

Article

Functional Expression of *Cannabis sativa* Polyketide Enzymes in *Chlamydomonas reinhardtii* Reveals Metabolic Constraints to Olivetolic Acid Accumulation

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Abstract

Plant specialized metabolites are valuable sources of pharmaceuticals, nutraceuticals, and industrial compounds. Transferring their biosynthetic pathways into microbial hosts remains a major challenge, particularly in photosynthetic microalgae. Here, we investigated the ability of the green microalga *Chlamydomonas reinhardtii* to functionally express *Cannabis sativa* polyketide biosynthetic enzymes involved in olivetolic acid synthesis, a key intermediate in cannabinoid biosynthesis. Nuclear transformants carrying the *C. sativa* tetraketide synthase (*CsTKS*) and olivetolic acid cyclase (*CsOAC*) genes were generated. Functional enzyme activity was confirmed by in vitro enzymatic assays using total protein extracts. In vitro olivetolic acid production was detected, verified, and quantified by high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry using authentic standards. However, in vivo olivetolic acid and downstream metabolites were not detected in algal biomass. This indicated that functional enzyme expression did not result in productive intracellular pathway flux. Untargeted metabolomic profiling revealed extensive metabolic rewiring and strain-specific metabolic perturbations following pathway introduction. These findings suggest that heterologous pathway expression triggered broad physiological responses. This study demonstrates that plant polyketide enzymes remain catalytically active in *C. reinhardtii*; however, intrinsic metabolic and cellular constraints limit in vivo metabolite accumulation. These findings provide important insights into the biological barriers that limit the functional implementation of plant polyketide pathways in photosynthetic microalgae *C. reinhardtii* and identify key targets for future metabolic and cellular engineering.

Keywords: microalgal biotechnology; metabolic engineering; polyketides; untargeted metabolomics; pathway bottlenecks; synthetic biology; biopharmaceutical

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1. Introduction

Plant specialized metabolism gives rise to an extraordinary diversity of structurally complex bioactive compounds with important ecological, pharmaceutical, and

biotechnological relevance. Among these pathways, polyketide biosynthesis occupies a central position, generating metabolites through iterative condensation reactions of acetyl- and malonyl-CoA-derived building blocks catalyzed by type III polyketide synthases. Reconstructing plant polyketide pathways in heterologous hosts [1] is therefore of strong interest, both to enable sustainable biosynthesis and to gain mechanistic insight into pathway function outside the native plant cellular context.

Olivetolic acid (OA) is a plant-derived polyketide intermediate widespread in fungi, lichens, and plants, where they function in chemical defense, stress tolerance, and specialized metabolism. It also serves as a key precursor of the therapeutically valuable cannabinoids from *Cannabis sativa* [2,3]. OA biosynthesis requires the coordinated activity of a type III polyketide synthase, tetraketide synthase (TKS), which catalyzes the condensation of three malonyl-CoA with hexanoyl-CoA to generate a linear polyketide intermediate, and olivetolic acid cyclase (OAC), which performs a C2–C7 aldol cyclization, thioester cleavage, and aromatization to yield OA (Figure 1) [4,5]. OAC is unique among plant enzymes, representing the only known plant polyketide cyclase and a rare example of a functionally characterized $\alpha+\beta$ barrel protein catalyzing ring formation in plant polyketide metabolism [6]. Together, TKS and OAC define a minimal and well-characterized enzymatic module [7] for studying plant polyketide biosynthesis.

OA occupies a central position in cannabinoid biosynthesis in *Cannabis sativa*, where it serves as the polyketide precursor for the formation of downstream cannabinoids. The primary objective of this study was to evaluate whether the upstream polyketide module comprising TKS and OAC could function in the photosynthetic microalga *Chlamydomonas reinhardtii*. To further investigate pathway performance, we also incorporated downstream cannabinoid biosynthetic enzymes and assessed cannabinoid formation. This extended pathway was included to determine whether downstream reactions could improve metabolic flux, stabilize upstream intermediates, or support product formation. Heterologous production of OA and downstream cannabinoids has been achieved in heterotrophic microbial hosts (e.g., *Escherichia coli*, *Komagataella phaffii*, *Saccharomyces cerevisiae*) [8–12]. These systems have demonstrated that heterologous expression of TKS and OAC is feasible, but they have also revealed recurrent limitations, including low OA yields, accumulation of side products such as olivetol and pyrones [5], and the need for extensive metabolic engineering to enhance precursor supply, particularly hexanoyl-CoA. In many cases, production relies on substrate supplementation and tightly controlled fermentation conditions, underscoring the metabolic burden associated with polyketide biosynthesis in non-native hosts.

Photosynthetic microalgae have been proposed as alternative platforms for the heterologous expression of plant specialized metabolic pathways, offering theoretical advantages such as light-driven reducing power, utilization of CO₂ as a primary carbon source, and reduced reliance on sugar feedstocks. In this context, *C. reinhardtii* has emerged as a genetically tractable model microalga with a well-annotated nuclear genome, established transformation methods, and an expanding molecular toolbox [13–16]. Previous studies have demonstrated its capacity to express heterologous enzymes and produce diverse classes of bioactive compounds, including terpenoids, polyamines, and recombinant proteins [17–25], supporting its potential as a chassis for plant-derived metabolism.

Despite these advantages, transferring complex plant polyketide pathways into photosynthetic microalgae presents fundamental biological challenges. Carbon partitioning in *C. reinhardtii* is tightly regulated to support photosynthesis, growth, and essential cellular functions, which may restrict flux toward non-native metabolic routes. In addition, differences in cellular compartmentalization, precursor availability, and regulatory architecture between plants and microalgae may limit intracellular accumulation of polyketide products, even when heterologous enzymes are expressed and catalytically active. These

constraints remain poorly characterized, particularly for type III polyketide pathways, and necessitate systematic experimental evaluation.

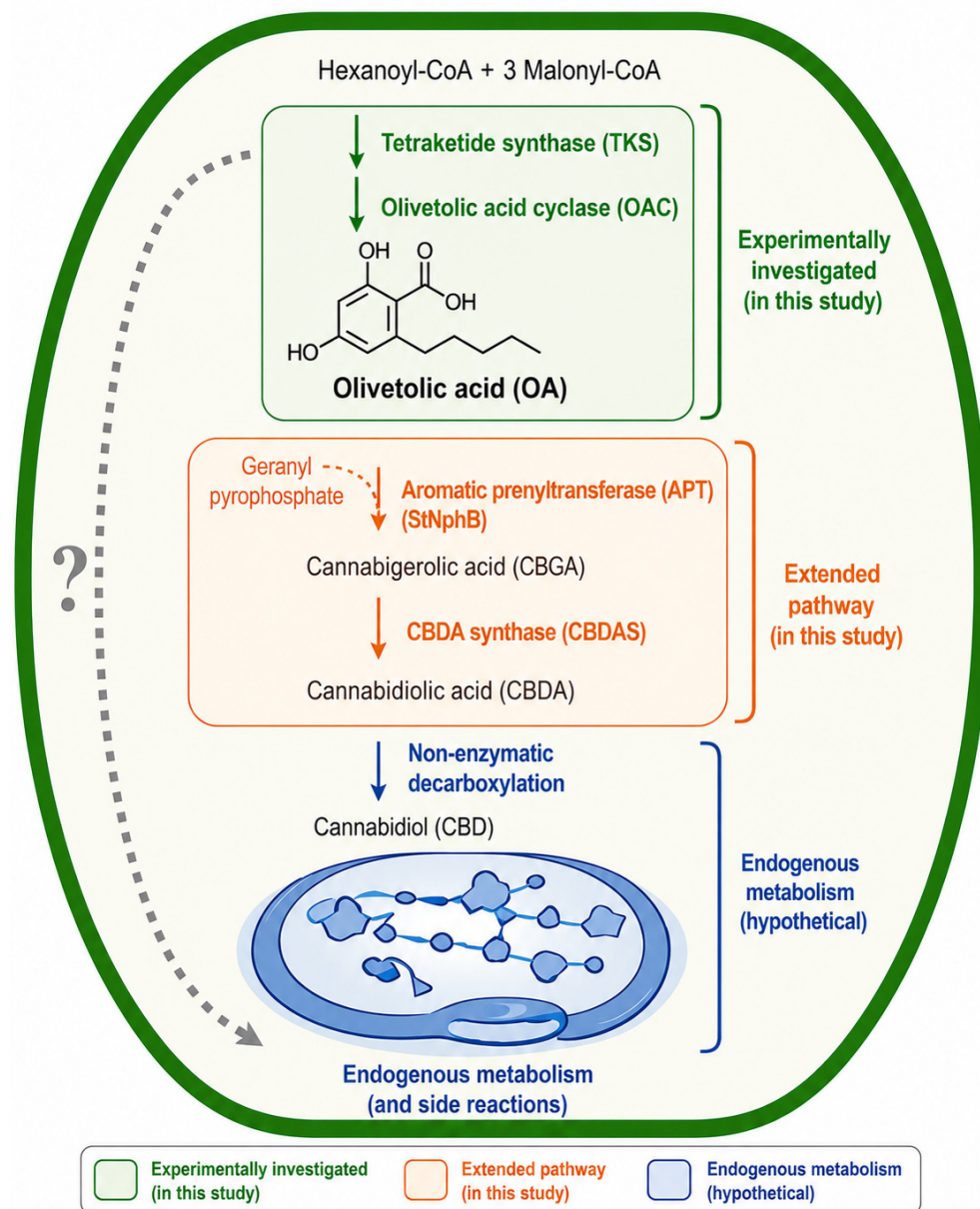


Figure 1. Overview of plant polyketide biosynthesis leading to OA and its position within the cannabinoid biosynthetic pathway. The green section highlights the experimentally investigated pathway in this study, comprising the coordinated action of TKS and OAC for OA biosynthesis. The orange section illustrates the extended cannabinoid biosynthetic pathway engineered in this study, including aromatic prenyltransferase (APT) and CBDASynthase (CBDAS). The blue/gray section represents downstream hypothetical reactions and the potential fate of pathway intermediates through endogenous metabolism in *Chlamydomonas reinhardtii*. This color-coded organization distinguishes the experimentally validated pathway from the proposed downstream reactions.

