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Promoter and N-Terminus Engineering of the *Chlamydomonas* Chloroplast Protein for Taxadiene Production

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Abstract

Taxadiene, a diterpene hydrocarbon, is the first committed intermediate in the biosynthesis of paclitaxel, a crucial anticancer drug. Its efficient production remains a key bottleneck for heterologous paclitaxel production. Although chloroplasts provide abundant terpenoid precursors, heterologous enzymes expressed in this compartment are rapidly degraded. Here we show that promoter engineering combined with N-terminal modification enables biosynthesis of taxadiene in the chloroplast of *Chlamydomonas reinhardtii* and accumulation in the dodecane overlay of a biphasic culture. Expressing TS under the *psaA* promoter rather than *atpA* increased taxadiene production 5.6-fold (1.9 to 10.7 μg/L). Adding methionine to the N-terminus of taxadiene synthase (TS) raised the titer to 26.7 ± 1.3 μg/L, a 14-fold increase over the *atpA* strain; the modified strain retained about 30% of TS at day 6, whereas the unmodified strains retained less than 10%, indicating that the modification slowed protein turnover. Optimizing light, acetate, temperature, inoculation density and methyl jasmonate supplementation increased the titer a further 6.9-fold to 185.5 ± 4.4 μg/L, about 98-fold above the initial *atpA* strain. N-terminal methionine engineering therefore provides a simple strategy to stabilize chloroplast-expressed enzymes.

Keywords: taxadiene; taxadiene synthase; chloroplast expression; N-terminal modification; *Chlamydomonas reinhardtii*

1. Introduction

Paclitaxel, a first-line chemotherapeutic for solid tumors including breast and ovarian cancer, remains prohibitively expensive, owing to its limited natural sources, extremely low abundance in plants, and laborious purification [1]. Total chemical synthesis of paclitaxel remains a challenge [2]; it requires more than 40 steps, relies on toxic reagents, proceeds in low overall yield and involves complex stereochemistry, and is therefore not industrially viable. Current production still relies on semi-synthesis from the naturally derived precursors baccatin III and 10-deacetylbaccatin III, with improved Taxus cell cultures remaining an important route to secure supply [3]. As a consequence, engineered biosynthesis of paclitaxel and its intermediates has attracted growing interest. Substantial advances regarding model microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* have already delivered heterologous production of the early taxane precursors taxadiene and taxadiene-5α-ol [4,5].

Paclitaxel biosynthesis originates from geranylgeranyl pyrophosphate (GGPP), which is built from the universal five-carbon (C5) precursors isopentenyl pyrophosphate (IPP)

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and dimethylallyl pyrophosphate (DMAPP). These C5 units are supplied by two pathways that differ in compartmentalization and phylogenetic distribution: the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, prevalent in plants, algae, and most bacteria, and the cytosolic mevalonate (MVA) pathway, found mainly in animals, fungi, and archaea [6]. Taxadiene, the first dedicated intermediate of the paclitaxel pathway, has been produced in model microorganisms—including *E. coli*, *S. cerevisiae*, and *Bacillus subtilis*—by overexpression of taxadiene synthase (TS), with titers up to 1 g/L reported in *E. coli* [4] and 390 mg/L in *Bacillus subtilis* [7]. Plants are also increasingly recognized as heterologous hosts, exploiting photoautotrophic growth, photosynthetic oxygen supply, and the capacity to support the membrane-bound cytochrome P450 enzymes required downstream; after transformation with TS, species such as *Arabidopsis thaliana* [8], *N. benthamiana* [9], *Physcomitrella* [10], and *Panax ginseng* [11] accumulate taxadiene at roughly 0.6–160 µg/g. Encouragingly, the pathway downstream of taxadiene has recently been reconstructed up to baccatin III [12] and paclitaxel in plant and yeast hosts [13,14]. Nevertheless, these reconstructions depend on the scarce plant precursor baccatin III and remain confined to low-yield, oxygenated intermediates, so the efficient and low-cost production of the earlier taxane scaffold—above all taxadiene—remains the key bottleneck for heterologous paclitaxel biosynthesis. Developing a high-yield, easily scalable chassis for taxadiene is therefore both a pressing goal and a rational entry point for the whole paclitaxel pathway.

To overcome this bottleneck, the green microalga *Chlamydomonas reinhardtii* has emerged as a highly promising chassis for taxadiene biosynthesis, primarily owing to its abundant supply of terpenoid precursors, a well-established genetic toolbox, and the favorable prokaryotic-like organization of its chloroplast genome [15]. As a photoautotroph, it harbors a single cup-shaped chloroplast occupying [16] ~40% of the cell volume [17], can also grow rapidly on exogenous organic carbon, and provides an abundant supply of terpenoid precursors. Its genetic toolbox is well developed: the nuclear, chloroplast, and mitochondrial genomes can all be engineered, and it has already been used to synthesize a range of terpenoids, bisabolene [18], limonene [19], 13R(+) manoyl oxide [20], and patchedoulol [21]. Moreover, the chloroplast genome exhibits prokaryotic polycistronic organization and limited post-translational modifications—features that favor the simultaneous, high-level expression of multiple heterologous genes. These properties suggest that the *C. reinhardtii* chloroplast may be a particularly suitable compartment for taxadiene synthesis.

