as $P < 0.05$. It is suggested that the chloroplast could provide a suitable microenvironment for FPP accumulation by the MEP pathway, which could improve patchoulol synthesis and accumulation.
Also, the production of patchoulol in *M. paleacea* was feasible (Figure 4).

The pathway marked in red was the patchoulol heterogeneous pathway introduced into *M. paleacea* via Agrobacterium transformation. Two terpenoid pathways could provide the common precursor IPP (Isopentenyl diphosphate) in different cell compartments: cytoplasm and chloroplast; it was feasible to produce patchoulol in *M. paleacea*. The compartmentalization strategy was performed using signal peptides; the cytoplasm lines and chloroplast lines were named as FPS + PTS and tpFPS + tpPTS groups, respectively (Figure 5).

**Cell Compartmentation Has No Significant Effect on the Transcription Level.** In the tpFPS + tpPTS and FPS + PTS groups, FPS appears to show a higher transcription level than PTS; it demonstrated that FPS is the committed enzyme in the plastid patchoulol pathway and regulated the PTS transcript level. Compared to others, 1–9 was the highest in these lines (Figure 6). As for the FPS + PTS group, FPS was higher than PTS in most samples, and 2–3 was the highest.

*Figure 4.* Mass spectrum of transgenic plants. *m/z* = 98, 138, 161, and 222 were selected as the diagnostic ions to quantify patchoulol.

*Figure 5.* Content of patchoulol in the FPS + PTS group and tpFPS + tpPTS group. The statistical analysis significance was calculated using the student’s test, defining the significant level as *P* < 0.05.
individual. In this study, Arabidopsis RuBisCO, a small subunit transit peptide, carried out the pathway plastid targeting and compartmentation strategy. However, the insertion position is the vital factor affecting the transcription level except the transcript units themselves. When comparing the FPS and PTS transcription levels in the tpFPS + tpPTS and FPS + PTS groups, it seemed that there was no significant difference between different groups, whether FPS or PTS. Overall, it was suggested that the compartmentation strategy was not the key factor affecting the transcription level in our study (Figure 7).

**Dynamic Changes in the Yield and Transcription Level.** To explore the dynamic changes during the plant growth period, we analyzed the relative expression and metabolism in 2−3 and 1−9 lines (Figure 8). With *M. paleacea* growth, FPS and PTS expression levels increased continually and FPS was higher than PTS in same lines as well as in different lines. The addition of plasmid signal peptide tp enhanced gene expression to a certain extent from 1 to 4 weeks. Because of aging, the RNA extraction seemed difficult for transcription level analysis after 4 weeks. However, the constitutive promoters 35s and CVM promoted the gene-sustained expression throughout the plant growth cycle. The patchoulol content increased for 5 weeks, 2−3 content declined at the 6th week, and 1−9 content declined gradually at 7th week and then declined rapidly. The highest accumulation of content happened at the 5th week in the FPS + PTS group and at the 6th week in the tpFPS + tpPTS group; the yields were 621.56 and 1006.45 μg/g, respectively. According to the abovementioned data, a regular pattern showed that the accumulated content of patchoulol synthesis transgenic liverworts reached its peak at 5−6 weeks and then declined (Figure 9).

**Pathway Matching: the Transcription Ratio Determined the Efficiency.** Although the patchoulol synthesis pathway is short and simple, it is a good way to explore the efficiency of pathway matching. First of all, every module in the pathway should be reasonably designed and matched. In detail, strict control of transcript unit’s (TU’s) transcription level in the modules might be accomplished via optimal DNA parts such as promoters, terminators, CDS, and even transcription factor. In this study, we guessed that the patchoulol content was related to the gene transcription level, which was reasonably matched in the pathway; however, the key point was not clear. Then, we established a scatter plot of yields and different gene expressions to describe the connection between them (Figure 10). It seemed that the transcript level ratio FPS/PTS plays a vital role. Many spots, as shown in the figure, were around 1, which demonstrated that the similar transcription level between FPS and PTS was the rational match and could contribute to the final product. We hypothesize that when the
ratio was about 1, the optimal product could be obtained. Then, a model was established to prove the hypothesis in this study.

According to the steady-state hypothesis of metabolites in metabolic flux analysis, we can assume that each metabolic reaction is in a steady state, that is, the consumption rate and production rate of each metabolite are equal. The production rate of the final product in the metabolic network is related to the rate-limiting step in the pathway. The rate-limiting step is the reaction with the lowest enzyme catalytic activity. The catalytic ability can be expressed by the reaction velocity $v$. The constant $k$ was used to describe the relationship between the reaction velocity $v$ and relevant gene transcription level.