In this study, we engineered *C. reinhardtii* to express the *Cannabis sativa*-derived enzymes TKS and OAC to evaluate their functional activity and the metabolic consequences of introducing a plant polyketide biosynthetic pathway into a photosynthetic microalgal host. Specifically, we investigated whether the heterologous enzymes were functionally active and whether their expression led to successful metabolic integration within the host. To address these questions, we combined in vitro enzymatic assays with untargeted

metabolomic profiling. This complementary approach enabled us to distinguish enzyme functionality from effective pathway integration, revealing biological and metabolic constraints that limit heterologous polyketide biosynthesis in *C. reinhardtii*. These findings provide a framework for future metabolic and cellular engineering strategies aimed at improving the production of plant specialized metabolites in microalgae.

2. Results

2.1. Stable Nuclear Integration of *Cannabis sativa* Polyketide Biosynthetic Genes in *C. reinhardtii*

To evaluate the suitability of *C. reinhardtii* as a host for OA biosynthesis, three expression constructs (C0, C1, and C2) encoding the *Cannabis sativa* enzymes CsTKS and CsOAC were designed and codon-optimized for nuclear expression (Figure 2; Appendix A—Tables A1 and A2). In all three constructs, CsTKS and CsOAC were expressed as a single bicistronic transcript separated by an extended FMDV-2A self-cleaving peptide and driven by the *HSP70A-RBCS2-i1* promoter, with the *RBCS2* 3' untranslated region serving as the transcriptional terminator (Figure 2; Appendix A—Tables A1 and A2).

The three constructs incorporated distinct design features that may influence heterologous protein expression in *C. reinhardtii* (Figure 2; Appendix A—Tables A1 and A2). Construct C0 contained the native bicistronic CsTKS–CsOAC cassette without epitope tags or introns and served as the baseline design. Construct C1 encoded an intron-free cassette but incorporated an N-terminal 6×His tag on CsTKS and a C-terminal Myc tag on CsOAC. Construct C2 retained the same tagged protein configuration as C1 but additionally included three engineered introns within the coding sequence (Figure 2; Appendix A—Tables A1 and A2).

Agrobacterium-mediated transformation of *C. reinhardtii* with the C0, C1, and C2 constructs generated hygromycin-resistant transformants for all three expression cassettes. Following three rounds of hygromycin selection, stable transformants were obtained for each construct, with transformation efficiencies ranging from 116 to 139 colonies per 10^8 cells (Table 1). Colony PCR confirmed successful integration of the complete TKS–OAC expression cassette in 19–31% of hygromycin-resistant clones, depending on the construct (Table 1; Appendix A—Figures A1 and A2).

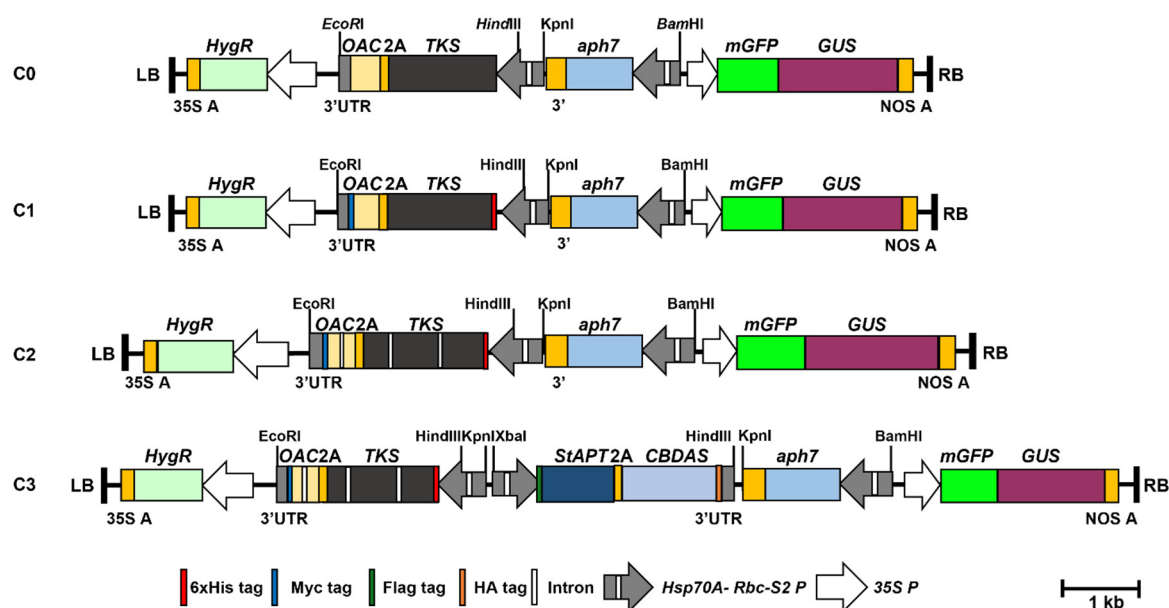


Figure 2. Expression cassettes (between LB and RB in T-DNA) used in this study. The minimal plant polyketide module includes cistron C0, C1 and C2 sequences inserted at the 3' region of the sequence

using EcoRI/KpnI restriction sites. Whereas downstream enzymes with cistron of C3 cassette was inserted at the 3' region of the sequence using XbaI/HindIII restriction sites. *mGFP*: gene of green fluorescent protein; *Hsp70A Rbc-S2p* hybrid constitutive promoter and *RBCS2* 3'UTR terminator (*RbcS2*: *C. reinhardtii* gene 2 for the small subunit of ribulose biphosphate carboxylase); *Aph7* is *Streptomyces hygroscopicus* Aminoglycoside Phosphotransferase gene mediates resistance against hygromycin B; *OAC*: *Olivetolic Acid Cyclase*; *2A*: *FMDV2A* (foot and-mouth-disease-virus 2A self-cleavage peptide); *TKS*: *Tetraketide Synthase*; *StAPT*: *Streptomyces Aromatic Prenyltransferase* gene (*NphB*^{Y288A/G286S}); *CBDAS*: *C. sativa* cannabidiolic acid synthase; *GUS*: β -glucuronidase reporter gene; *HygR*: hygromycin B resistance gene used as the plant selectable marker.

Table 1. Summary of number of transformants obtained and characterization of the PCR-positive clones. EA⁺: Positive enzymatic activity.

Name of Construct	Transformation	Stable Clones on Hygromycin	¹ PCR+/Hygromycin+ (%)	Clone EA+
C0	<i>Agrobacterium</i>	116	14/45 (31)	C0-30 ²
C1	<i>Agrobacterium</i>	139	16/59 (27)	C1-10
C2	<i>Agrobacterium</i>	119	9/48 (19)	C2-17
C3	Electroporation	300	2/300 (0.7)	C3-47 and C3-83

¹ PCR⁺ clones/hygromycin⁺ clones screened after three rounds of selection. C0, C1, and C2 represent the three different cistron constructs harboring *TKS-2A-OAC*; C3 contains two cistrons harboring *TKS-2A-OAC* and *NphB-2A-CBDAS* used in this study. ² “C0-30” refers to clone 30 of construct C0.

To extend the pathway beyond OA biosynthesis, a fourth construct (C3) was designed by combining the C2 *CsTKS*–*CsOAC* bicistronic cassette with a second bicistronic cassette expressing a soluble aromatic prenyltransferase (*StAPT*:*NphB*^{Y288A/G286S}) from *Streptomyces* sp. strain CL190 [26] and *C. sativa* cannabidiolic acid synthase (*CsCBDAS*) (Figure 2; Appendix A—Tables A1 and A2). Owing to the larger size and increased complexity of this dual-cistron construct, C3 was introduced into *C. reinhardtii* by electroporation rather than *Agrobacterium*-mediated transformation. Colony PCR confirmed successful integration of the complete dual-bicistronic expression cassette in only 0.7% of hygromycin-resistant clones, corresponding to two complete expression cassette-harboring transformants (Table 1; Appendix A—Figure A2). These transformants maintained hygromycin resistance during prolonged culturing, indicating stable integration of the construct.