In this study, we combined chloroplast transformation with promoter engineering and N-terminal modification to construct taxadiene-producing chloroplast-engineered strains of *C. reinhardtii*. We delivered the TS gene into the chloroplast genome, improved TS transcript/protein abundance and stability through promoter and N-terminal optimization, and then systematically tuned culture conditions—acetate concentration, illumination regime, and temperature—to maximize taxadiene accumulation. Our results confirm the feasibility of chloroplast-based taxadiene production in *C. reinhardtii* and provide a practical reference for the design and chloroplast-level production of other high-value terpenes.

2. Results

2.1. Construction of the Chloroplast-Engineered Strains

In chloroplast engineering of *C. reinhardtii*, exogenous genes are usually integrated into the chloroplast genome by homologous recombination. To facilitate the selection of transformants, we used CC5168—which cannot grow photoautotrophically because of a deletion of the endogenous *psbH* gene by the spectinomycin resistance gene *aadA*—so that successful homologous replacement of the *psbH* locus by the vector-borne *psbH* cassette restores photosynthetic competence (illustrated in Figure 1A) [22]. A codon-optimized TS gene from *T. brevifolia* was inserted between the *atpA* promoter and the rbcL 3′ UTR

of vector pASapI to generate the expression vector pASap-TS. After transformation and screening, four engineered strains (A1–A4, *atpA*-TS transformants) were obtained, and the empty vector pASapI was transformed in parallel to generate the negative-control strain (C1, empty-vector transformant).

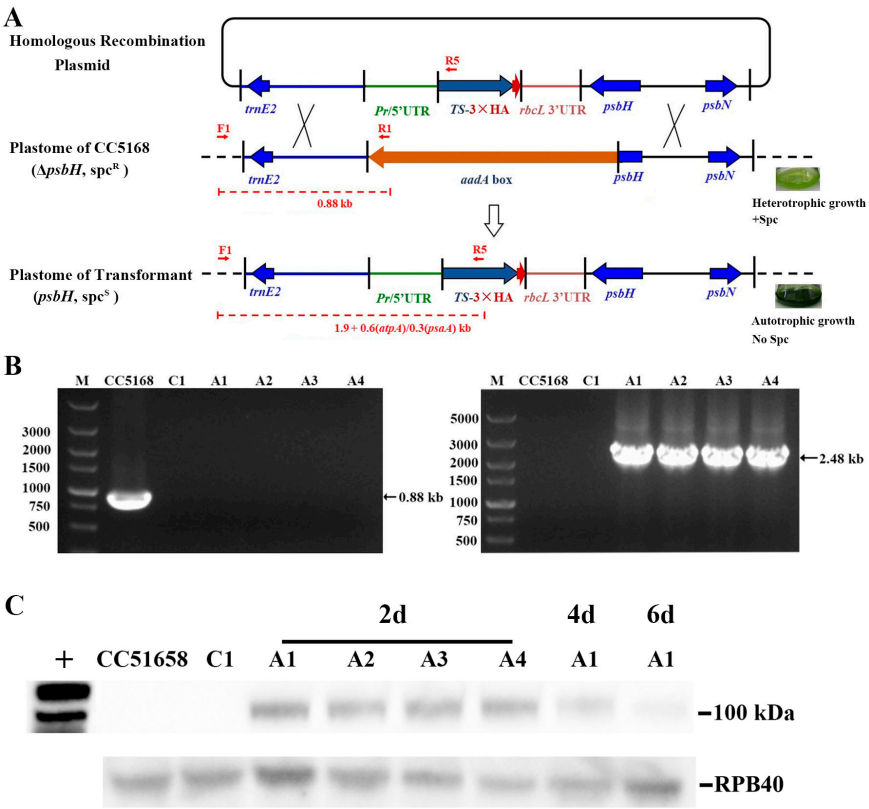


Figure 1. Construction and analysis of chloroplast-engineered *C. reinhardtii* strains transformed with the TS gene. (**A**) Schematic of strain construction by homologous recombination. In the parental strain CC5168, the *aadA* cassette replaces most of *psbH*, so CC5168 cannot grow photoautotrophically. The expression plasmid carries the TS cassette flanked by sequences homologous to the chloroplast genome and a complete *psbH* gene. Homologous recombination restores *psbH*, and the transformants regain photoautotrophic growth. Red arrows F1, R1 and R5 indicate the binding sites of primers. (**B**) Homogenization analysis of the *atpA* promoter strains. (**Left**), PCR with primers F1/R1; R1 anneals within the *aadA* cassette in strain CC5168. (**Right**), PCR with primers F1/R5; R5 anneals within TS. C1, the empty-vector transformant; A1–A4, transformants carrying the *atpA* promoter. (**C**) Western blot analysis of TS accumulation using an anti-HA antibody. For each strain, lanes correspond to samples harvested at 2, 4 and 6 days. CC5168, parental strain; C1, empty-vector transformant; A1–A4, transformants carrying the *atpA* promoter; +, positive control.

