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MYB Transcriptional Factors Affects Upstream and Downstream MEP Pathway and Triterpenoid Biosynthesis in *Chlamydomonas reinhardtii*

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Abstract: Terpenoids are enormous and different types of naturally occurring metabolites playing an important role in industrial applications. Cost-effective and sustainable production of terpenoids at commercial scale is the big challenge because of its low abundance from their natural sources. Metabolic and genetic engineering in microorganisms provide the ideal platform for heterologous overexpression protein systems. The photosynthetic green alga *Chlamydomonas reinhardtii* is considered as a model host for the production of economic and sustainable terpenoids, but the regulation mechanism of their metabolisms is still unclear. In this study, we have investigated the genetic and metabolic synthetic engineering strategy of MYB transcriptional factors (MYB TFs) in terpenoids' synthesis from *C. reinhardtii* for the first time. We heterologous overexpressed MYB TFs, specifically SmMYB36 from Salvia miltiorrhiza in *C. reinhardtii.* MYB upregulated the key genes involved in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Expression of the SQS gene, which is involved in the downstream triterpenoid synthesis pathway, highly accumulated in MYB-overexpression lines of *C. reinhardtii*. The contents of squalene increased about 90.20 μg/g in MYB-overexpressed lines. Our results propose the rerouting of the carbon flux toward the biosynthesis of triterpenoids upon overexpression of MYB TFs in *C. reinhardtii.* Our study suggests imperative novel understandings into the regulation mechanisms of *C. reinhardtii* triterpenoid metabolism through MYB TFs in photosynthetic green microalgae *C. reinhardtii.* The role of MYB TFs is investigated for the first time in *C. reinhardtii*, and provides a prodigious potential for recognizing important transcriptional regulators of the MEP pathway as goals for prospective metabolic and genetic manipulation investigation for increased production of triterpenoids.

Keywords: squalene; triterpenoids; MEP pathway; *C. reinhardtii*; synthetic biology; MYB transcriptional factor; heterologous overexpression

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Citation: Anwar, M.; Wang, J.; Li, J.; Altaf, M.M.; Hu, Z. MYB Transcriptional Factors Affects Upstream and Downstream MEP Pathway and Triterpenoid Biosynthesis in *Chlamydomonas reinhardtii*. *Processes* **2024**, *12*, 487. https://doi.org/10.3390/pr12030487

Academic Editor: Hao Song

Received: 5 December 2023
Revised: 12 February 2024
Accepted: 21 February 2024
Published: 28 February 2024

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1. Introduction

Globally, the population is rapidly increasing, which ultimately leads to accelerated demand for food, pharmaceutical compounds, and energy. Alternative sources are crucial to fulfill the demand for these bioproducts. In this perspective, the cultivation of photosynthetic green microalgae might be an attractive opportunity due to their reduced water footprint than those of agriculture crops and the best option of cultivating them in diverse environmental conditions consuming wastewater as a phosphorous and nitrogen source. Existence mechanisms of carbon concentration in photosynthetic green microalgae facilitate to assimilate CO_2 efficiently and liberate O_2 as a byproduct through the process of photosynthesis [1]. The biomass created by microalgae contains relatively high concentrations

of sterols, phenolic compounds, and others [2]. Thus, microalgae could be deliberated to increase CO_2 sequestration, able to recover nutrients from waste and yield valuable compounds [3]. Terpenoids are enormous and a different class of naturally occurring metabolites playing an important function in various industrial applications [4]. Most commercially available terpenoids are obtained from higher plants, but their cost-effective and sustainable production for industrial purposes is still challenging because of their low abundance of production from natural sources, extraction and purification obstacles, and degree of labor intensity [5]. In response to these confronts, bioengineering of microorganisms in controlled bioreactors for terpenoid production has appeared as an ideal solution. Microorganisms including bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*) are unable to naturally produce plant metabolites. So these organisms are usually selected as heterologous ideal hosts for the production of terpenoids owing to the advantages of complete information regarding their metabolism and genetic tractability [6]. *E. coli* and *S. cerevisiae* need sources of organic compounds for their optimized growth. Therefore, the production of terpenoids from these organisms at a commercial level also presents economic challenges. Currently, genetically modified photosynthetic green microbial hosts have become a carbon-neutral alternative to a generally utilized fermentative system. These carbon-neutral alternatives have the potential to transform CO_2 into highly valuable bioproducts [7]. Previous studies have provided evidence of heterologous terpenoid expression for *Synechocystis* sp. PCC 6803 and *C. reinhardtii* [8,9].

C. reinhardtii is a single-celled eukaryotic green alga, known as "photosynthetic yeast" due to its simple cultivation conditions, short growth cycle, rapid growth, and high photosynthetic efficiency. It has a complex post-translational modification system that can accurately process and modify eukaryotic proteins to ensure that recombinant proteins have the same activity as natural proteins [10]. The growth and metabolism of Chlamydomonas reinhardtii do not produce toxic waste, and the extraction and purification process of the product is relatively simple. There is no risk of environmental pollution from by-products. These advantages indicate that it is a promising terpenoid production system [7].

Squalene is a critical intermediate in the biosynthesis of sterol in a range of organisms from bacteria to humans [11]. Squalene C30 has been considered for various biological functions, such as a natural antioxidant, anticancer, dietary complement, auxiliary for vaccines, and skin moisturizer for therapeutic, cosmetic, and pharmacological uses as well as a protective response to cardiovascular diseases [12,13]. Shark liver oil is the main source of squalene, with a content of up to 80% [14]. Among vegetable sources of squalene, extra virgin olive oil (EVOO) is one of the richest ones, containing squalene in 0.1–1.2%, along with amaranth oil that contains a higher amount (7–8%) but is produced with lower yields compared to olive oil [14,15]. The determination of squalene content in olive oil by transmethylation and GC analysis is 2.88 $\pm$ 0.08 (mg g^{-1}) [16]. Eating olive oil is beneficial to prevent colon cancer and breast cancer, and also has the ability to reduce blood pressure [17]. Microorganisms, including microalgae, have also been investigated for the biosynthesis of squalene at a commercial level [18]. Compared with extracting squalene from vegetable oil, the production cycle of squalene using microorganisms is shorter, the cost is lower, it is economical, environmentally friendly, and easier to regulate [19]. To assure a sustainable supply of squalene and fulfill its industrial demand, substitute sources are required, such as marine organisms. The amount of squalene in microorganisms varies greatly depending on the species. The contents of squalene obtained from *Torulaspora delbrueckii* and *S. cerevisiae* were 0.24 and 0.04 μg/mg, respectively [20]. A higher amount of squalene (171 μg mg^{-1} dry weight) in the cells of *Aurantiochytrium* sp. strain 18W-13a was accumulated [21]. Previous studies reported that *Euglena* and *Botryococcus braunii* were able to accumulate squalene in their cells [11]. Functionally, the characterization of a few genes, which are considered for the metabolism of squalene, has been documented in microalgae [22].