$v_{FPS}$: FPS reaction velocity; $v_{PTS}$: PTS reaction velocity; $v$: patchoulol production velocity; $P$: patchoulol content; TR: transcript level; $a$: actual value; and $t$: theoretical value.

In the patchoulol pathway, the production velocity of the final product was determined by lower reaction velocity between $FPS$ and $PTS$.

$$v = \begin{cases} v_{FPS}, & v_{FPS} < v_{PTS} \\ v_{PTS}, & v_{FPS} \geq v_{PTS} \end{cases}$$  \hspace{1cm} (1)

$$v = \min(v_{FPS}, v_{PTS})$$  \hspace{1cm} (2)

then we defined $v$ as $v = k \cdot TR$

$$v = \int \min[k_{FPS}TR_{FPS}, k_{PTS}TR_{PTS}] \, dt \approx \min[k_{FPS}TR_{FPS}, k_{PTS}TR_{PTS}]$$  \hspace{1cm} (3)

Then we calculated the $\min(P_{av} - P_{tv})^2$ by solver function in the office and got the minimum value, which means the highest patchoulol content could be obtained when the transcript level ratio was about 1.12. This is consistent with our previous assumption.

Two Methods to Test the Pathway Matching Hypothesis in Patchoulol Synthesis. To test the applicability of the pathway matching conclusion derived from the hypothesis and the model, two kinds of vectors were constructed and introduced into $M. paleacea$ via Agrobacterium-mediated stable and transient transformation.

The 35S-3 promoter was used to construct the $FPS$ and $PTS$ fusion protein vector and the same promoter–promoter vectors. Because of the time and transformation difficulty, relevant data were collected on a transient transformation group: p35s-3-FPS-linker-PTS-T35s, p35s-3-FPS-T35s:p35s-3-PTS-T35s; and stable transformation group: p35s-3-tp-FPS-T35s:p35s-3-tp-PTS-T35s. We found that in the transient transformation group, the fusion protein line and p35s-3-FPS-T35s:p35s-3-PTS-T35s line could produce the same level of patchoulol content, about 15.34 μg/g (dry weight) (Figure 11), and higher than the original pathway (5.03 μg/g). What is more exciting is that the stable transformation group produced a maximum content of about 3250.30 μg/g (dry weight) patchoulol (Figure 12). It demonstrated that the fusion protein and the same promoter–promoter pathway might have the same effect, and the stable transformation of these two lines
might provide higher yields than p35s-3-tp-FPS-T35s:p35s-3-tp-PTS-T35s lines. Thus, the pathway matching hypothesis could be verified by fusion protein and the same promoter—promoter pathway.

**Discussion**

Synthetic biology of important terpenoids generally focuses on the microorganism chassis, and compared with microbial synthetic biology, plant synthetic biology has great prospects but also has bottlenecks. *M. paleacea*, which is of small genetic size and which grows fast, is a platform in our study because of the low genetic redundancy and mature and convenient genetic transformation method. Many attractive synthetic technological tools have been developed for studying *M. paleacea*, including those that improve the robust Loop assembly vector systems for nuclear and chloroplast transformation and genome editing. Here, we engineered the patchoulol pathway via compartmentalizing FPS and PTS in the chloroplasts of *M. paleacea*. Combining the compartmentalization strategy with enhancement of precursors, we successfully obtained 1006.45 and 621.56 μg/g dry weight of patchoulol in tpFPS + tpPTS and FPS + PTS groups of *M. paleacea* thalli. In both tpFPS + tpPTS and FPS + PTS groups, although the same expression vectors and retargeting and overexpression strategies were used, the examination on the expression level of all lines revealed the low expression level, which explains the low yield. The positional effects on the *M. paleacea* genome are much less understood. On the whole, the production of sesquiterpene patchoulol in *M. paleacea* was feasible and had potential.