All strains (A1–A4 and C1) grew photoautotrophically on HSM medium [17], confirming restoration of photosynthetic competence, whereas none retained spectinomycin resistance. PCR analysis confirmed correct integration: the primer pair F1/R1 (outer region and *aadA* internal primer) amplified a product from the parental strain CC5168 but not from A1–A4 or C1, whereas the pair F1/R5 (outer region and an internal TS primer) amplified a product from A1–A4 but not from CC5168 or C1 (Figure 1B). These results demonstrate that the TS gene was integrated at the intended locus and that all engineered strains were fully homogenized. RT-PCR of total RNA from A1–A4 further confirmed that TS was transcribed normally in the chloroplasts (Supplementary Figure S1). To assess protein expression, total cellular protein from the engineered strains was analyzed by Western blotting. All four

strains (A1–A4) showed a clear band near 100 kDa. However, the expression levels of TS gradually declined after 4 and 6 days of culture in A1 (Figure 1C).

Having confirmed that TS is functionally expressed in the chloroplasts, we collected the dodecane phase from an 8-day culture of A1 and analyzed it for taxadiene production. The extract from A1 displayed an additional peak at a retention time of 34.2 min, matching that of the taxadiene standard (Supplementary Figure S2). Strain A1 produced taxadiene at a volumetric titer of 1.9 ± 0.1 μg/L and a cellular titer of 0.1 ± 0.005 fg/cell. Taxadiene was detected exclusively in the dodecane phase and not in the cell pellet, indicating that the product crosses both the chloroplast envelope and the plasma membrane and accumulates extracellularly. These results indicate that the chloroplast-expressed TS is biocatalytically active and can convert GGPP into taxadiene.

2.2. Promoter Optimization and N-Terminal Modification to Improve Taxadiene Production

Promoter strength strongly influences the expression and accumulation of heterologous proteins in the chloroplast. To improve taxadiene production, we replaced the *atpA* promoter with the stronger *psaA* promoter. The vector containing the TS expression cassette driven by a strong *psaA* promoter was transformed into a chloroplast to generate two engineering strains (P1 and P2, *psaA*-TS transformants). Both grew photoautotrophically and were fully homogenized (Figure 2A). Western blotting showed that P1 and P2 produced a TS band at 100 kDa at essentially equivalent levels. Consistent with the earlier results, TS accumulation in both strains still gradually declined at days 4 and 6 (Figure 2B). As expected, after replacement of the promoter, TS abundance in P1 was markedly higher than in A1 (Figure 3B). Accordingly, GC–MS analysis showed that strain P1 produced more taxadiene: after 8 days of culture, the volumetric titer reached 10.7 ± 0.4 μg/L and the cellular titer 0.6 ± 0.02 fg/cell, corresponding to 5.6- and 6.7-fold increases over strain A1, respectively.

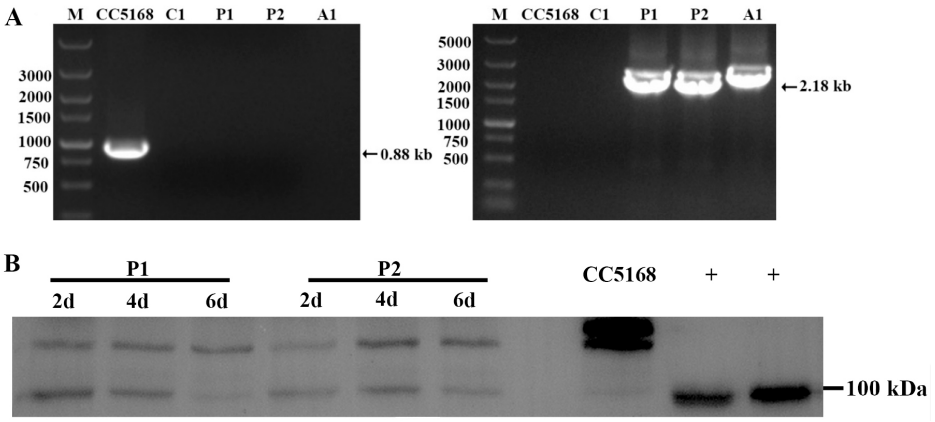


Figure 2. Homogenization and TS accumulation in the engineered strains expressing TS under the *psaA* promoter. CC5168, the parental strain; C1, the empty-vector transformant; A1, a strain transformed with TS under the *atpA* promoter; P1 and P2, strains transformed with TS under the *psaA* promoter. (**A**) Analysis of strain homogenization. (**Left**), PCR with primers F1/R1; R1 anneals within the *aadA* cassette, which is present in the genome of the parental strain CC5168. (**Right**), PCR with primers F1/R5; R5 anneals within TS. (**B**) Western blot analysis of TS accumulation in the algal strains using an anti-HA antibody. +, positive control. For each strain, the three lanes correspond to samples cultured for 2, 4, and 6 days.