The two general biosynthetic pathways, including the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway, are involved in the production of squalene in plants, while green algae entertain the MEP biosynthetic pathway only [23]. IPP and DMAPP are the universal precursors of terpenoids, which are regulated by the MEP/MVA biosynthetic pathway. The building blocks of particular terpenoids are the GPP, FPP, and GGPP, which are formed by the condensation of IPP and DMAPP. The heterologous terpenoid synthesis usually involves the whole biosynthetic pathways or specific enzyme expressions, which link to endogenous terpenoid metabolism and change the substrate of prenyl phosphate into the required product [24]. In order to obtain higher yields of the desirable product, it is essential to modify the upstream terpenoid biosynthetic pathways (MEP) through genetic and metabolic engineering [6]. In microalgae, to increase sustainable terpenoid production, metabolic engineering is required in the MEP pathway in order to improve the carbon flow toward the synthesis of terpenoids [25]. In the current study, we have investigated MYB transcriptional factors as a prospective elicitor of the MEP/MVA biosynthetic pathway in the plants. R2R3-MYB transcriptional factors are the largest family in plants and play an important role in the regulation of secondary metabolites and terpenoids. VvMYB5b transcriptional factors increased the accumulation of terpenoids, as previously described [26]. *SmMYB97* TFs belong to medicinal plants (*S. miltiorrhiza*) and increase the contents of the terpenoids (tanshinones) in their overexpression lines [27]. *SmMYB98* belong to the R2R3-MYB subgroup 20, which are involved in the biosynthesis of phenolic acid and terpenoids. *SmMYB98* accumulate the terpenoids (tanshinones) in overexpressed lines [28]. In light of the above evidence, MYB transcriptional factors play an important role in the synthesis of terpenoids in higher plants. In microalgae, a few studies have been reported on the regulation of MYB transcriptional factors in terpenoid synthesis. For example, *MYB44* positively regulate the carotenoid biosynthesis in *U. prolifera*, which is known as marine green algae [29]. However, the role of MYB transcriptional factors in the regulation of terpenoids in *C. reinhardtii* is not yet functionally characterized.

In *C. reinhardtii*, the biosynthesis of squalene is excepted through the MEP pathway [30]. In the initial stage, squalene is synthesized by squalene synthase (SQS) from farnesyl diphosphate (FPP). A two-step reaction produces squalene by reductive dimerization of FPP with SQS. This reaction continues through head-to-head coupling of two FPP molecules to produce squalene through a stable cyclopropylcarbinyl diphosphate intermediate [31].

In the current study, by using advanced synthetic biotechnology approaches, heterologous overexpression of MYB TFs was achieved in *C. reinhardtii*, which might play an important role in the production of squalene (Figure 1). To optimize MYB TFs' expression in *C. reinhardtii*, it was completely redesigned. The vectors were constructed with the goal of expressing and accumulating the targeted protein in *C. reinhardtii*'s nucleus.