In our study, we found that the introduction of the exogenous pathway may match or interfere with the endogenous terpenoid regulatory network, and the matching could determine the synthesis efficiency. In addition, the compatibility of exogenous pathways and plant endogenous pathways is an ideal state for efficient synthesis. A reasonable pathway consists of different optimal modules, and the biological element selection and match are the base of suitable modules. The biological elements include CDS, promoters, terminators, transcript factors, and so on and affect the pathway transcription level and even the translation level. In this study, we found that when the same transcription level was in the patchoulol pathway, the optimal yield was obtained in the tpFPS + tpPTS and FPS + PTS groups, which was similar to that of patchoulol in *S. cerevisiae*, and the yield could increase by five times via the fusion of FPS and PTS proteins because the fusion CDS could afford the same transcription level. It might be that the same transcription level between FPS and PTS was the reasonable match. The reasonable match means that the pathway could lead to consumption of FPP produced by overexpression in time to avoid triggering the chassis’ own FPP regulatory network because FPP was strictly regulated in the cell. We guessed that the optimal yield will be obtained when the FPS and PTS transcription levels are same, then the hypothesis was verified via mathematical and consisted with the guess. After that, the fusion protein and same promoter—promoter vectors were constructed for transient or stable transformation. It is found that fusion protein afforded the same patchoulol yields with the same promoter pathway in the cytoplasm, higher than those with the original unoptimized pathway in the cytoplasm. Compared with the different promoter pathway, the same promoter pathway in plastid could increase the patchoulol yields by 3 times to about 3250.30 μg/g (DW). Thus, according to the transient and stable transformation data, we can infer that pathway matching could significantly improve the patchoulol production in *M. paleacea*.

Usually, overexpression of *FPS* to accumulate FPP in the cell sometimes might not work as we expected. FPP, which has been demonstrated to have toxicity in the *E. coli* cell, could inhibit cell growth, and the introduction of FPP sensor regulators could balance the endogenous regulatory network and heterogeneous pathway. Protein farnesylation is a post-translational modification and central to molecular cell biology. Farnesyl transferase inhibitors are potential cancer anticancer agents, which correct aberrant genome organization in Hutchinson–Gilford progeria syndrome fibroblasts. As for plants, many mechanisms of farnesylation signaling are still unknown. And the mutant of plant protein farnesyl transferase caused meristem or ganization and mediate brassinosteroid biosynthesis to regulate abscisic acid responses. Thus, FPP was strictly regulated because it plays a critical role in the growth and development of organisms. Meanwhile, the codon-optimized genes have previously been shown to be useful for increasing the translation level. However, whether it is useful for the transcription level or not is not clear.

The isoprene is production via MVA and MEP pathway in plant. Generally, the crossflow between the MVA and MEP pathway is restricted; nevertheless, the systematic analysis of isoprenoids in 86 plant species shows that strict compartmentalization of biosynthesis occurred in triterpenoids, tetraterpenoids, and diterpenoids. Contrary to that, monoterpenes, diterpenes, hemiterpenes, sesquiterpenes, and polyterpenes could be derived by both pathways in the case of specific environmental conditions. Compartmentalized metabolic engineering in *Nicotiana* spp. and *P. patens*, especially targeting the isoprenoid synthase, has proven to be an effective strategy for improving sesquiterpene production such as patchoulol and artemisinin in plants. Also, the possible reason for retargeting terpenoids to FPS as the substrate in plastids that can effectively improve the yield may be the fact that the sesquiterpene production mainly relies on the MVA pathway operation on plastids and retargeting it to plastids could provide a less FPS-competitive environment. In this study, we co-expressed FPS and PTS in the cytoplasm and chloroplast. The chloroplast targeting of FPS and PTS lines afforded a high maximum yield compared to the cytoplasm lines, which is consistent with the patchoulol production in *P. patens*. Although the plastid-targeting lines showed a high average
yield, there was no significant difference between two groups according to the t-test. Meanwhile, we noticed a phenomenon that the cell compartmentation strategy has no significant effect on the transcription level because the signal peptide works at the translation level and not at the transcription level.

Overall, the reasonable collocation of modules inside the pathway, coordination or matching, and compatibility between the pathway and the chassis itself are the initial factors we should focus on, and these strategies might help us to better understand plant chassis and design plant synthetic biology.
**Experimental Section.** Plant Materials and Growth Conditions. A female strain of *M. paleacea*, FSN-2, was isolated from Hubei province (china) and maintained on 1/2 Gamborg media (0.5X strength Gamborg B5 medium plus vitamins, Duchefa Biochemie G0210, pH 5.8) and 1% (w/v) agar under sterile conditions, at 22 °C, with 10,000 lx light (16 h light/8 h dark), in a 100 mL Erlenmeyer flask.