2.2. Functional Activity of Heterologously Expressed TKS and OAC Demonstrated by In Vitro Enzymatic Assays

To determine whether heterologously expressed *CsTKS* and *CsOAC* were enzymatically active, total soluble protein extracts were prepared from PCR-positive transformants harboring the complete expression cassette and subjected to in vitro enzymatic assays using hexanoyl-CoA and malonyl-CoA as substrates. Reaction products were analyzed by high-performance liquid chromatography with diode array detection (HPLC–DAD) and liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Among the PCR-positive transformants screened, only one clone from each expression construct exhibited detectable in vitro enzymatic activity. Total protein extracts from these clones C0-30, C1-10, and C2-17, representing the C0, C1, and C2 constructs, respectively, produced OA in in vitro enzymatic assays (Figure 3; Appendix A—Figures A3 and A4). Product identity was confirmed by matching the retention time, UV absorption spectrum, and MS/MS fragmentation pattern with those of an authentic OA standard (Appendix A—Figures A3 and A4). Quantification of the enzymatic reaction products showed OA concentrations of 19.5, 16.5, and 28.5 mg L^{−1} for extracts from clones C0-30, C1-10, and C2-17, respectively (Figure 3).

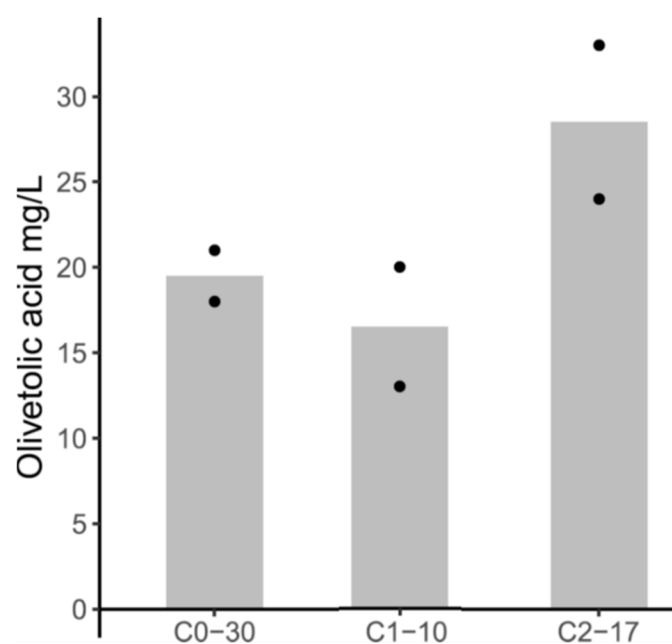


Figure 3. Quantification of OA generated during in vitro enzymatic assays using total protein extracts from one enzymatically positive *C. reinhardtii* transformant representing each expression construct (C0-30, C1-10, and C2-17), each expressing a different TKS–OAC cassette. OA formed in the reaction mixtures was quantified by HPLC. Bars represent the mean of two technical replicates (N = 2) performed using protein extracts from a single biological transformant (clone), and black dots represent the individual technical replicate measurements.

No OA was detected in assays performed with protein extracts from empty-vector control strains (Appendix A—Figure A4), confirming that product formation resulted from heterologous enzyme activity. These results demonstrate that plant-derived CsTKS and CsOAC remain functionally active when expressed from the nuclear genome of *C. reinhardtii* and can catalyze OA synthesis in vitro.

2.3. Absence of Detectable OA and Downstream Products In Vivo

Despite clear in vitro enzymatic activity, OA was not detected in vivo in metabolite extracts obtained from algal biomass of CsTKS–CsOAC-expressing strains using targeted HPLC–UV and LC–MS/MS analyses. Similarly, no downstream pathway intermediates or products, including Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabigerol (CBG), or cannabigerolic acid (CBGA), were confidently detected in biomass extracts from strains harboring the dual-cistron construct encoding the extended pathway using the targeted metabolomic analysis.

To further evaluate pathway progression, in vitro assays were performed using total protein extracts from the two transformants harboring the complete dual-bicistronic C3 construct (C3-47 and C3-83). Among these, only clone C3-47 exhibited detectable OA formation in the initial enzymatic step (Appendix A—Figure A5). However, no prenylated intermediates or cyclized downstream products were detected in subsequent reactions. These results indicate that, although the initial step of the pathway was functionally active in one C3 transformant, this activity did not translate into complete pathway progression or intracellular accumulation of downstream metabolites.

The absence of in vivo detectable OA and downstream metabolites in algal biomass suggests that intracellular accumulation is constrained, potentially due to limited precursor availability, rapid metabolic turnover, or competition with endogenous metabolic processes.

2.4. Untargeted Metabolomic Analysis Reveals Extensive Metabolic Rewiring Associated with Pathway Introduction

To assess whether heterologous pathway expression influenced endogenous metabolism, untargeted metabolomic profiling was performed on engineered strains, along with wild-type and empty-vector control strains, specifically *C. reinhardtii* strains EV, WT, C0-30, C3-47, and C3-83, using both reversed-phase (RP) and hydrophilic interaction chromatography (HILIC) coupled to high-resolution mass spectrometry.

Across all strains, this metabolomic analysis detected a total of 10,924 analytes in RP mode and 6083 analytes in HILIC mode (Figure 4). Of these, 349 analytes were annotated by comparison with MS-DIAL[®] “MSMS-Pos-MassBank” spectral library, with several of them matching the same metabolites in different analysis modes (Figure 4B; Supplementary File S1—Data S1 and S2). Among the analytes putatively annotated, we found alkaloids (such as hirsutine was only detected in the C0-30 strain), several amino acids and their derivatives, and peptides, terpenoids, sesquiterpene lactones, and diterpene lactones (such as andrograpanin, only detected in C3-83 strain), flavonoids (including luteolin 3',4'-di-O-beta-D-glucopyranoside, only present in C3-47 and C3-83), and other chemical group (Supplementary File S1—Data S2).

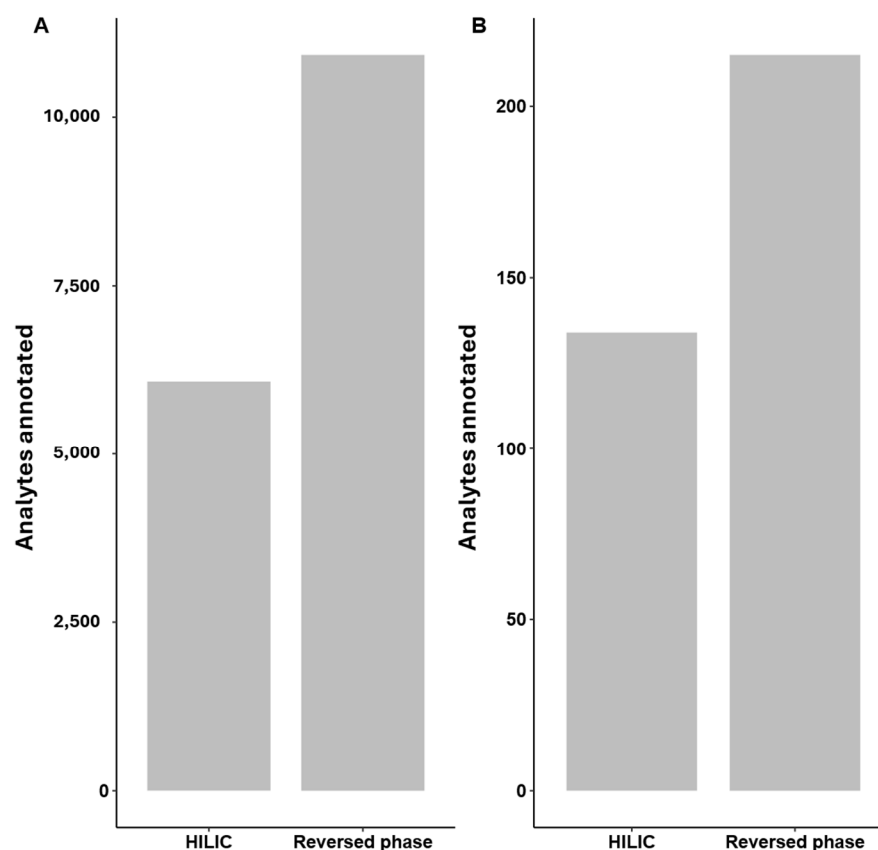


Figure 4. Analytes detected (A) and annotated (B) in each analysis. Analytes were considered detected if their peak area in at least two replicates of one or more strains was $3\times$ the blank[®] peak. Annotation was considered if the MS/MS profile matched, and the total annotation score was $>70\%$ against MS-Dial[®] ESI⁺ spectral library.