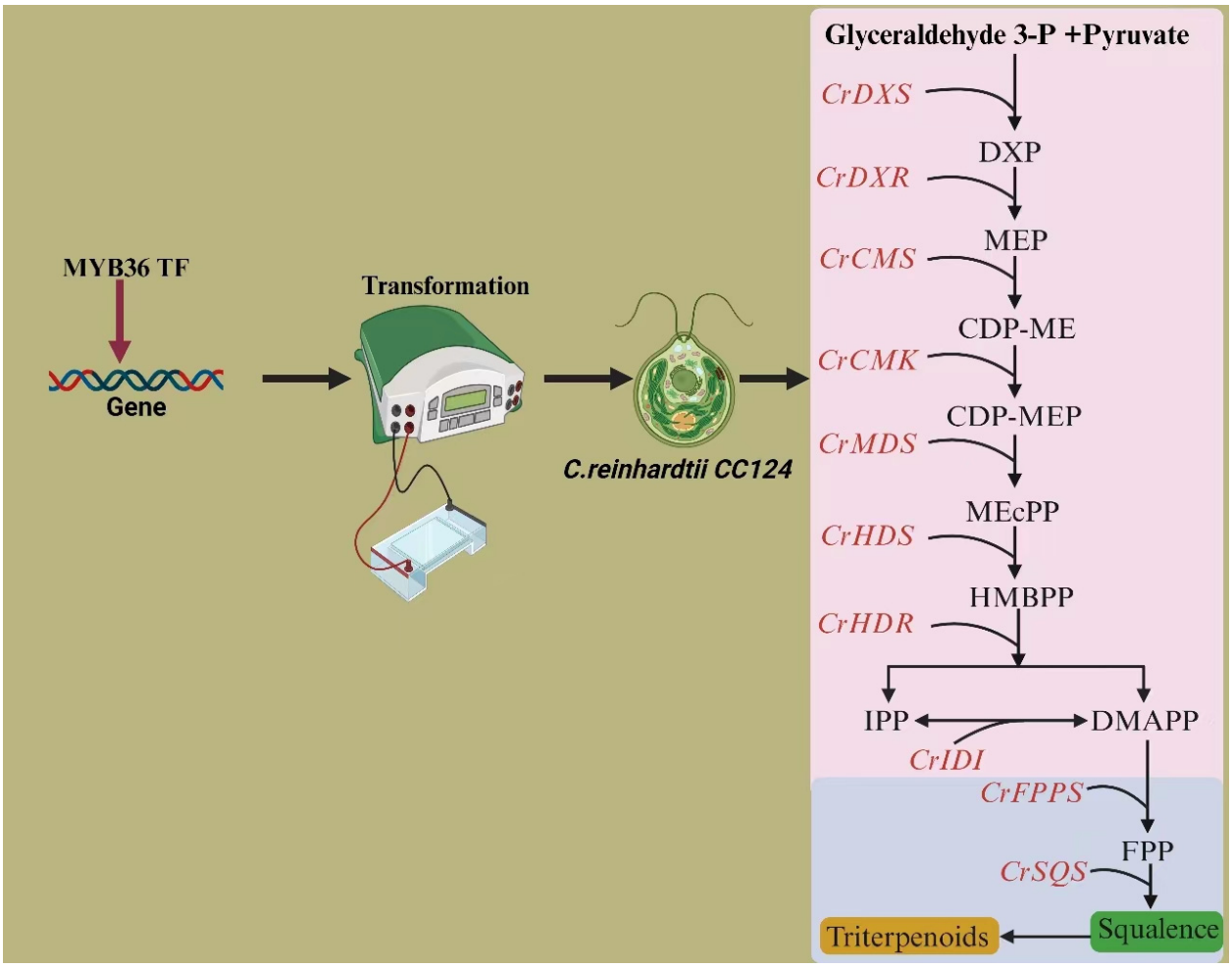

Figure 1. Proposed model for heterologous overexpression of MYB transcriptional factors in *C. reinhardtii*. Role of MYB TFs affect the MEP biosynthetic pathway, indicated in pink box, and the downstream biosynthesis of triterpenoids are indicated by sky blue box.

2. Materials and Methods

2.1. Strain of C. reinhardtii and Culture Condition

Chlamydomonas reinhardtii (CC-124) was obtained from Guangdong Technology Research Center for Marine Algal Bioengineering, Shenzhen University, and was cultivated in media (Tris-Acetate-Phosphate (TAP)) under a photoperiod of 16/8 h light and dark cycle with the photon fluence rate of 50 µmol m^{-2} s^{-1} in a growth chamber at 25 °C. The maintenance of transgenic *C. reinhardtii* cells were carried out in the TAP media supplemented with respective antibiotics. Transgenic *C. reinhardtii* cells were chosen on the agar plate (TAP) supplemented with zeocin (8 µg/mL) (Invitrogen, Carlsbad, CA, USA). The transgenic lines and wild type cells of *C. reinhardtii* were cultured to 1 × 10^6 cell/mL cell density. In order to harvest the cells of transgenic and wild type of *C. reinhardtii* CC124, centrifugation at 6000× *g* for 5 min was carried out.

2.2. The Construction of Plasmid and Expression Screening

The coding sequence of SmMYB36 was optimized according to the codon bias of *C. reinhardtii* in order to achieve high transformation efficiency and the expression of foreign genes. The synthesis and redesign of nucleotides for its optimization were carried out by the company GenScript Biotech Corp. (Nanjing, China). The optimized nucleotide sequence was introduced into the expression vector in order to acquire the RPL23-SmMYB98-RPL23-ble plasmid (Figure 2). This vector contains the RPL23 promoter to induce the expression of

the gene of interest. The designated vector was multiplied in *E. coli* on LB media, which was supplemented with ampicillin antibiotic (100 µg/mL). The optimized codon synthesized sequence of SmMYB36 contains *BamH1* and *EcoRI* restriction sites, respectively, and were cloned into the RPL23-SmMYB98-RPL23-ble vector, which carries bleomycin resistance. The confirmation of nucleotide sequence accuracy of each plasmid was carried out by sending the samples to the company. The plasmid RPL23-SmMYB98-RPL23-ble was constructed and was used to transform *Chlamydomonas* cells.

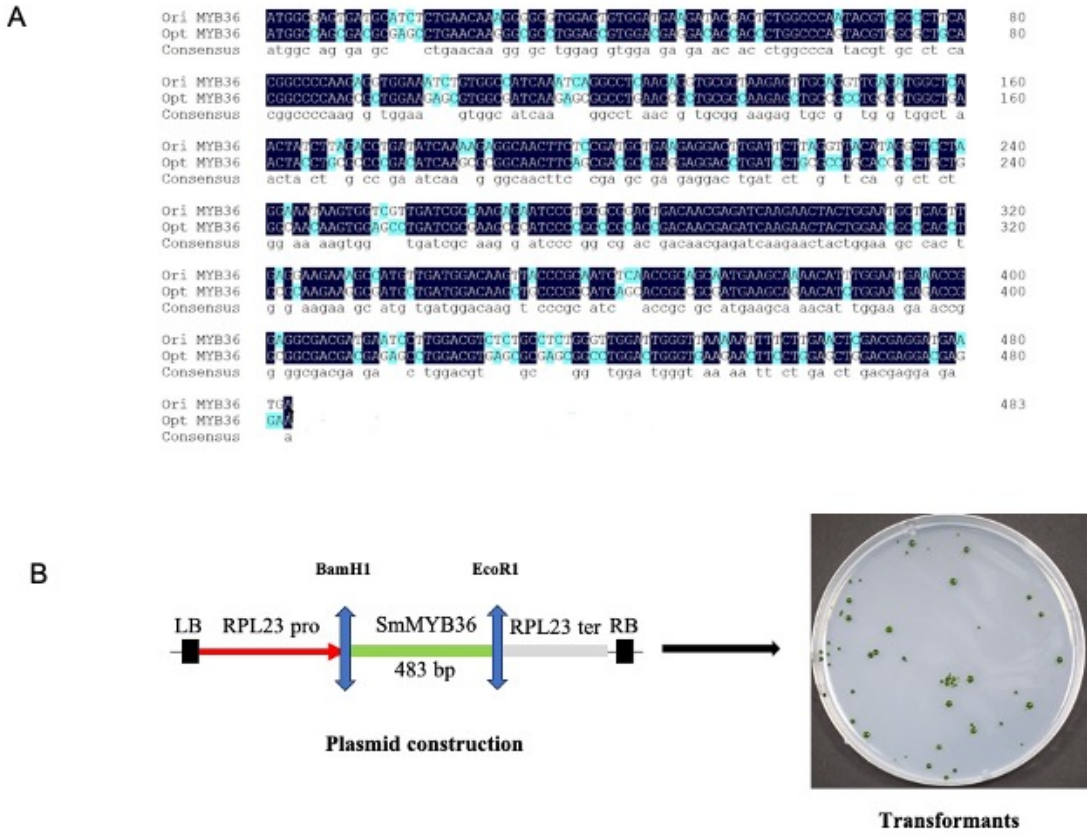

Figure 2. The alignment between the original codon and codon-optimized MYB36 gene. Ori MYB36 denotes the original codon of MYB36 gene, while Opt MYB36 denotes the optimized MYB36 gene (**A**). Plasmid construction and screening of transformation (**B**).