N-terminal modification can inhibit protein degradation and thus increases protein accumulation. To this end, an additional methionine codon was introduced immediately before the start codon of TS in vector pSRSap-TS, generating vector pSRSap-TSM. Transformation of pSRSap-TSM into the chloroplast yielded four engineered strains (PM1–PM4, *psaA*-driven N-terminally modified TS transformants), all of which grew photoautotrophi-

cally and were fully homogenized (Figure 3A). To compare the effect of different optimization strategies on TS accumulation, strains A1, P1 and PM1 at 2, 4 and 6 days of culture were examined (Figure 3B). The TS abundance in PM1 was obviously higher than that in P1 and A1 throughout the culture period. At day 2, TS in PM1 was 4.4-fold that of P1 and 5.1-fold that of A1 (Supplementary Figure S3). As observed above, TS declined progressively with culture time in all strains, indicating continuous degradation of the protein in the chloroplast of *C. reinhardtii*. Notably, however, degradation was substantially slower in PM1 than in the other two algae. At day 6, PM1 still retained about 30% of the protein, whereas P1 and A1 retained less than 10%. These results demonstrate that introducing an additional methionine at the N-terminus of a chloroplast-expressed proteins in *C. reinhardtii* markedly inhibits protein degradation. Consistent with this increased accumulation, taxadiene production by PM1 was significantly higher, with a volumetric titer of 26.7 ± 1.3 μg/L, approximately 2.5-fold that of P1 and 14-fold that of A1, and a cellular titer of 2.2 ± 0.3 fg/cell, approximately 3.6-fold that of P1 and 22-fold that of A1.

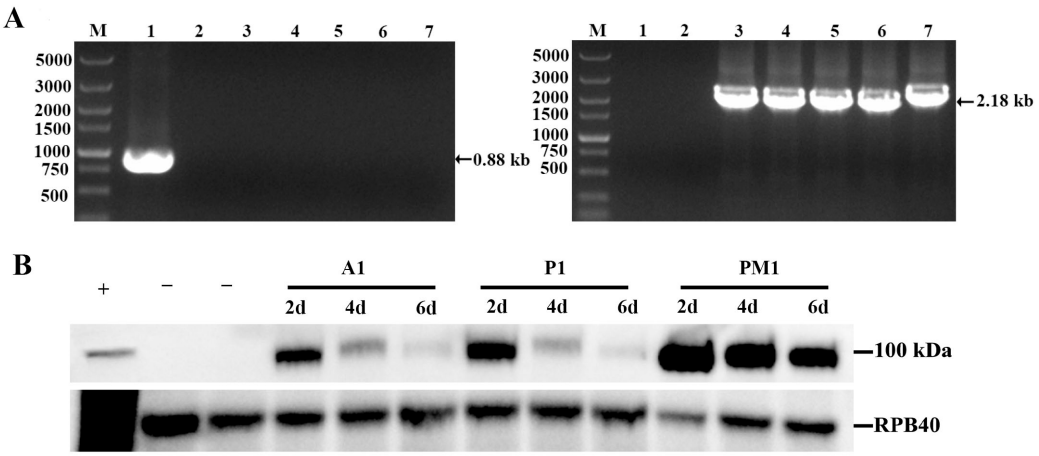


Figure 3. Homogenization and TS accumulation in the N-terminally modified engineered strains. (**A**) Analysis of strain homogenization. (**Left**), PCR with primers F1/R1; R1 anneals within the *aadA* cassette, which is present in the genome of the parental strain CC5168. (**Right**), PCR with primers F1/R5; R5 anneals within TS. Lane 1, CC5168; lane 2, the empty-vector transformant; lanes 3–6, the transformants PM1–PM4. (**B**) Western blot analysis of TS accumulation in the algal strains using an anti-HA antibody. +, positive control; −, negative control. A1, strain expressing TS under the *atpA* promoter; P1, strain expressing TS under the *psaA* promoter; PM1, strain expressing the N-terminally modified TS. For each strain, the three lanes correspond to samples cultured for 2, 4, and 6 days.

2.3. Optimization of Engineering Strains

Culture conditions strongly influence algal growth and product accumulation. We therefore examined the effects of light regime, acetate concentration, temperature, and initial cell density on taxadiene production by the engineered strains. We first compared continuous light with a light/dark cycle (Figure 4A). Under continuous light at 25 °C, the volumetric taxadiene titers of A1, P1 and PM1 were 1.9 ± 0.1 μg/L, 10.7 ± 0.4 μg/L and 26.7 ± 1.3 μg/L, respectively. While under the light/dark cycle, they decreased to 1.0 ± 0.2 μg/L, 7.9 ± 0.4 μg/L and 23.1 ± 1.1 μg/L in the same strains. This showed that continuous light significantly increased taxadiene production in all engineered algae compared to the light/dark cycle by 35.4% in P1 and a 15.6% in PM1 ($p < 0.01$).