Despite sharing most detected analytes (12,002 in total), the engineered strains exhibited unique metabolomic profiles, with 1302 analytes detected in a single strain (Figure 5A). Excluding analytes detected exclusively in the WT or EV strains revealed 1014 analytes specific to the engineered strains. Of those, 695, 325, and 584 analytes were present

in the C0-30, C3-47, and C3-83 strains, respectively (Figure 5B), with 96 analytes detected in all three strains. Notably, an analyte putatively annotated as CBD (comparing with MS-Dial® ESI⁺ spectral library) was detected only in strain C3-47. LC-MS/MS validated the presence of a peak corresponding to the mass of CBD, but compared to the standard, the signal may have been a misidentification because only one of the three MS transitions showed ion intensity above a signal-to-noise ratio of 5×10^1 (Appendix A—Figure A6).

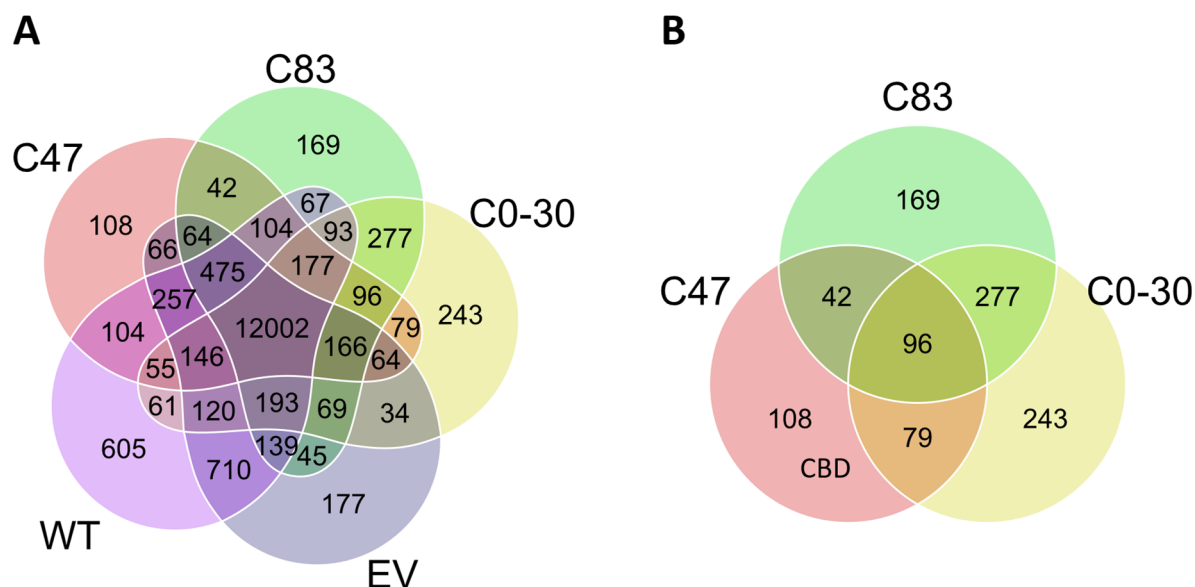


Figure 5. Cannabidiol (CBD) was only detected in the strain C3-47. Venn diagram of the differentially abundant analytes, detected with HILIC and reversed-phase modes in ESI⁺, in five *C. reinhardtii* strains. (A) Analytes detected in each strain exclusively or shared between strains. (B) Analytes detected in either wild-type (WT) or empty-vector (EV) strains were removed to focus on those detected only on bioengineered strains with one cistron (C0-30) or two cistrons (C3-47 and C3-83).

After the exclusion of analytes differentially abundant in the EV compared to the WT strain, we found 1266 analytes whose abundance was significantly altered—either increased or decreased—referred to as “deregulated” in the engineered strains compared to each other or to the WT and EV controls (Figure 6). There were few analytes deregulated between C3-83 and C3-47, indicating their genetic similarity. Bioengineered strains C0-30 and C3-83 showed common down-regulation of more than 280 analytes compared to the WT, suggesting a metabolic similarity between these strains. In RP mode, several metabolites were found downregulated in engineered strains (C0-30, C3-47, and C3-83) compared to the EV strain, indicating that these strains are consuming more/producing less of these analytes compared to the EV (Figure 6). Of the 349 analytes annotated, 39 were deregulated either in HILIC or in RP mode (Figure 7, Appendix A—Figure A7). Principal component analysis (PCA) of the normalized abundances of all analytes was performed with exclusion (Appendix A—Figure A8A) or inclusion of the cannabinoid standard and WT-spiked samples (Appendix A—Figure A8B). In both PCA plots, C0-30 replicates show greater heterogeneity between themselves in PC1 compared to the replicates of C3-47 or C3-83. C0-30 and C3-83 samples show the same distribution in PC2, with two replicates more separated from the other samples. This similar distribution could explain the common deregulation of analytes in these two strains compared to WT. All strains are similarly spread in PC1. In the case of C3-47, this result agrees with the different abundance of CBD in each replicate.