2.3. Transformation and Screening

The wild type strain *C. reinhardtii* (CC124) was transformed following electroporation protocol. The cells of wild type *C. reinhardtii* CC124 strain were cultured in TAP liquid media at 25 °C under continuous light intensity (100 $\mu E \cdot m^{-2} \cdot s^{-1}$). The incubator shaker was adjusted at 110 rpm. Cells of *C. reinhardtii* (1~2 × 10^7 cell/mL) were collected in a 50 mL centrifuge tube. The centrifugation was carried out at 6000× *g* for 5 min to harvest the fresh cells. TAP liquid medium was used to adjust the density of cells and then carried out the transformation. Electroporation method was used for the transformation. After transformation, the cells were placed on TAP plates consisting of zeocin (15 µg/mL) to screen the positive colonies of transformed cells of *C. reinhardtii*.

2.4. Genomic DNA and RNA Extraction and cDNA Synthesis

In order to conduct genomic DNA and RNA extraction, 50 mL fresh cells of *C. reinhardtii* after cultivation were harvested by centrifugation at 5000× *g* for 5 min at 4 °C. Then the cells were frozen in liquid nitrogen and stored at −80 °C for further investigation. Genomic DNA was isolated from the cells of *C. reinhardtii* CC124 and *MYB36*-overexpression lines by following the instructions of Plant Genomic DNA extraction kit (Transgene, Shen-

zhen, China). The total RNA from *C. reinhardtii* CC124 and *MYB36*-overexpression lines were extracted by using the Plant RNA Extraction Kit (Trangene, China). Aliquots of 2 μg RNA were used to synthesize the first strand of cDNA by following the instructions of Prime ScriptTM RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The quantity of RNA was examined by using a nanodrop. The quality of RNA was checked using electrophoresis on a 1% agarose gel.

2.5. The Analysis of qPCR and Genomic PCR

The green colonies, which were obtained after transformation, were transferred to a fresh TAP media harboring zeocin (8 μg/mL) and 100 ampicillins (μg/mL), and cultured for 3–5 days. Genomic PCR was carried out to detect the transgenic cells of *C. reinhardtii*. We have selected 200 transformed cells of *C. reinhardtii* and confirmed by genomic PCR as previously described.

The genes' relative expressions were investigated by RT-qPCR in three technical replicates using SYBR Premix Ex Taq GC (Takara, Dalian, China). The cDNA from *C. reinhardtii* CC124 and MYB36-overexpression lines was diluted (1:20). A total of 20 μL volume per reaction was exploited with specific primer (Table 1). For an internal control, the β-actin gene was used. The relative expressions of genes were calculated as previously described [32]. The pairs of primers used for qRT-PCR in this study are listed in Table 1.

Table 1. List of primers used in this study.

Genes	Forward (5′-3′)	Reverse (5′-3′)
CrDXS	GACGGTGGCTATGCACTATG	GAAATCGAGGTGGAGCTGTG
CrDXR	CCATCTTCCAGGTGATGCAG	CCAGACCCTTGTTCATGAGTG
CrCMS	GCACTACTGCTCCTACCAAG	GAACGTCTCCAGCGAGTATG
CrCMK	ACGCTGCAGACCATGTACTA	GTTGGAGAAGAAGACCGAGATG
CrMECPS	CTCTGTGCCTCCCAGACATC	GTTGCGGATGTTCTCCTTGTG
CHDS	GCTGATTGAGGAGACCTTAC	GCAGAAGACGAAGTTGTGGTAG
CrHDR	CTGACTGACTTCAAGGAGAAGG	GATGTAGTTGGAGGCGAAGG
CrIPPI	TCCTCCTTCTCCTTCCTCAC	GCCATCATGGACTGAAGCTC
CrIPP2	TTCCGCAACAAGGGATTCAG	GAACCAGACCGTGTAGTCCT
CrGPS	GTGCTGTCGCTCAATACCAG	AGTAGGTCTTGGCCAGGTAG
CrFPS	CCGAGGATGAGGTGTTCAAG	CGCTTGAGGATGGAGTAGATG
CrGGPS	CCATGGACAACGACGACTTC	TTGCCCAGCTCCATGATTAC
CrSQS	TGGCAGCATGCTACAACAAC	GAACTGCAGGAACCAGGTGT

2.6. Measurements of Squalene Contents

For triterpenoid analysis, fresh cells from the *C. reinhardtii CC124* strain and MYB36-overexpression lines were harvested. The fresh cells of *C. reinhardtii CC124* strain and MYB36-overexpression lines were then cultured (1×10^7/mL) in 50 mL TAP media for 2 days and transferred to a 1 L conical flask containing 400 mL TAP media for optimal growth. The cells of *C. reinhardtii CC124* strain and MYB36-overexpression lines were harvested followed by centrifugation at 8000 rmp for 5 min at 4 °C. The collected cells were rinsed once with TAP medium and sterilized distilled water, snap frozen, and stored at −80 °C. The collected cells of each treatment were freeze-dried, and the dry weights were recorded. The extraction and analysis of squalene were performed by GCMS. Squalene was (Sigma-Aldrich, St. Louis, MO, USA) used as an internal control. The squalene samples were analyzed on Thermo Fisher TRACE GC1300 MS ISQ LT equipped with Thermo Fisher TG-5ms 30 × 0.25 mm × 0.25 μm column (Thermo Fisher Scientific, Waltham, MA, USA). Helium was used as a carrier gas with a flow rate of 0.8 mL/min. Injection volume was

2 µL with 300 °C and split ratio was 40:1. Mass spectrometry was performed in an electron impact approach at an ionization voltage of 70 eV. The ion source temperature was 230 °C. The squalene was identified in samples by its relevant retention time.