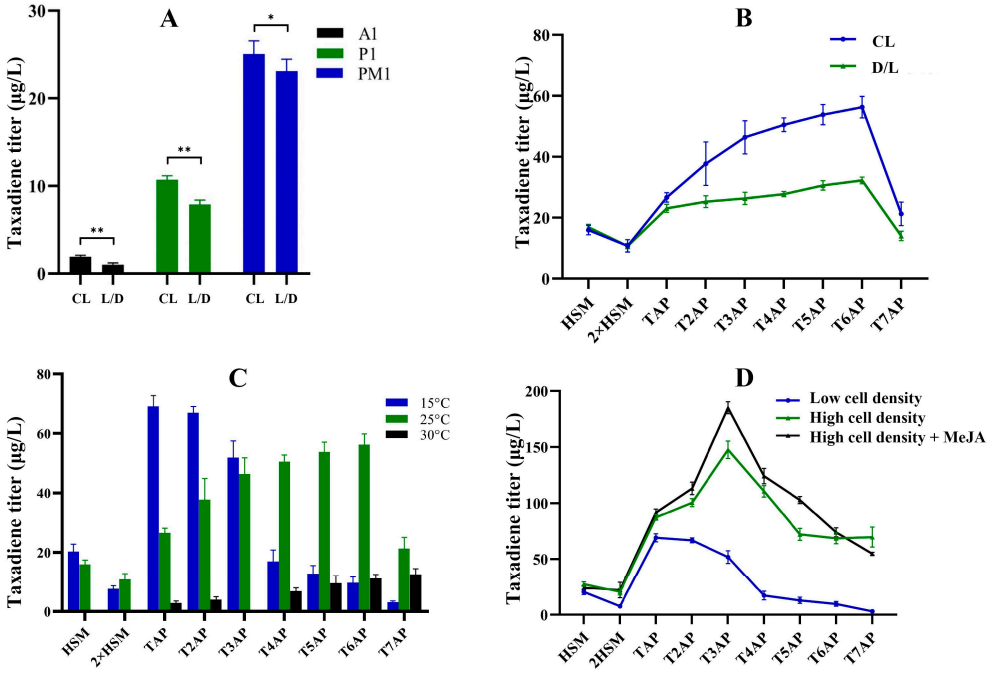


Figure 4. Optimization of culture conditions to improve taxadiene production in the chloroplast-engineered strains. (**A**) Effect of light regime on taxadiene production. (**B**) Effect of acetate concentration in the medium on taxadiene production in PM1. (**C**) Effect of culture temperature on taxadiene production in PM1. (**D**) Effect of inoculation density on taxadiene production in PM1. Values are mean ± SD ($n = 3$). Significance was calculated by the two-tailed Student's *t*-test. * and ** represent $p < 0.05$ and $p < 0.01$, respectively. A1, transformant carrying the *atpA* promoter; P1, transformant carrying the *psaA* promoter; PM1, transformant carrying the N-terminal modification; CL, continuous light; L/D, light–dark cycles; HSM, high-salt medium; TXAP, TAP medium containing X-fold the acetate concentration of standard TAP medium.

Because the carbon source concentration generally influences product formation, we next investigated taxadiene production by strain PM1 using an acetate gradient. As shown in Figure 4B, under continuous light at 25 °C, taxadiene production by PM1 increased progressively with increasing acetate supplementation, peaking at 6-fold acetate (T6AP medium), where the volumetric and cellular titers reached 56.3 ± 2.9 μg/L and 3.5 ± 0.4 fg/cell, respectively, and declined thereafter. Under the light/dark cycle, PM1 showed a similar trend, again peaking in T6AP with a volumetric titer of 32.3 ± 0.9 μg/L, about 57.4% of that obtained under continuous light.

We then examined the effect of temperature on taxadiene production. The titers of PM1 grown for 8 days in the acetate gradient at 15 °C, 25 °C and 30 °C are shown in Figure 4C. At 15 °C, PM1 produced relatively high levels of taxadiene in the low-acetate media (TAP, T2AP, and T3AP) but low levels in the high-acetate media, reaching the highest volumetric and cellular titers of 69.2 ± 2.9 μg/L and 6.5 ± 0.3 fg/cell, respectively, in TAP medium. At 25 °C, by contrast, PM1 produced the most taxadiene in the high-acetate media (T4AP, T5AP, and T6AP), indicating that the optimal acetate concentration shifted upward as the temperature increased from 15 to 25 °C. At 30 °C, taxadiene production decreased markedly, with the maximum titer less than 20% of that at 15 °C. Accordingly, 15 °C was selected as the optimal culture temperature for the subsequent experiments.

Initial cell density affects both the growth rate and product accumulation. We therefore compared taxadiene production in strains inoculated at a low density (5×10^5 cells/mL) and a high density (7×10^6 cells/mL) at 15 °C for 8 days. As shown in Figure 4D, a high initial cell density significantly increased taxadiene production and required more acetate. At 7×10^6 cells/mL, the highest titer, 146.4 ± 2.1 μg/L, was obtained in T3AP. In addition, we

examined the effect of methyl jasmonate (MeJA) treatment, and found that 0.5 mM MeJA significantly increased taxadiene production, consistent with the jasmonate-responsive enhancement of terpenoid accumulation previously reported in this alga [23]. Through the abovementioned optimization, the highest taxadiene titer, 185.5 ± 4.4 μg/L, was achieved at 15 °C with a high initial cell density in T3AP medium supplemented with 0.5 mM MeJA.