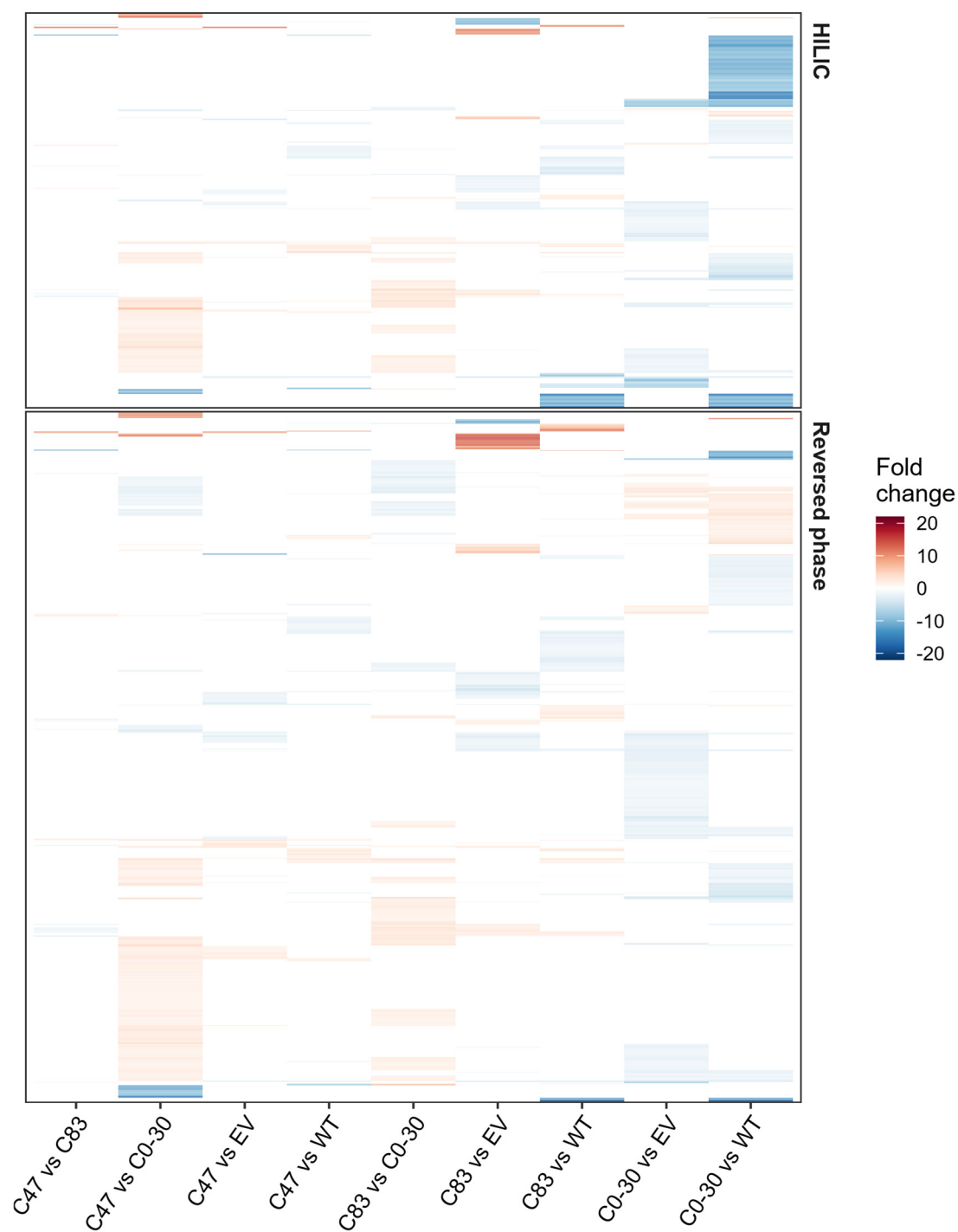
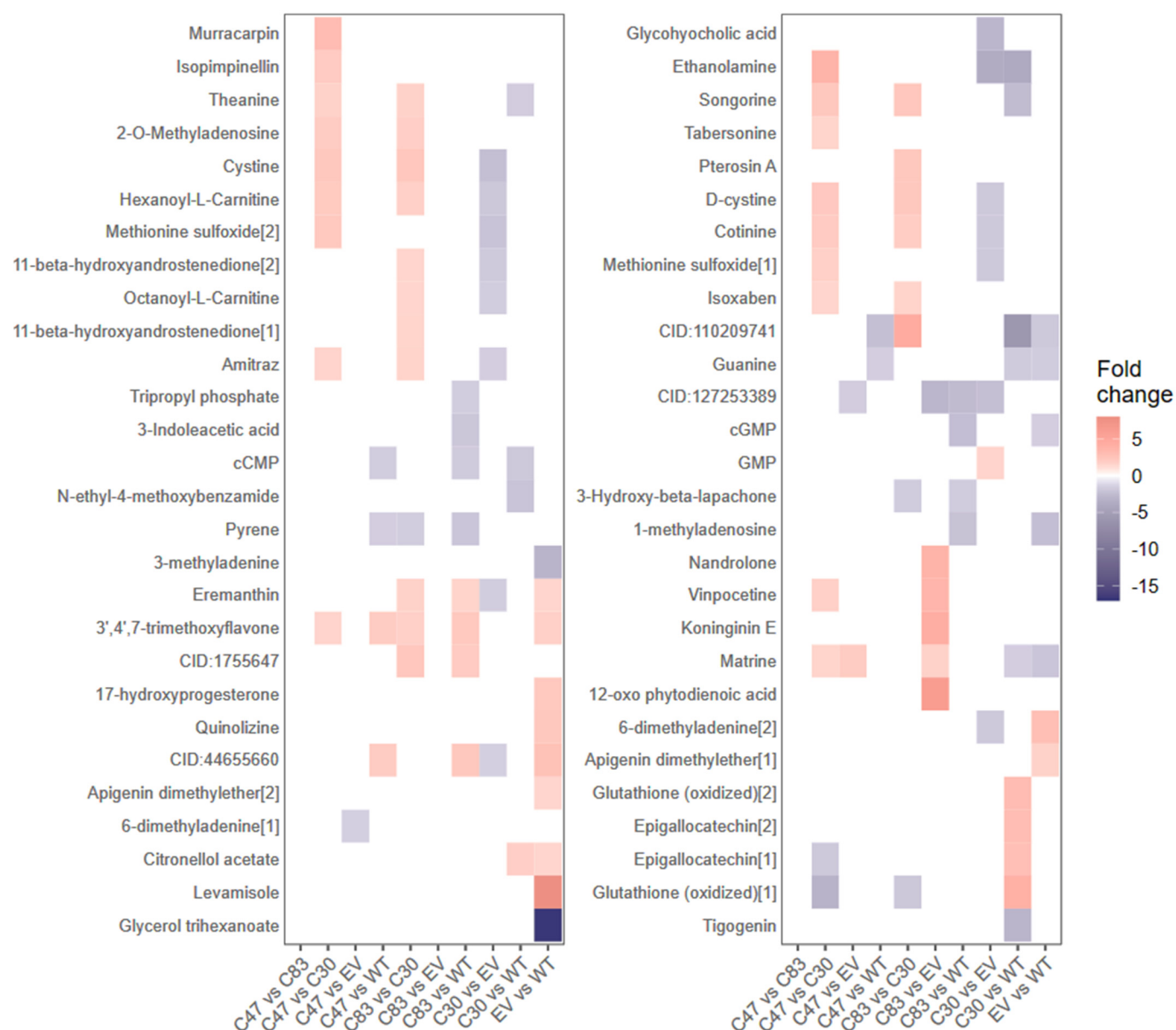


Figure 6. Differential abundance of analytes in *C. reinhardtii* strains detected with HILIC and reversed-phase modes (top and lower panel, respectively) in ESI⁺. Overview of analytes deregulated in engineered strains (C0-30, C3-47 and C3-83), excluding those also deregulated in the Empty-Vector (EV) strain compared to the Wild-Type (WT) strain.



Thus, differential abundance analysis revealed extensive metabolic perturbations associated with pathway introduction, including strain-specific changes and shared patterns of deregulation across engineered lines (Figures 6 and 7, Appendix A—Figure A7). The PCA further highlighted separation between engineered strains and controls, indicating that expression of plant polyketide enzymes induced broad metabolic reprogramming in *C. reinhardtii*. Although a small number of analytes exhibited mass spectral features tentatively matching known bioactive compounds; however, these annotations were not confirmed by authentic standards and should be interpreted cautiously. Collectively, the metabolomic data indicate that heterologous pathway expression significantly reshapes cellular metabolism, consistent with metabolic partitioning and diversion of precursors away from polyketide accumulation.

Together, these results demonstrate that plant polyketide enzymes can be stably expressed and remain enzymatically active in *C. reinhardtii*, while intracellular accumulation of pathway products is constrained. Untargeted metabolomic analyses reveal extensive metabolic responses to pathway introduction, providing insight into biological limitations that shape heterologous polyketide metabolism in photosynthetic microalgae.

3. Discussion

A principal finding of this study is that the *C. sativa* enzymes CsTKS and CsOAC were stably expressed from the nuclear genome of *C. reinhardtii* and remained catalytically active in vitro. The formation of OA confirms that both enzymes were properly folded and functional in the algal cellular environment. Furthermore, transformants retained enzymatic activity over an extended period under selective conditions, demonstrating the stable expression of heterologous plant enzymes in *C. reinhardtii*.

Despite clear in vitro enzymatic activity, neither OA nor downstream pathway products were detected in vivo in algal biomass extracts. This disconnect between enzyme functionality and intracellular metabolite accumulation indicates that successful expression of CsTKS and CsOAC alone is insufficient to establish productive metabolic flux in *C. reinhardtii*. Similar observations have been reported for other heterologous biosynthetic pathways and have been attributed to limitations in precursor availability, metabolic competition, subcellular localization, and rapid turnover of pathway intermediates [27–29].

We observed no significant differences among the three constructs in their transformation efficiency (Table 1). A limitation of the present study is that detailed enzymatic characterization was performed using one enzymatically active transformant from each construct. Independent transformants can differ in transgene integration site, copy number, and expression level, which may influence enzyme activity and metabolite production. Consequently, the present work should be considered a proof-of-concept demonstrating that CsTKS and CsOAC remain enzymatically active following heterologous expression in *C. reinhardtii*. The C2 construct clone C2-17 exhibited the highest OA level in the enzymatic assay (28.5 mg/L), compared to C0-30 (19.5 mg/L) and C1-10 (16.5 mg/L), respectively. Future studies examining multiple independent biological transformants will be required to quantify transformant-to-transformant variability and determine whether the observed differences among constructs are reproducible. The low transformation efficiency (0.7%) in the C3 construct is likely due to its large size, containing two cistrons, and the electroporation method used. Since we did not directly compare the transformation efficiency of the C3 construct using *Agrobacterium*-mediated transformation and electroporation, we cannot conclusively determine whether the observed low transformation efficiency is due to the transformation method, the large size of the construct, or potential toxicity. Endonucleases can degrade foreign DNA before genome integration [30], with larger constructs being more susceptible. We suggest that future studies evaluate single-gene insertions under constitutive or inducible promoters as a strategy

to improve transformation efficiency and transgene expression. The low efficiency could also be strain-specific, as UV mutant strains (e.g., UVM4, UVM11) have shown higher insertion and transformation rates [31]. Future studies with improved strains may enhance DNA integration. Another approach is using minichromosomes in the chloroplast for integrating larger DNA fragments as independent replicating elements [32]. Finally, the use of inducible promoters may facilitate coordinated transgene expression and improve transformation efficiency. In this study, *Agrobacterium*-mediated transformation was selected because it promotes stable, low-copy T-DNA integration with reduced gene silencing and minimal DNA damage, making it well suited for the wild-type *C. reinhardtii* strain used in this study [33]. For the larger C3 constructs, electroporation was also evaluated and showed higher transformation efficiency than the glass bead method under our experimental conditions [34]. Future studies using methods like biolistic transformation or other direct DNA delivery techniques will help determine whether these approaches offer better insights into gene expression and stability.