2.7. Statistical Analysis

Three biological replications of each treatment were carried out for this study. The results are indicated as standard deviation (SD). The difference of significance was analyzed by using one-way ANOVA. The statistical significance was represented by $p < 0.05$ or $p < 0.01$.

3. Results

3.1. The Design Expression Cassette of MYB36

The full-length CDS sequence of MYB36 (KF059390.1) was acquired from the National Center for Biotechnology Information (NCBI) website. MYB36 transcriptional factors for heterologous overexpression in *C. reinhardtii CC124* were synthetically redesigned by codon optimization to improve transgene expression, as previously described [33]. After codon optimization, the MYB gene's GC contents increased from 48% to 65% (Figure 2). The MYB36 gene was then inserted into the vector in order to obtain the RPL23-SmMYB-RPL23-ble plasmid (Figure 2).

3.2. The Screening of Transgenic Strain of C. reinhardtii

The RPL23-SmMYB98-RPL23-ble plasmid was transformed into the cells of *C. reinhardtii* using the electroporation method. After 14 days of recovery, we have observed visible green colonies of transformed cells of *C. reinhardtii* and we then transferred them to fresh TAP media containing 100 µg/mL ampicillin and 8 µg/mL zeocin. We have obtained more than 200 colonies and a frequency of transformation (3×10^{-8}) was noticed. After that, in order to screen positive transformants, genomic PCR targeting the MYB36 gene was carried out. According to PCR results, no target bands were obtained in non-transgenic cells of *C. reinhardtii* (Figure S1).

3.3. MYB36 Transcriptional Factors Upregulate the MEP Biosynthetic Pathway in Overexpression Lines of C. reinhardtii

As MYB transcriptional factors have a critical role in the production of terpenoids in plants, we hypothesized that our candidate MYB36 gene stimulates the biosynthesis of terpenoid precursors in the cells of *C. reinhardtii*. The general building blocks of entire terpenoids are isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). In *C. reinhardtii*, these precursors are uniquely synthesized by the MEP pathway, while the MVA biosynthetic pathway is absent in *C. reinhardtii* [34]. So, transcript levels of key structural genes involved in the MEP pathway were assessed in MYB36-overexpression lines by qPCR analysis. The transcript level of gene encoding enzymes *CrDXS*, *CrDXR*, *CrGPS*, *CrGPPS*, *CrFPS*, *CrFPPS*, *CrIPP2*, *CrIPP1*, *CrIDS*, *CrCMS*, and *CrCMK* were investigated by qPCR (Figure 3). The outcomes from this study indicated that the expression levels of the above-mentioned genes were higher than the wild type of *C. reinhardtii* CC124. *DXS* and *DXR* play a key function in the regulation of flux in the MEP pathway of land plants [35]. Overexpression of *DXS* accumulates in the isoprenoid production in transgenic Arabidopsis, suggesting that DXS catalyzes one of the rate-limiting steps involved in the biosynthetic pathway of MEP [36]. *DXR* and *HDR* are other enzymes that play a function as rate-limiting factors in the formation of IPP and DMAPP [37]. Recently, investigations on DXS have attained more attention in order to modify terpenoid metabolisms. Methyl jasmonate (MeJ)-treated *C. reinhardtii* cells increase in the expression of *DXS* leading to an increase in the triterpenoid squalene synthesis [38]. Heterologous overexpression of *Salvia pomifera DXS* in *C. reinhardtii* improved flux toward diterpenoid production via the MEP biosynthetic pathway [24]. MEP plays a crucial function in the regulation of the MEP biosynthetic pathway. The MEP is the initial obligated precursor of the pathway

and triggers the MDS gene transcript level [39]. The change in metabolism regarding the IPP production implies a promising accumulation of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) in the MYB36-overexpression lines. The expression levels of FPP and GGPP were remarkably higher in the MYB36-overexpression *C. reinhardtii* lines compared to parent lines. Our results were similar to the previous study, which indicated that the accumulation of GGPP expression in MeJ-treated cells of *C. reinhardtii* improved the terpenoid production [38]. The incidence of a metabolic change toward the production of triterpenoids, such as squalene, is also supported by the accumulation of FPP.