3. Discussion

Chlamydomonas reinhardtii relies on the plastidial MEP pathway to supply the precursors of all terpenoids. The chloroplast envelope encloses a relatively closed space that effectively separates cytosolic proteins from chloroplast proteins [24]. Only a few small precursors, such as IPP and DMAPP, can cross it, although the transport mechanism remains unclear [25]. This compartmentalization is a double-edged feature for terpenoid synthesis in chloroplasts. On the one hand, it limits contact between cytosolic enzymes and chloroplast-derived isoprenoid precursors (e.g., GGPP), preventing the unnecessary depletion of these precursors. On the other hand, it complicates the design and operation of chloroplast engineering. The intracellular distribution of precursors must be considered carefully, and the spatial and temporal organization of the enzymes must be arranged deliberately. In addition, the chloroplast lacks certain post-translational modifications that may affect the activity of heterologous proteins; for example, Zedler was able to detect cis-abienol synthase in *C. reinhardtii* chloroplasts, but failed to find the target product [26]. However, more recent work has shown that plastid-targeted sesquiterpenoid synthases and cytochrome P450s can be functional in the *C. reinhardtii* plastid, with functionalization efficiencies reaching 80% [27].

Two strategies have generally been adopted to construct cell factories that use chloroplastic isoprenoid precursors efficiently. In the first, enzymes encoded in the nuclear genome are directed into chloroplasts by transit peptides. This approach has been used to produce bisabolene [18], limonene [19], manoyl oxide [20], casbene [20], taxadiene [9,20], patchoulol [21], and sclareol [28]. In the second, the target genes are encoded directly in the chloroplast genome, such as for labdane-type abienol [26] and diterpene [29]. Nuclear transformation is relatively mature, and a variety of techniques are available to enhance protein expression, including multi-copy transformation, intron-mediated expression enhancement, and the use of strong promoters [30,31]. By contrast, fewer tools are available for chloroplast genome transformation, although exogenous proteins expressed from the chloroplast genome tend to be more stable and to accumulate to higher levels.

In this study, the endogenous chloroplast promoter *psaA* proved much more active than *atpA*. TS accumulation driven by *psaA* was about 10% higher than that driven by *aptA*, and taxadiene production increased by 5.6-fold. Several strong chloroplast promoters, such as *psbA*, *psbD*, and 16S/*atpA*, have been used to express heterologous proteins efficiently, and libraries of native and synthetic chloroplast regulatory elements are now available for tuning their strength [32,33]. Each of them may therefore further improve taxadiene production in *C. reinhardtii*.

Besides promoter strength, the stability of the TS enzyme in the chloroplasts had an essential influence on taxadiene production. Adding an additional methionine to the N-terminus of TS markedly improved the stability of the protein and simultaneously increased taxadiene production. We attribute this effect to the prokaryotic-type N-end rule (N-degron) pathway. After synthesis in the chloroplast, the initiator methionine can be removed by methionine aminopeptidase. The residue thereby exposed at the N-terminus may act as an N-degron that is recognized by the adaptor protein ClpS1 and delivered to the ClpC–ClpP protease complex for degradation [34]. In tobacco, placing Met, Glu, or Val at the penultimate position of a chloroplast protein gives the highest protein accumulation [35],

and this Met-based N-terminal strategy has likewise been shown to increase the accumulation of triterpene synthases in the *C. reinhardtii* chloroplast [36]. Our results further indicate that this effect acts on protein turnover rather than on protein synthesis. Transcript levels were comparable among all engineered strains, and TS accumulation declined continuously over 6 days in every strain, indicating that the protein is under constant turnover in the chloroplast. The additional methionine substantially slowed this degradation but was not sufficient to achieve complete stabilization. Other determinants, including internal sequence features, the folding or assembly state, and the wider N-terminal sequence context, may also contribute to protein turnover and thereby to the final level of taxadiene [35,37].

Efflux of the product can reduce the metabolic burden on the cell, relieve feedback inhibition or toxicity, and thereby promote product accumulation. This makes efflux an important route to higher productivity in *C. reinhardtii* cell factories. In this study, taxadiene was detected only in the dodecane phase and not in cells, suggesting that taxadiene can cross the lipid bilayers of the chloroplast envelope and cell membrane. No terpenoid transporter has yet been reported in microalgae, but we speculate that taxadiene efflux may involve ABC transporters. Overexpression of the ABC transporter SNQ2 in *Rhodosporidium toruloides* increased extracellular β-carotene 4.04-fold [38], and engineering efflux systems in *Yarrowia lipolytica* substantially increased the secretion of abscisic acid, a sesquiterpenoid-derived product [39]. In contrast, disruption of the ABCG1 transporter in *Marchantia polymorpha* altered sesquiterpene accumulation in oil bodies [40]. These studies together suggest an association between taxadiene efflux and ABC transporters. Consistent with this, acetate concentration strongly influenced taxadiene production in our experiments, with the peak titer obtained when acetate was increased to 6-fold (T6AP medium) at 25 °C. We further examined the transcripts of the ABC transporters CrABCG3 and CrABCG4 at 1-, 4-, and 6-fold acetate and found that they increased significantly with acetate concentration. Although acetate also feeds the isoprenoid precursor pool and the two effects are therefore not mutually exclusive, we conclude that the effect of acetate dosage on taxadiene yield may be at least partly attributable to altered expression of these transporter proteins. Although the chloroplast chassis offers clear advantages over chemical synthesis, the titer obtained here remains modest compared with that achieved in well-established microbial chassis. For instance, metabolically engineered *Escherichia coli* has reached taxadiene titers of approximately 1 g/L [4], which is over three orders of magnitude higher than the 185.5 μg/L obtained in this study. While such bacterial systems benefit from highly optimized precursor supply and rapid growth, the chloroplast-based approach reported here offers a distinct, photosynthetic route that may complement existing microbial platforms for taxane production. Further improvement in our system will require a stronger precursor supply and more efficient product efflux. Modulating ABC transporters to promote the efflux of heterologous terpenoids may offer a new strategy for enhancing terpenoid production in microalgae.