The absence of detectable OA production *in vivo* could be due to limitations by precursors for polyketide synthesis, such as hexanoyl-CoA and malonyl-CoA, as reported in bacteria and yeast [9,11,35], or a result of gene silencing [36]. Although CsTKS was shown to be catalytically active *in vitro*, efficient OA biosynthesis also depends on adequate intracellular pools of its CoA-bound substrates, hexanoyl-CoA and malonyl-CoA, as well as effective metabolic flux through the engineered pathway. The untargeted metabolomic approach employed in this study was not designed to detect or quantify CoA thioester intermediates, precluding direct assessment of precursor availability or substrate utilization by CsTKS. Therefore, the absence of detectable OA and downstream metabolites *in vivo* may reflect limitations in precursor availability and metabolic flux rather than a lack of enzyme functionality. Future studies combining targeted analysis of CoA ester pools with metabolic flux analysis will be valuable for identifying the primary bottlenecks limiting heterologous polyketide biosynthesis in *C. reinhardtii*. Another potential reason is that any produced OA may be rapidly converted into other metabolites by endogenous enzymes before detection. A similar observation was previously reported [37], where similar challenges in OA detection were encountered. To address this issue in future studies, increasing the pool of precursors could be achieved by introducing genes or entire pathways responsible for the synthesis of hexanoyl-CoA and malonyl-CoA, or by expressing the enzymes individually under inducible systems to better control their expression. Inducing enzyme expression at specific growth stages when the precursor pool is abundant could enhance OA production. While we cannot completely rule out the possibility of low enzyme expression or insufficient enzyme activity due to the fusion of both enzymes in a single cistron, future studies focusing on controlled separate expression and optimization of conditions may help clarify the reasons for the absence of detectable OA. Additionally, targeting or expressing these enzymes in the chloroplast could improve access to both precursors and may enhance OA detection, given the chloroplast's role in isoprenoid biosynthesis and abundance of geranyl pyrophosphate (GPP) and fatty acids. Future studies utilizing CRISPR/Cas9 to develop novel *C. reinhardtii* strains with reduced gene silencing, such as UVM4 and UVM11 [31], will enable us to explore the potential for higher expression of biosynthetic genes by directly addressing gene silencing issues. This approach will provide valuable insights into whether enhanced gene expression can be achieved *in vivo*, potentially leading to increased production and more effective detection of end-products.

As summarized in Table 2, heterologous production of OA has been demonstrated in bacterial, yeast, fungal, and photosynthetic hosts; however, productive biosynthesis generally requires extensive metabolic engineering to overcome limitations in precursor availability and pathway flux. Across these systems, increasing the intracellular supply of hexanoyl-CoA and malonyl-CoA, optimizing carbon flux, and improving cofactor

availability have consistently enhanced OA production [9,35]. In contrast, although CsTKS and CsOAC remained catalytically active in *C. reinhardtii*, OA was detected only in vitro, indicating that productive intracellular pathway flux is constrained by host metabolism. These findings suggest that, similar to other heterologous platforms, future engineering efforts in *C. reinhardtii* should focus on improving precursor availability and redirecting carbon flux. In addition, alternative enzymatic systems, such as fungal polyketide synthases, may provide complementary strategies for enhancing polyketide biosynthesis in photosynthetic microalgae [38].

The extended pathway from OA, incorporating downstream enzymes for cannabinoid formation in *C. reinhardtii*, is hypothesized to occur through the fusion of an undetectable OA with the endogenous GPP [39], facilitated by the heterologous soluble prenyltransferase (*StNphB*), with subsequent oxidative cyclization by the CBDAS (Figure 1). This process, followed by a non-enzymatic decarboxylation step, results in the production of CBD. A feature tentatively annotated as CBD was detected in one engineered strain (C3-47); however, confirmation using authentic standards and diagnostic MS/MS transitions was insufficient. This signal is therefore not interpreted as evidence of intracellular cannabinoid accumulation. This finding is interesting compared to what was obtained in diatoms using similar constructions, which led to the successful in vivo production of cannabinoids when the cultures were supplemented with 2 mM OA and 2 mM GPP [26]. In contrast, when this pathway was engineered in filamentous fungi *Penicillium chrysogenum*, only low detectable levels of cannabinoids were produced using 1 mM OA and 0.1 mM GPP as substrates. These observations, together with our findings, suggest that precursor availability and metabolic flux remain major constraints for heterologous cannabinoid biosynthesis. Further metabolic engineering will be required to determine whether precursor availability, enzyme localization, or competition with endogenous metabolism limits pathway performance in *C. reinhardtii*.

Untargeted metabolomic profiling provided additional insight into the cellular response to pathway introduction. Engineered strains exhibited extensive metabolic rewiring compared with wild-type and empty-vector controls, indicating that expression of the heterologous pathway altered endogenous metabolism despite the absence of detectable OA accumulation. These perturbations suggest that host metabolic constraints extend beyond a single enzymatic step and involve broader cellular adaptation to heterologous pathway expression.

Although pathway enrichment analysis can provide valuable biological insight, it was not performed in the present study because only a limited number of differentially abundant metabolites could be putatively annotated. Furthermore, metabolite identification was based primarily on spectral matching against the MassBank database and should therefore be interpreted cautiously. Under these conditions, pathway enrichment analysis could introduce bias and lead to overinterpretation of the data. Future studies combining higher-confidence metabolite identification, authentic standards, and improved metabolite coverage will enable robust pathway-level analyses of the metabolic consequences associated with heterologous polyketide biosynthesis.

Although several mass spectral features were tentatively annotated as known bioactive compounds, these assignments could not be confirmed using authentic standards and were therefore not interpreted further. The combined use of enzymatic assays and untargeted metabolomics enabled us to distinguish functional enzyme expression from productive intracellular pathway flux. Collectively, our findings demonstrate that, while *C. sativa* polyketide enzymes remain functional in *C. reinhardtii*, host metabolism limits productive accumulation of pathway metabolites, providing a foundation for future metabolic engineering efforts.

Table 2. Comparison of heterologous platforms for OA biosynthesis.

Host Organism	Engineering Strategy	Reported OA Production	Major Bottleneck Identified	Principal Conclusion	Ref.
<i>E. coli</i>	Heterologous expression of CsTKS and CsOAC	Up to 80 mg L ⁻¹	Limited hexanoyl-CoA and malonyl-CoA supply	Demonstrated microbial OA production and the importance of precursor engineering.	[9]
<i>S. cerevisiae</i>	Complete cannabinoid pathway including CsTKS and CsOAC	~1.5 mg L ⁻¹	Low pathway flux	Demonstrated successful integration of OA biosynthesis into a complete cannabinoid pathway.	[10]
<i>Yarrowia lipolytica</i>	Codon-optimized CsOLS/CsOAC with precursor and cofactor engineering	0.11–9.18 mg L ⁻¹	Carbon flux, precursor availability and cofactor supply	Demonstrated OA production in an oleaginous yeast through extensive host metabolic engineering.	[35]
<i>Phaeodactylum tricornutum</i>	Expression of CsTKS and CsOAC	0.6–2.6 mg L ⁻¹	Limited precursor availability; precursor supplementation required for cannabinoid production	First demonstration of OA production in a photosynthetic microalgal host.	[37]
<i>Aspergillus nidulans</i>	Alternative fungal polyketide synthase system	80 mg L ⁻¹	Efficiency of OA-precursor formation	Fungal polyketide synthases may provide an alternative enzymatic route for OA production	[38]
<i>C. reinhardtii</i> (This study)	Nuclear expression of CsTKS/CsOAC; extended cannabinoid pathway constructs; in vitro enzymatic assays and untargeted metabolomics	OA detected only in vitro (up to 28.5 mg L ⁻¹ in enzymatic assays); no detectable intracellular OA or cannabinoids	Host metabolic constraints limiting productive intracellular pathway flux	Demonstrated that CsTKS and CsOAC remain catalytically active in <i>C. reinhardtii</i> , but enzyme activity alone is insufficient for intracellular OA accumulation, highlighting the need for improved precursor supply and host metabolic engineering.	This study

4. Materials and Methods

4.1. Chemicals

Commercial standards were used in this study. Olivetolic acid (OA, CAS #491-72-5) and olivetol (OL, CAS #500-66-3) standards were obtained from Cedarlane Laboratories (Burlington, ON, Canada) and Millipore Sigma Aldrich (Oakville, ON, Canada), respectively. Δ^9 -tetrahydrocannabinol (Δ^9 -THC, CAS #1972-08-3), cannabidiol (CBD, CAS #13956-29-1), Δ^9 -tetrahydrocannabinolic acid (Δ^9 -THCA, CAS #23978-85-0), cannabidiolic acid (CBDA, CAS #1244-58-2), cannabigerolic acid (CBGA, CAS #25555-57-1), cannabigerol (CBG, CAS #25654-31-3), were purchased from Agilent Technologies (Québec, QC, Canada). Hexanoyl-CoA (CAS #103476-19-3) and malonyl-CoA (CAS #108347-84-8) were purchased from Millipore Sigma-Aldrich (ON, Canada). Geranyl pyrophosphate (GPP, CAS #104715-14-2) was obtained from Cedarlane Laboratories. Stock solutions of each standard were prepared at 100 mg/L (100 ppm) and stored in the dark at -20°C . Analytical HPLC-UV and LC-MS grade methanol (99.9%) (CAS #67-56-1) and formic acid (99%) (CAS #540-69-2) were purchased from Fisher Scientific (Nepean, ON, Canada).