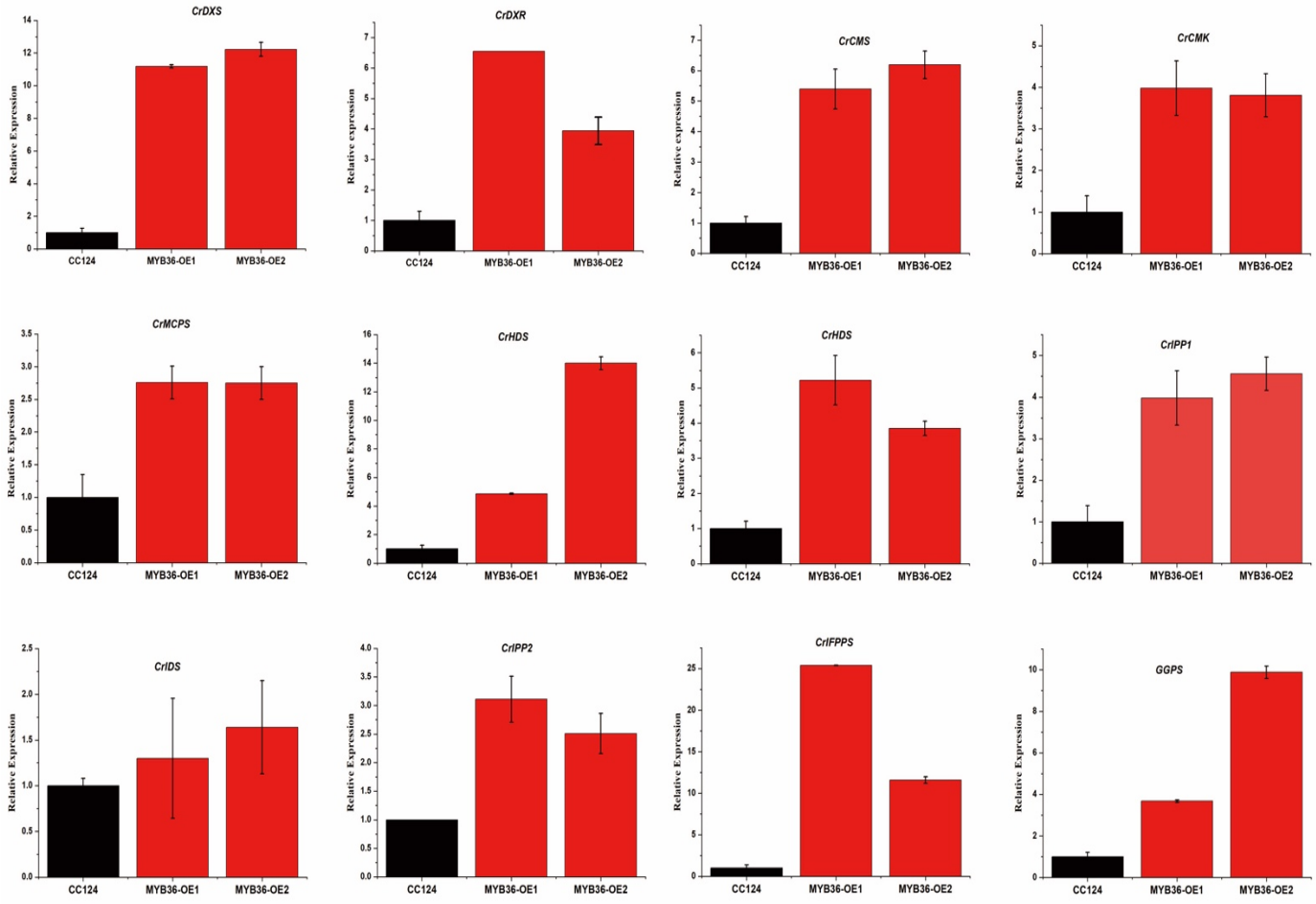

Figure 3. Heterologous overexpression of MYB36 TF gene in *C. reinhardtii*. MYB36 TFs affect the MEP biosynthetic pathway encoding genes. CC124 is the wild type of *C. reinhardtii* and MY36-OE are the transgenic lines of *C. reinhardtii*. The error bars stand for the SE of three biological replicates.

3.4. MYB 36 Affect the Endogenous Squalene Biosynthetic Pathway Genes

To check the functional characterization of the *MYB36* transcriptional factor in the squalene biosynthesis, the *R2R3-MYB36* gene was overexpressed in the cells of *C. reinhardtii*. A plasmid, which is used for the overexpression of the *R2R3-MYB36* gene, consisting of the coding region of the *R2R3-MYB36* gene under the control of the constitutive *RPL23* promoter in *C. reinhardtii* CC124 strain, was appropriate for the effective and stable expression of foreign genes. qRT-PCR was carried out to check the total expression levels of endogenous *CrSQS*. Expression levels of *CrSQS* were higher in the *MYB36*-overexpression lines compared to the wild type *C. reinhardtii* CC124 strain (Figure 4A).