**Vector Construction.** GoldenBraid Kit 2, a kind of synthetic tool, based on the type IIS restriction enzymes, was used to assemble DNA elements. Three kinds of plant DNA part used in this paper are promoter with 5′ UTR (PROM5), coding sequence with start and stop codons (CDS), and 3′ UTR with terminator (3TERM). The 5′ end and 3′ fusion sites on each part were designed, following the common genetic syntax of phytobricks, which enables directional assembly of these three parts into the transcription unit (TU) in one reaction. All DNA parts were cloned into a universal acceptor plasmid called pUPD2, which has one pair of divergent BsmBI sites to clone a DNA part and one pair of convergent Bsal sites to assemble parts into a transcription unit.

Two strong PROM5 parts, PCVM-6 and P35S-3, were built based on the genome sequence of the Cassava vein mosaic virus and cauliflower mosaic virus by the synthetic strategy (protocol in the Supporting Information). The expression level of these new promoters was measured by the Dual-Luciferase Reporter Assay System (Promega), following the recommended conditions (Figure S1).

CDS parts encoding the FPP synthase (FPS) and patchoulol synthase (PTS) were domesticated based on the genome of *Gallus gallus* and *P. cabiln* by the synthetic strategy (protocol in the appendix). The sequence encoding *Arabidopsis Rubisco*, a small subunit transit peptide, was fused to the 5′ terminal of these CDS to build two new plastid-targeting CDS (Figure 13), called as tp + FPS and tp + PTS. CDS of the hygromycin phosphotransferase gene (HYG) provided by the GoldenBraid kit were used as a selectable marker for Marchantia.

T3SS, provided by the GoldenBraid kit, was used as a terminator part in this paper. In a single step reaction with Bsal and T4 ligase, three parts, PROMOTERS, CDS, and 3TERM, can be assembled into a transcription unit and cloned into α-level destination vector PDGB3. Two TUs, P35S-3-FPS-T3SS and PCVM-6-PTS-T3SS, harboring in α1 and α2 PDGB3, respectively, can be assembled together into Ω1 PDGB3. In the same way, the hygromycin resistant TU, P35S-3-HYG-T3SS, was assembled with Twister plasmid pDGB1alpha2_SF (GB0107) and cloned into Ω2 PDGB3. Then, DNA assemblies harbored in these two ΩPDGB3 can be joined together and cloned into α1 PDGB3 in a single step reaction with BsmBI and T4 ligase. The binary assembly can be done recursively, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step, and the number of TU assembled doubles every step. The transformed cells were sequenced to verify the correct assembly (Figure 14).

Agrobacterium-containing plasmids were introduced into the leaf of *M. paleacea* by transient transformation. Scale bar indicates 30 μm; YFP: yellow fluorescent protein.

**Transformation Procedures.** The plasmid-harboring patchoulool pathway was introduced into Agrobacterium tumefaciens GV3101, by which the T-DNA between RB and LB was transformed into Marchantia via the Agar-Trap method. Gemmae produced in a 3-week thallus gemma cup were isolated by the TPS method. The primers are shown in Table 1.

**Table 1. Primer Sequence Used in PCR Analysis**

| type      | primer                     | sequence (5′-3′)                                    |
|-----------|----------------------------|-----------------------------------------------------|
| PCR       | FPSF                       | ATGCAGCCCCCATTACATCATATA                             |
|           | FPSR                       | GGGTCCCCAAAAGCATTCCAGGTTAA                           |
|           | TPF                        | ATGCTCTCTCTATGTCTTC                                  |
|           | PTSF                       | ACATCAGTTCATGCGAAGGCAAA                              |
|           | PTSR                       | ACATCAGTTCATGCGAAGGCAAA                              |
|           | FPSQF                      | GAAGGATTGTCGAGAAGCCCTGCGAGT                        |
|           | FPSQR                      | ATGAGGTCGCAAGAATCCCGGCAAGG                           |
| RT QPCR   | PTSQF                      | GATTGGGTGTTCTCAGCGACCCCTCT                          |
|           | PTSQR                      | TCTTTTGAATAAACCTCAGATGTTCCGGA                       |
|           | ACTINQF                    | AGCAAGATGAAAGATTAAGGTTG                             |
|           | ACTINQR                    | CCTTGAGATCAGTTG                                    |