4.2. Chlamydomonas Strains and Culture Conditions

The wild-type cell wall-proficient strain of *C. reinhardtii* (cc137, mating type +) was purchased from the Chlamydomonas Resource Center (Minneapolis, MN, USA; <http://chlamycollection.org>). *C. reinhardtii* cells were grown photo-mixotrophically in liquid Tris-acetate-phosphate (TAP) medium at pH 7.0 and 1.5% agar *w/v* was added for cells grown on solid state [40]. TAP medium was prepared per liter by combining 20 mL of 1 M Tris base, 1.0 mL of Phosphate Buffer II (10.8 g K_2HPO_4 and 5.6 g KH_2PO_4 in 100 mL water), 10.0 mL of Solution A (containing 20 g NH_4Cl , 5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, and 2.5 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ in 500 mL water), 1.0 mL of Hutner's trace elements (Chlamydomonas Resource Center, Minneapolis, MN, USA; <http://chlamycollection.org>), and 1.0 mL of glacial acetic acid. The final volume was brought to 1 L with distilled water, and the pH was adjusted to 7.0. The medium was sterilized by autoclaving. The cells were cultured at 22°C with mild agitation (100 rpm) and under fluorescent white light at $50 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light intensity and a photoperiod of 16 h:8 h (light:dark).

4.3. Bacterial Strains

Agrobacterium tumefaciens strain GV3101 (with C58 chromosomal backbone and Ti plasmid pMP90, carrying rifampicin and gentamicin resistance, respectively) and was provided by Professor Guido Sessa, Tel Aviv University. This strain was grown at 28°C on *Agrobacterium* (AB) minimal medium supplemented with rifampicin 25 $\mu\text{g/mL}$ and gentamycin 50 $\mu\text{g/mL}$. AB-minimal medium was prepared by dissolving 2.5 g of glucose and 7.5 g of agar in 450 mL of double-distilled water, followed by autoclaving. After cooling to $50\text{--}60^\circ\text{C}$, 25 mL of 20 $\times$ AB salts (containing 2.0 g NH_4Cl , 0.6 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.3 g KCl, 0.3 g CaCl_2 or 0.31 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, and 0.005 g $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ per 100 mL) and 25 mL of 20 $\times$ AB buffer (6.0 g K_2HPO_4 and 2.0 g NaH_2PO_4 , or 2.6 g $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$ per 100 mL, pH 7.0) were added aseptically. Appropriate antibiotics were added before pouring the medium into plates. *E. coli* DH5 α strain harboring the binary vector pCambia1304 (Figure 2), which contains a bacterial *HygR* gene driven by a 35S promoter and a *gfp::uidA* fusion as a reporter gene controlled by the same promoter, was used for transformation of the empty-vector pCambia1304 and the recombinant plasmids. Transformed *E. coli* strains were grown at 37°C on Luria–Bertani (LB) supplemented with 50 $\mu\text{g/mL}$ kanamycin used for the maintenance of the plasmids. LB medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L of distilled water. The pH was adjusted to 7.0.

using NaOH, and the medium was sterilized by autoclaving at 121 °C for 15 min. For solid LB medium, 15 g of agar was added before autoclaving.

4.4. Construction of the Expression Plasmids

Synthetic coding sequences of all genes were codon optimized for expression in *C. reinhardtii* C137 and were purchased without signal peptides from Biobasic (Markham, ON, Canada). The oligonucleotides used in this study were synthesized by Integrated DNA Technologies (San Diego, CA, USA). All DNA and primer (forward and reverse) sequences used in this study are listed in Table 3. Recombinant plasmids pCambia1304 with one cistron (C0, C1, and C2) or two cistrons (C3) were constructed (Figure 2) and contain an extended FMDV-2A (foot-and-mouth-disease-virus 2A self-cleavage peptide) [41]. Cistrons of the constructs carried the *heat shock 70A-Rubisco small* subunit two fusion promoter with Rubisco small subunit intron 1 (collectively *HSP70A-RBCS2-i1*) and *RBCS2* 3' untranslated region (3' UTR) as regulatory elements [42].

Table 3. Primers used in this study. Bold and underlined letters indicate restriction sites.

Primer	Sequence (5'–3')	Gene Description
HPH_FP	CTATTTCTTTGCCCTCGGACGAGTGC	<i>E. coli</i> Hygromycin phosphotransferase
HPH_RP	ATGAAAAAGCCTGAACTACCGCGA	
GUS_FP	AGTTTACGTCCTGTAGAAACCCCAACC	<i>GUS: β-glucuronidase A</i>
GUS_RP	GCGAGTGAAGATCCCTTTCTTGTTACC	
mGFP_FP	ATGGTAGATCTGACTAGTAAAGGAGAAG	<i>Green fluorescent protein</i>
mGFP_RP	AGCTTTGTATAGTTCATCCATGCCATG	
TKS-OAC_FP_KpnI	AACGGGTACCAGCGAGTCAGTGAGCGAGGAAG	<i>6His-TKS-2A-OAC-Myc</i>
TKS-OAC_RP_EcoRI	ACGCGAATTCGTGTCATAGCGCAAGAAAGACATTC	
NphB-CBDAS_FP-XbaI	AAGTTCTAGAGTATTTGAAGCGGGATCCTTCGAACGTAC	<i>Flag-NphB-2A-CBDAS-HA</i>
NphB-CBDAS_RP-HindIII	TACTAAGCTTTGTCAAGCCTCAGCACGCGTATTTAA	
Aph7_FP	ATCAAGCTGTTTCGGCGAGC	<i>Streptomyces hygroscopicus</i> aminoglycoside phosphotransferase
Aph7_RP	CGAAGTCGTGCAGGAAGGTG	
PGK_FP	AGTCCGAACAACCCACTTAC	<i>Phosphoglycerate kinase</i>
PGK_RP	CAGAGCTCAGGAGGTGAAATAG	

Cassettes of constructs C0 (*CsTKS-extFMDV2A-CsOAC*), C1 and C2 (*6His-CsTKS-extFMDV2A-CsOAC-Myc*) were inserted in the binary vector pCambia1304 at the 3' region of the sequence using EcoRI/KpnI restriction sites. For C0 and C1, *CsTKS-extFMDV2A-CsOAC* were designed without introns, whereas construct C2 has three introns (Appendix A—Table A1). Construct C3 is composed of two cistrons; the first is the same as the C2 construct and the second cistron (*Flag-StAPT-extFMDV2A-CBDAS-HA*), designed without introns, was inserted in the binary vector pCambia1304 at the 3' region of the sequence using XbaI/HindIII restriction sites (Figure 2). Since the hygromycin resistance gene (*HygR/HPH*) in pCambia1304 is not codon-optimized for *C. reinhardtii* and is driven by the 35S promoter, we cloned a *C. reinhardtii*-optimized *aphVII* (*aph7*) gene, which confers hygromycin B resistance, from the pOpt_mRuby2_Hyg construct [16]. The *aph7* gene, along with the *Hsp70A-Rbc-S2p* promoter and terminator, was amplified from the pOpt_mRuby2_Hyg construct and inserted as a BamHI/KpnI fragment into the respective site of pCambia1304, generating the final vectors for transformation. All constructs (C0, C1, C2, and C3) contained two hygromycin resistance genes in the transfer DNA (T-DNA) region: the plant-optimized *HygR/HPH* gene driven by the 35S promoter and the *aph7* gene driven by the hybrid constitutive *Hsp70A-Rbc-S2* promoter (Figure 2). *A. tumefaciens* strain GV3101 was transformed with the constructs and then screened by colony PCR by amplification of four genes (*β-glucuronidase A* (*GUS*), *Green fluorescent protein* (*mGFP*),

Hygromycin phosphotransferase (HygR/HPH), and *TKS-extFMDV2A-OAC*) as shown in Appendix A—Figure A9.

4.5. *Agrobacterium*-Mediated Transformation

A. tumefaciens strains GV3101, *C. reinhardtii* C137 strain and binary vector pCAMBIA 1304 (empty or recombinant vector) were used for nuclear gene insertion. The transformation was done following the methodology described by P Mini, OC Demurtas, S Valentini, P Pallara, G Aprea, P Ferrante and G Giuliano [15]. *Agrobacterium* transformed with pCAMBIA1304 vectors (empty or recombinant containing the gene of interest) was inoculated in 15 mL of AB-minimal medium (pH 7) containing 0.5% (*w/v*) glucose, 30 µg/mL rifampicin, and 100 µg/mL kanamycin (in 50 mL tubes). The culture was incubated at 28 °C overnight under agitation at 220 rpm. After 24 h, the bacterial culture was centrifuged at 2700 g for 30 min and the pellet was resuspended in 40 mL of AB induction medium (AB medium containing 0.5% glucose, 20 µM MES pH 5.6 and 100 µM acetosyringone) and grown overnight at 28 °C. *Agrobacterium* transformants were confirmed for all the intact genes, namely *GUS*, *mGFP*, *HPH*, and *TKS-OAC*, by colony PCR using different primers (Table 3, Appendix A—Figure A9). In parallel, 50 mL of *C. reinhardtii* culture was grown for 2 days in TAP medium under continuous white-fluorescent light at an intensity of 50 µmol m⁻² s⁻¹. *C. reinhardtii* cells were harvested at the early exponential phase by centrifugation at 1500 g for 5 min, then resuspended in 5 mL of AB-minimal medium at a final cell density of 10⁸ cells/mL. *Agrobacterium*-mediated transformation was selected for its ability to deliver lower-copy number T-DNA insertions with reduced gene silencing risk, particularly relevant for our wild-type *C. reinhardtii* strain.