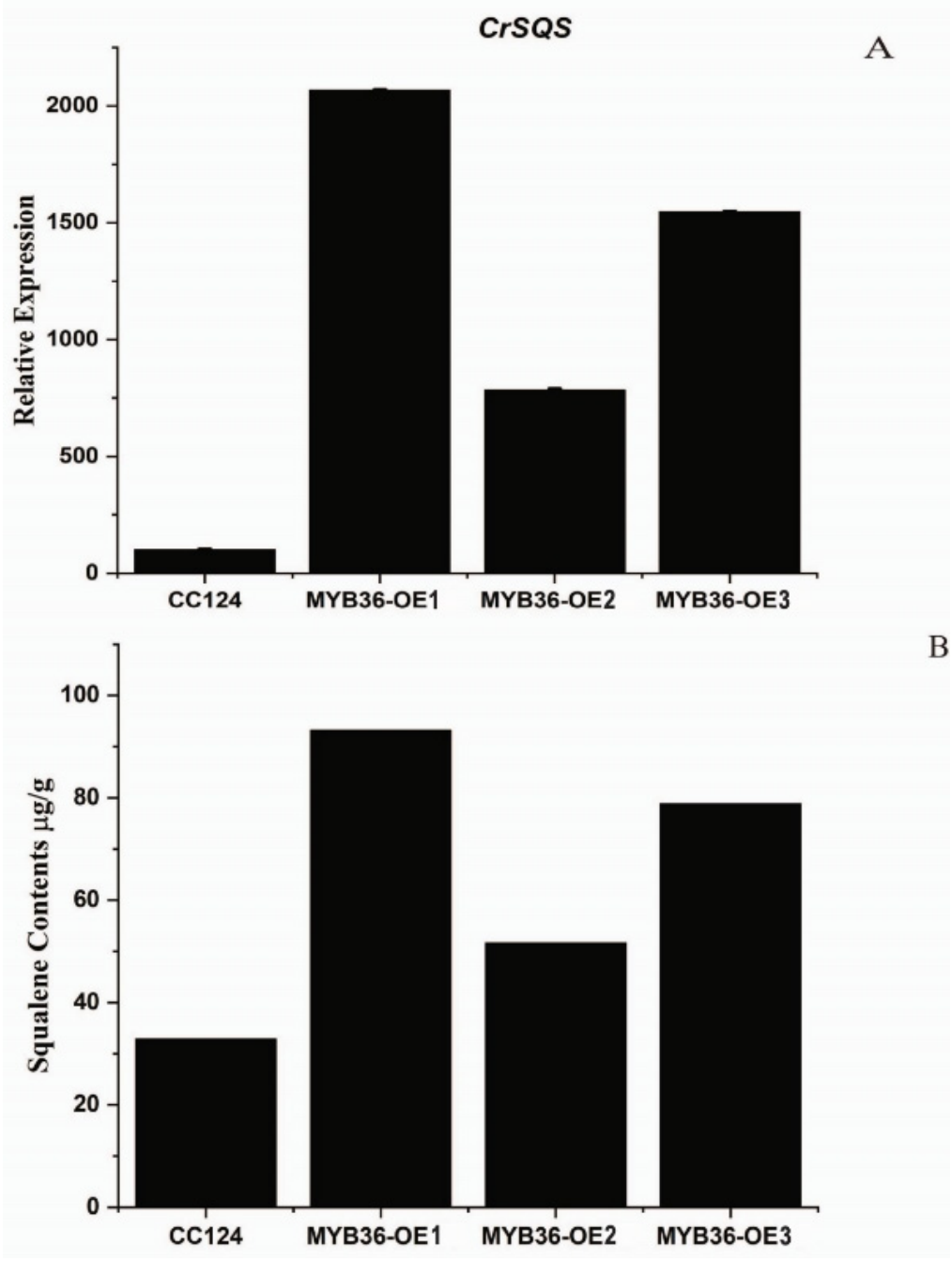

Figure 4. Overexpression of MYB36 TF gene in *C. reinhardtii* squalene biosynthesis. CC124 is the wild type of *C. reinhardtii* and MY36-OE32, 35, and 36 are the transgenic lines of *C. reinhardtii*. The error bars stand for the SE of three biological replicates. (**A**) The expression level of CrSQS. (**B**) The contents of squalene μg/g.

3.5. Accumulation of Squalene on MYB-Overexpression Lines

The membranes of *C. reinhardtii* consist of sterols. They are produced from squalene by a pathway similar to higher plants [40]. Squalene (C30 precursor of triterpenoids) is the product of the condensation of two FPP molecules. The determination of squalene was determined by the GC-MS method from the harvested cells of *MYB36*-overexpressed lines of *C. reinhardtii* and wild type strain *C. reinhardtii* CC124 (Figures 4 and S1). The peak with a retention time of 15.61 is the squalene (Figure 5). The squalene contents determined in the MYB36-overexpreesed lines (OE-1, OE-2, and OE-3) were 93.20, 52, and 80 μg/g, respectively. The squalene contents were higher in MYB36-overexpreesed lines

compared to wild type strain *C. reinhardtii* CC124 (Figure 4B). The squalene's increasing contents were correlated with the improved accumulation of the CrSQS encoding gene. The accumulation of SQS transcript levels led to the increase in the squalene contents, as previously described [41]. The entire sterol biosynthetic pathway upregulation could indicate higher levels of the intermediate upstream of squalene.

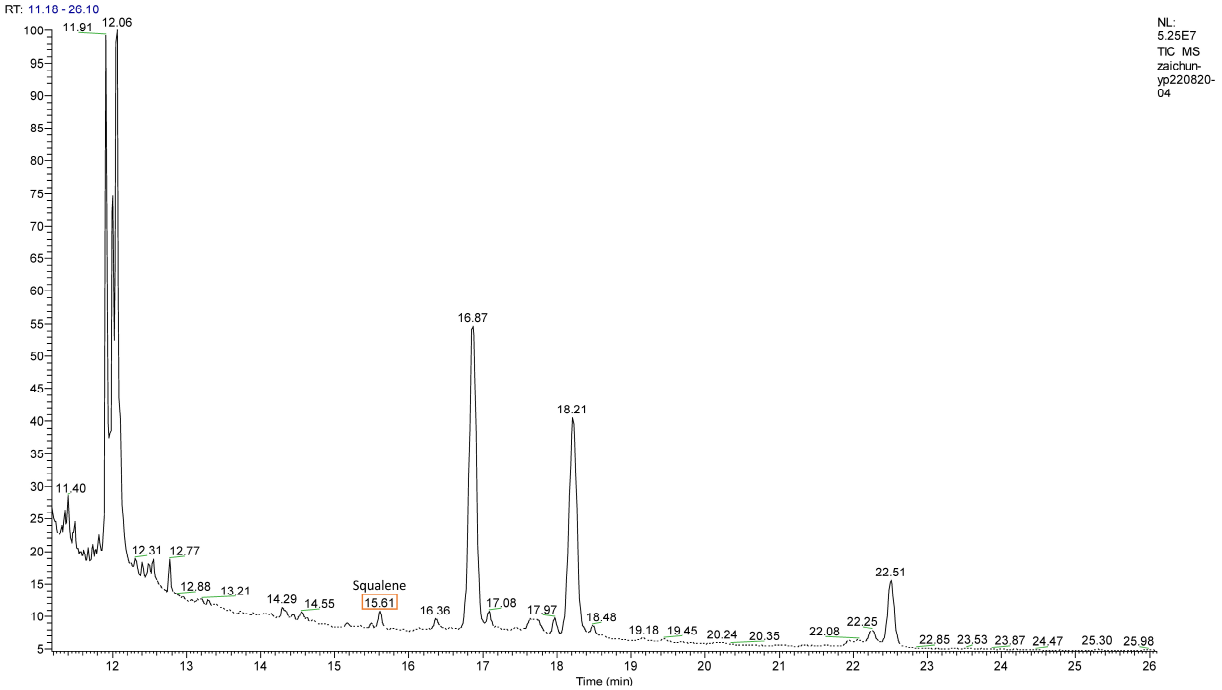

Figure 5. Concentration of squalene in MYB36-overexpression lines. The peak with 15.61 retention time is the squalene.

4. Discussion

The MYB TFs are a large transcriptional family found in plants and play an important role in the regulation of plant secondary metabolites and other functions [42,43]. In previous studies, some MYB TFs have been described, which play key roles in the regulation of terpenoid biosynthesis in plants [44,45] and green microalga [29,46]. In *Tripterygium wilfordii*, *BpMYB21* TFs are involved in the regulation of squalene through the upregulation of squalene epoxidase a and Cycloartenol synthesis [47]. *CiMYB42* TFs from citrus regulate the triterpenoids and transcript level of *SQS* involved in squalene synthesis [48]. By comparison, evidence on the transcriptional regulation of triterpenoids synthesis by MYB TFs in *C. reinhardtii* is extremely limited.