4. Materials and Methods

4.1. Strain and Culture

The *C. reinhardtii* strain CC5168 (also designated TN72) was obtained from Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN, USA). As a recipient for chloroplast transformation, this mutant features a partial deletion of the endogenous *psbH* gene, which is replaced by an *aadA* cassette in the same orientation. Consequently, the strain exhibits a PSII-deficient phenotype and relies on exogenous acetate for growth, rendering it incapable of photoautotrophic growth, while conferring resistance to spectinomycin. The strain was routinely maintained in Tris-acetate phosphate (TAP) at 25 °C in the dark [22]. Transformants were cultured in high salt medium (HSM) at 25 °C under a light intensity

of 70–120 μmol/(m^2·s) [17]. *E. coli* was cultured in LB medium at 37 °C with shaking at 200 rpm.

4.2. Vector Construction

The vector pASapI [22] carried the chloroplast *atpA* promoter together with its 5′ UTR, the rbcL 3′ UTR, and a homologous-recombination cassette containing the *psbH* gene of *C. reinhardtii*. This vector can complement *psbH* in CC5168 and restore photoautotrophic growth. The *TS* gene (GenBank accession no. U48796) from *taxus brevifolia* was codon-optimized for the high-frequency codons of the *C. reinhardtii* chloroplast genome and synthesized with a 3×HA a C-terminal 3×HA tag. The optimized *TS* was digested with *Sap* I and *Pst* I and ligated into pASapI to generate expression vector pASap-TS.

To construct pSRSap-TS, which harbors the *psaA* promoter, total DNA of *C. reinhardtii* was extracted by the phenol–chloroform method and used as templates to amplify the promoter *psaA* and its 5′ UTR using primers psaA-F and psaA-R under the following cycling conditions: 98 °C for 3 min; then 30 cycles of 98 °C for 10 s, 47 °C for 5 s, and 68 °C for 15 s. The amplified product was digested with SapI and ligated with the SapI-digested TS gene. The ligation product served as a template to amplify the *psaA*–TS fragment using primers psaA-F and TSR1, and the fragment was then double-digested with MluI and BamHI and ligated into pASapI to generate pSRSap-TS. Vector pSRSap-TSM was constructed by introducing another methionine (start) codon at the 5′ terminus of TS in pSRSap-TS using an In-Fusion cloning kit (Takara Bio, Dalian, China). Primers used are listed in Supplementary Table S1.

4.3. Chloroplast Transformation and Transformants Screen

The chloroplast transformation was performed by the glass bead method as described previously [22]. CC5168 was inoculated in TAP at 5 × 10^5 cells/mL and incubated at 25 °C in the dark until the culture reached 1–2 × 10^6 cells/mL. Cells from 300 mL culture were collected by centrifugation at 3000× *g*, washed 1–2 times with HSM, and resuspended to a density of 2 × 10^8 cells/mL. Aliquots of 300 μL of concentrated algal cells, 0.3 g of sterilized glass beads (400–625 μm), and 10 μg of vectors linearized with Eco147I enzyme were combined in a sterile 1.5 mL centrifuge tube and vortexed at maximum speed for 15 s. The cells were then collected, plated onto HSM agar, allowed to dry, and incubated upside down in the dark overnight. The plates were subsequently cultured at 25 °C with a light intensity of 70–120 μmol/(m^2·s); for 4–6 weeks, green transformants appeared. Transformants were transferred to fresh HSM plates, streaked for single colonies, and incubated under light until they grew. This step was repeated once or twice to promote homogenization. Colony PCR with primer pairs TSJF1/TSJR1 and F1/R1 was used to detect the target gene and to assess the degree of homogenization, respectively.

4.4. Western Blot

For Western blotting, algae in the mid-to-late logarithmic phase were diluted 1:20 with fresh TAP medium and incubated under continuous light, with samples collected every two days. For total protein extraction, 100 mL of the algal culture was harvested by centrifugation at 5000× *g* for 6 min at 4 °C and washed once with an appropriate volume of phosphate-buffered saline (PBS). The cell pellet was resuspended in 1 mL of lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 μg/mL leupeptin; 0.5 mM EDTA; 2 mM DTT; 1 mM PMSF), kept on ice, and sonicated (60 W power; 2 s on, 2 s off) for 10–20 min. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was transferred to a clean 1.5 mL tube. Protein concentration was determined with a Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA), and samples were stored at −80 °C. The 3×HA tag fused to TS was detected using a mouse monoclonal anti-HA antibody (Abcam,

Cambridge, UK) as the primary antibody and a horseradish peroxidase (HRP)-labeled goat anti-mouse IgG as the secondary antibody. RBP40 was used as an internal reference [41]. Because the *psaA* and *atpA* promoters are also active in *E. coli*, total *E. coli* protein from a strain carrying the TS-expression plasmid was used as a positive control, whereas CC5168 transformed with the empty vector pASapI was used as a negative control.