Expression Profiling in *M. paleacea*. Two weeks old thallus (100 mg) was extracted by an RNAprep pure Plant Kit (TIANGEN BIOTECH, Beijing China) according to the protocol provided. The RNA quality and concentration were determined by a Nanodrop2000 Spectrophotometer (Thermo Fisher Scientific). Real-time quantitative PCR was performed under the normal growth conditions mentioned above. Precultured solid medium consisted of 0.5X Gamborg media, 1% sucrose, and 1% agar and was autoclaved. For each interest plasmid, a single Agrobacterium colony was incubated into 1 mL LB medium without antibiotics and then inoculated into 15 mL LB medium plus the antibiotics at 28 °C with shaking at 200 rpm for 1 day. The Agrobacterium cultures, induced for 3 h with 150 μM acetylsyringone, were centrifuged for 10 min at 4000 g, resuspended in 10 mL transformation solution, and then incubated for 3 h at 28 °C at 200 rpm; OD600 was adjusted between 0.5 and 0.8. The transformation solution consisted of 10 mM MgCl2, 10 mM MES–NaOH, 0.01% Tween 20, 150 μM AS. Then, the resuspended Agrobacterium was mixed with precultured gemmae and kept still for 10 min. The infected gemmae were placed on the 0.5X Gamborg B5 media and 150 μM AS plates and, after 2 days dark coculture with 1 mL of screen solution and 100 μg/mL cefotaxime and 20 μg/mL hygromycin, were spread on the medium to screen the transformants. After about 2 weeks, the transformants (T0 generation) were transferred to the subculture 0.5X Gamborg B5 media and 100 μg/mL cefotaxime and 10 μg/mL hygromycin. T0 generation gemmae were then cultured with low humidity and strong light; after 1 month, the surviving grams were treated as homoyzygous transgenic plants (T1 generation).

Transient transformation was used to detect the promoter activity, the subcellular localization of the signal peptide, and the effect of the vector reconstructed according to the matching hypothesis. The thallus was cut into pieces and then infected with Agrobacterium according to the stable transformation process. After 2 days of cocultivation, the Dual-Luciferase data and patchoulool content data were collected. As for the subcellular localization, gemmae cocultured for 2 days were washed with sterile water and then observed using a confocal microscope.

Screening of Transformants with PCR. Two weeks old fresh liverwort samples were harvested, and the transgenic liverwort was identified by PCR analysis using genomic DNA isolated by the TPS method. The primers are shown in Table 1.
using a SuperReal PreMix Plus (SYNR Green) kit (TIANGEN BIOTECH, Beijing, China) according to the protocol provided and run at 95 °C for 15 min, 40 cycles at 95 °C for 20 s followed by 55 °C for 20 s and 72 °C for 20 s on a lightcycler96 System (BioRad, Germany). qPCR was performed with three biological replicates for each sample and three technical replicates for each biological sample. Primers used are listed in (Table 1). *M. palustris ACTIN* was used as the reference gene, and the transcript level was calculated as follows: \[ \Delta CT = CT(GOI) - CT(ACTIN) \]. \( \Delta CT \) was normalized using \( \Delta CT \), and the relative change in gene expression is calculated by the \( 2^{-\Delta \Delta CT} \) method.

**Quantification of Patchoulol Alcohol by GC–MS.** Four weeks old fresh thallus was harvested, cut into small pieces, snap-frozen, and then ground into fine powder; 500 mg of powder was extracted with 3 mL of ethyl acetate, followed by vacuum filtration, and then ground into fine powder; 500 mg of this fine powder was extracted with 3 mL of ethyl acetate, followed by vacuum filtration and dried at 75 °C for 24 h, and dry weights were measured to calculate the sample water contents. The GC–MS analysis method was obtained from the work by Zhang. GC–MS analysis was performed on a GC–MS 7890/5975C (Agilent) equipped with an LTM column module (DB–1MS) (10 m × 0.18 mm i.d. × 0.18 μm). The samples (1 μL) were injected with a split ratio of 25:1 into an LTM (DB–1MS) column using the following temperature program: 50 °C (held for 1 min), 50–320 °C (30 °C/min, held for 1 min), and the total time is 11 min (2 min solvent delay). The oven temperature was 200 °C (held for 11 min). The injector temperature of GC was 250 °C. The ion source temperature of the mass spectrometer was 230 °C, and the transfer line temperature was set at 250 °C. Helium was used as a carrier gas at a constant flow rate of 0.7 mL/min. Data were acquired by EI+ with Selected Ion Monitor (SIM) mode. Diagnostic ions: 98, 138, 161, 222 and RT (5.00–9.00) min were selected as the detail of the SIM method to quantify patchoulol. Statistical analysis significance was calculated using the student’s test, defining the significant level as \( P < 0.05 \).

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**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acsomega.0c04391](https://pubs.acs.org/doi/10.1021/acsomega.0c04391).

All the DNA elements’ sequence and vector construction methods and the Dual-Luciferase data (PDF)

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Notes

The authors declare no competing financial interest.

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