In the current study, we have investigated the role of heterologous overexpression of *MYB36* in the regulation of triterpenoids in *C. reinhardtii* and its effect on the upstream and downstream of the MEP pathway and its derivative biosynthetic pathway. We have obtained the *R2R3 MYB36* (KF059390.1) TFs from the National Center for Biotechnology Information (NCBI) website. R2R3 MYB TFs belong to the medicinal plant *Salvia miltiorrhiza*. *S. miltiorrhiza* contains various biologically active compounds, which have great potential to treat different diseases, including cerebrovascular diseases and others [49]. MYB36 showed an imperative role in the regulation of terpenoids, especially tanshinones and phenolic acid [49].

In order to improve transgene expression, *MYB36* transcriptional factors were re-designed synthetically by codon optimization for heterologous expression in *C. reinhardtii*, as previously described [33]. After codon optimization, the MYB gene's GC contents increased from 48% to 65%. In order to analyze the foreign transcriptional factor, R2R3-MYB36 overexpressed in *C. reinhardtii*. We have found that the genes encoding for the

upstream MEP biosynthetic pathway, including *CrDXS*, *CrDXR*, *CrCMS*, *CrCMK*, *Cr. HDS*, and *Cr. HDR* genes, were upregulated in the MYB36-overexpression lines. Previously similar studies showed that *SmMYB97* TFs belong to the medicinal plant *S. miltiorrhiza* and affect the MEP biosynthetic encoding genes in its overexpression lines [27]. SmMYB98 TFs also upregulate upstream MEP biosynthetic encoding genes in transgenic lines [28]. In higher plants, *DXS* and *DXR* have a significant task in the regulation of flux in the MEP pathway [35]. Overexpression of *DXS* accumulated the isoprenoid production in transgenic Arabidopsis, suggesting that catalyzing of DXS is one of the rate-limiting steps involved in the MEP biosynthetic pathway [36]. *DXR* and *HDR* are other enzymes that play a function as rate-limiting factors in the formation of IPP and DMAPP [37]. Recently, investigations on DXS have attained more attention in order to modify terpenoid metabolisms. Methyl jasmonate (MeJ)-treated *C. reinhardtii* cells increase the expression of *DXS* leading to increase the triterpenoids' squalene synthesis [38]. Heterologous overexpression of *DXS* from *S. pomifera* in *C. reinhardtii* improved flux toward diterpenoid production via the MEP biosynthetic pathway [24]. MEP plays a crucial function in the control of the MEP biosynthetic pathway. MEP triggers the MDS gene expression, which is the initial obligated precursor of the pathway [39]. MYB36-overexpression lines may accumulate geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) as a result of an altered metabolism toward IPP. The expression levels of FPP and GGPP were significantly higher in the MYB36-overexpression *C. reinhardtii* lines compared to parent lines. Our results were similar with a previous study, which indicated that accumulation of GGPP expression in MeJ-treated cells of *C. reinhardtii* improved the terpenoid production [38].

The FPP (C15) is formed by the condensation of IPP and DMAPP. FPP (C15) further transformed into the squalene (C30 triterpenoid precursor), which was catalyzed by squalene synthase (SQS). The first precursor of triterpenoids is squalene. SQS is positioned at a key branch point and entertains as a switch. Therefore, SQS has a potential regulatory role in the synthesis of triterpenoids [50]. In our study, the squalene contents in the MYB36-overexpreesed lines (OE-32, OE-35, and OE-36) were 93.20, 52, and 80 μg/g, respectively, and were higher in MYB36-overexpreesed lines compared to wild type strain *C. reinhardtii* CC124. The squalene's increasing contents was correlated with the improved accumulation of the *CrSQS* encoding gene. The accumulation of *CrSQS* transcript level leads to an increase in the squalene contents, as previously described [41]. The previous study showed that *CiMYB42* regulates the *CiSQS* gene [50].

5. Conclusions

Most commercially available terpenoids are obtained from higher plants, but their cost-effective and sustainable production for industrial purposes is still challenging because of their low abundance production from natural sources, extraction and purification obstacles, and degree of labor intensity. In this study, we have redesigned and codon-optimized the MYB36 TFs by using the advanced synthetic biotechnology approaches and heterologous overexpressed in *C. reinhardtii* CC124 in order to analyze its potential role in triterpenoid biosynthesis for the first time. We have found that *MYB36* TFs positively affect the MEP biosynthetic pathway encoding genes in MYB36-overexpressed lines compared to the wild type of *C. reinhardtii* strain CC124. The first precursor of triterpenoids is squalene. Our results showed that the transcript level of *CrSQS* involved in squalene synthesis increased, and ultimately led to an increase in the contents of squalene in MYB36-overexpressed lines of *C. reinhardtii.* So, *MYB36* TFs also trigger the accumulation of the downstream pathway of triterpenoid biosynthesis genes. Our study provides new insights into the heterologous overexpression system of transcriptional factors in microalgae to modified and metabolic engineering in triterpenoid biosynthesis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12030487/s1, Figure S1. Concentration of Squalene in WT type (CC124) and MYB36 Overexpression lines (MYB36-OE1 and OE2).

Author Contributions: Conceptualization, Z.H.; Methodology, M.A.; Software, M.A.; Validation, Z.H.; Formal analysis, M.A., J.W., J.L. and M.M.A.; Investigation, J.L.; Data curation, M.A.; Writing—original draft, M.A. and Z.H.; Writing—review & editing, M.A.; Project administration, Z.H.; Funding acquisition, Z.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Chinese National Key R&D Project for Synthetic Biology (2018FA0902500). National Science Foundation of China (32273118), The Guangdong Key R&D Project (2022B11111070005). Shenzhen Special Fund for Sustainable Development (KCXFZ20211102164013021) and Shenzhen University 2035 Program for Excellent Research (2022B010) to Z.H.

Data Availability Statement: Data will be available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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