4.5. The Production and Detection of Taxadiene

To promote the accumulation of taxadiene and facilitate product recovery, engineered algae were cultivated in a biphasic culture system in which 500 µL of dodecane was overlaid on 4.5 mL of medium in six-well plates with 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 8 days at 25 °C as default [20]. Microalgae in the logarithmic phase were collected and re-inoculated into this biphasic system at a default density of 5×10^5 cells/mL. The effects of light regime, acetate concentration, and culture temperature on taxadiene yield were examined, together with the influence of 0.5 mM methyl jasmonate. The conditions tested were as follows: (1) light regime—continuous light (L = 24 h) or a 16 h:8 h light/dark (L:D) cycle; (2) acetate concentration—the concentration in TAP medium was used as the baseline (T1AP), and media containing 1- to 6-fold-higher acetate (T2AP–T7AP) were prepared to establish an acetate gradient; (3) culture temperature—15, 25, and 30 °C; and (4) inoculation density—5.0×10^5 or 7×10^6 cells/mL.

The dodecane phase was collected, centrifuged at 12,000× *g* for 2 min, filtered through a 0.22 µm nylon-66 membrane and then detected by gas chromatography–mass spectrometry (GC–MS; Agilent 7890A gas chromatograph, Agilent Technologies, Santa Clara, CA, USA; ISQ mass spectrometer, Thermo Fisher Scientific, Waltham, MA, USA). Separation was performed on a 30 m × 0.25 mm (Agilent J&W) HP-5ms column (Agilent Technologies, Santa Clara, CA, USA) coated with 5% biphenyl–95% dimethylpolysiloxane (0.25 µm film thickness). The injector, interface, and ion-source temperatures were 250, 250, and 220 °C, respectively. A 1 µL aliquot was injected in splitless mode and carried by helium at a constant flow of 1 mL/min. The oven temperature was held at 80 °C for 1 min, increased to 120 °C at 10 °C/min, then to 160 °C at 3 °C/min and held for 10 min, and finally to 270 °C at 10 °C/min and held for 2 min. After elution of the dodecane peak (approximately 12 min), mass spectra were recorded at a scan rate of 15 Hz over an m/z range of 50–750. Both full-scan and selected-ion-monitoring (SIM) modes were used, with ions of m/z 91.0, 97.0, 107.0, 122.0, and 272.0 monitored as the principal qualifier/quantifier ions of the extracted ion chromatograms (XICs). Taxadiene was quantified from its total-ion peak area relative to a taxadiene standard kindly provided by Prof. Liyan Wang.

4.6. Statistical Analysis

All experiments were conducted in three biological replicates with data shown in mean ± standard deviation (SD). Two-group comparisons were made with the two-tailed Student's *t*-test. Comparisons among three or more groups were made by one-way ANOVA followed by Tukey's multiple-comparison test [42]. * and ** represent $p < 0.05$ and $p < 0.01$, respectively. All statistical analyses and graphical visualizations were performed using GraphPad Prism (version 9.0; GraphPad Software, San Diego, CA, USA).

5. Conclusions

In this study, we showed that taxadiene production in the *C. reinhardtii* chloroplast was limited by two factors that could be improved independently: the strength of the promoter driving TS expression and the lifetime of the TS protein. Replacing the *atpA* promoter with *psaA* markedly increased TS accumulation and taxadiene production. Adding an additional methionine at the N-terminus further slowed protein degradation and raised the

titer to 26.7 µg/L. Because the two modifications act at different steps, their effects did not overlap, and the improvement was greater than that obtained with either modification alone. Culture conditions then increased the titer to 185.5 µg/L. The optimal acetate concentration changed with temperature and initial cell density, indicating that these parameters should be optimized together. Since the additional methionine acts on a degradation pathway that recognizes the N-terminus of any protein synthesized in the plastid, this modification is not specific to TS and requires no additional regulatory element. It may therefore be applicable to other diterpene synthases expressed in this organelle.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/md24110381/s1, Supplementary Figure S1: RT-PCR analysis of TS transcription in the engineered chloroplast strains; Supplementary Figure S2: GC–MS analysis of taxadiene produced by the chloroplast-engineered strains; Supplementary Figure S3: Relative levels of the TS protein in the chloroplast of engineered strains cultured for 2, 4, and 6 days. Table S1: Primers used in this study.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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Abbreviations

The following abbreviations are used in this manuscript:

A1-A4	engineered strains carrying the *atpA*-TS cassette
C1	negative-control strain carrying the empty vector
P1-P2	engineered strains carrying the *psaA*-TS cassette
PM1-PM4	engineered strains carrying the *psaA*-TSM (N-terminally modified TS) cassette
ABC	ATP-binding cassette
CL	continuous light
L/D	light–dark cycles
HRP	horseradish peroxidase
HSM	high-salt medium
TAP	tris-acetate phosphate
TXAP	TAP medium containing X-fold the acetate concentration
MeJA	methyl jasmonate
GC-MS	gas chromatography–mass spectrometry
GGPP	geranylgeranyl pyrophosphate
IPP	isopentenyl pyrophosphate
DMAPP	dimethylallyl pyrophosphate
MEP	2-C-methyl-D-erythritol 4-phosphate(pathway)
MVA	cytosolic mevalonate
TS	taxadiene synthase

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