For co-cultivation, 100 µL of microalgae corresponding to 10⁷ cells and 40 µL of *Agrobacterium* were spotted on 100 mm 1% agar AB induction medium plates. In total, 14 spots, corresponding to 10⁷ *C. reinhardtii* cells, were plated on each 1% agar AB induction medium plate and then incubated at 30 µmol m⁻² s⁻¹ for 48 h. The transformed cells were then harvested from each plate, washed twice with liquid TAP medium containing 500 µg/µL cefotaxime, centrifuged at 1500 g for 5 min, resuspended in liquid TAP medium at a density of 5 × 10⁷ cells/mL and plated on two 100 mm TAP agar plates containing the appropriate antibiotic selection (10 µg/mL of hygromycin), also supplemented with 500 µg/µL cefotaxime and 500 µg/µL carbenicillin. After two weeks, antibiotic-resistant colonies were transferred for three subculture rounds (7–10 days each) on 1.5% TAP agar plates containing the selective antibiotics and then analyzed by high-throughput colony PCR (HT-colony-PCR) as described previously [34].

4.6. *Chlamydomonas* Electroporation

The electroporation process was performed as described previously [34] with a few modifications. *C. reinhardtii* wild-type cells were cultured in 50 mL liquid TAP media and were grown in 125 mL Erlenmeyer flasks with an initial optical density (OD_{750nm}) of 0.1 to a final of 0.7 (~7 × 10⁶ cells/mL) for a one-week incubation. Cultures were incubated under 100 rpm agitation at a temperature of 22 ± 0.5 °C, photoperiod of 16 h:8 h (light:dark) at a light intensity of 50±10 µmol photons m⁻² s⁻¹ and relative humidity of 50%. These conditions were used during the electroporation process and after transformation. Cells were harvested by centrifugation at 7000 g for 5 min at 4 °C, then the pellet was washed 3 times with 5 mL of Invitrogen MAX Efficiency™ transformation Reagent for Algae (Burlington, ON, Canada). After the final centrifugation, 250 µL (~10⁵ cells/mL) of *C. reinhardtii* cells were incubated on ice for 5 min with 1 µg of linearized plasmid DNA with ScaI (linearized plasmid DNA was purified by gel extraction). Then, 250 µL of the cell-DNA mix was transferred into an ice-cold 4 mm cuvette for transformation. Electroporation parameters, on the Bio-Rad Gene Pulser Xcell™ electroporation system (Saint-Laurent, QC, Canada),

were used as follows: 0.5 kV, capacitance at 5 μ F and resistance at 800 Ω . Transformed cells were transferred into 5 mL TAP liquid medium supplemented with sucrose (50 mM) and incubated for 18 h at room temperature; then they were centrifuged at 7000 g for 5 min. The pellet was solubilized in 250 μ L of MAX Efficiency™, plated into TAP agar media with 10 μ g/mL hygromycin and incubated as described above for two weeks.

4.7. PCR Screening of Positive *Chlamydomonas* Transformants

Primer sequences used for the amplification of all the genes cited above are listed in Table 3. Selection of the positive clones after transformation and after 3 rounds of antibiotic selection (Appendix A—Figure A1) was done by HT-colony-PCR as described previously [34]. Multiplex PCR was done by the amplification of the integration *Pro::TKS-OAC:3'UTR* (Appendix A—Figure A1) of the three different constructs (with and without intron) of 2488 bp, 2584 bp and 3019 bp, *HYG^R* gene (506 bp) and the nuclear phosphoglycerate kinase (*CrPGK*) gene (as reference and positive control of genomic DNA extraction) at a size of 855 bp.

4.8. Total Protein Extraction and Enzymatic Assay

To verify if the produced heterologous enzymes are active, total proteins were extracted, and in vitro enzymatic assays were performed. For this, microalgae *C. reinhardtii* WT and transformants were cultured in 50 mL liquid TAP media without and with 10 μ g/mL of hygromycin, respectively, and were grown in 125 mL Erlenmeyer flasks with an initial OD_{750nm} of 0.1 to a final OD_{750nm} of 0.7, in approximately one week. Temperature condition of 22 ± 0.5 °C under a photoperiod of 16 h:8 h (light:dark) at a light intensity of 50 ± 10 μ mol photons $m^{-2} s^{-1}$ and relative humidity of 50%. Total protein extraction was done as described by L Soto-Sierra, LR Wilken and CK Dixon [43]. Cells from 25 mL culture were harvested by centrifugation at 3500 g for 5 min at 4 °C. The pellet was washed with 500 μ L solubilization buffer containing 51.4 mM Tris-HCl pH8, 0.75 mM lithium dodecyl sulfate, 0.02 mM EDTA and 10% glycerol. The solubilized cell pellet samples were flash frozen in liquid nitrogen and then stored at -80 °C for further use. To proceed with protein extraction, samples were thawed on ice, a volume of 5 μ L of protease inhibitor (NEB, cat. #5871S) was added and 50 μ L of PMSF (Sigma, cat. #P7626) was added. The samples were then sonicated at an amplitude of 35% 6 times: 30 s ON-30 s OFF for 3 min using Fisherbrand™ Model 505 Sonic Dismembrator (Thermo Fisher Scientific, Saint-Laurent, QC, Canada). The sample was then centrifuged at 14,000 g at 4 °C for 30 min. After centrifugation, the supernatant from each sample was transferred to a new Eppendorf tube. Protein quantification was done using the DC Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. For the detection of OA, the enzymatic assay mix was prepared for a final volume per reaction of 100 μ L as follows: 200 μ M hexanoyl-CoA (Sigma, cat. #H2012, Oakville, ON, Canada), 2 mM malonyl-CoA (Sigma, cat. #M4263), 5 mM DTT (Fisher, cat. #BP172-5, Nepean, ON, Canada), 200 μ g total protein extract, 50 mM HEPES buffer (pH 7.5) (Sigma, cat. #83264, Oakville, ON, Canada). After 16 h at 25 °C, the reaction was stopped by adding 10 μ L of 1.224 mM trichloroacetic acid (TCA) (Fisher, cat. #AC152130010, Nepean, ON, Canada). Substrates without protein extract and total protein extracts from transformed clones and wild-type (WT) without substrates were used as negative controls. Enzymatic assays of cannabinoids (CBG, CBGA, CBD and CBDA) were all carried out using 200 μ g of total protein extract, as mentioned above. The enzyme reaction for CBGA production was composed of 100 mM HEPES (pH 7.5), 25 mM MgCl₂, 2 mM OA, and 2 mM GPP, and was incubated for 16 h at 30 °C for CBGA production, while the reaction for CBDA detection was composed of 5 mM MgCl₂, 5mM KCl and 1mM FAD + 0.27 mM substrate CBGA in citrate buffer pH 5. The reactions were stopped by adding 10 μ L of 1.224 mM TCA. Targeted metabolites were

then analyzed by high-performance liquid chromatography coupled with a diode-array detector (HPLC-DAD). Metabolite identification was confirmed by LC-MS/MS mass spectrometry.

4.9. Targeted Metabolomics

A targeted metabolomic analysis was performed to detect OA, OL and selected cannabinoid (THC, CBD, THCA, CBDA, CBGA, CBG) pathway metabolites using authentic standards and predefined MRM transitions.

Extraction—A volume of 30 mL of cell culture was centrifuged at 3500 g for 10 min at 4 °C; the supernatant was discarded, and conical tubes were inverted to dry. Extractions were performed by adding ethanol 95% (cat. #P016EA95) at a ratio of 1 mL per 100 mg of biomass; the sample was vortexed for 10s and incubated overnight at −20 °C. The extract (supernatant) was then separated from the pellet by centrifugation at 3500 g for 10 min at 4 °C; samples were filtered using 0.22 µm nylon syringe filters (Thermo Scientific, cat. #CH2225-NN) and were stored at −20 °C for further analysis.

Metabolite detection—Metabolite extracts from *C. reinhardtii* transformants and WT were detected and analyzed compared to standards by an HPLC-DAD system (i.e., Shimadzu Prominence-I LC-2030C) equipped with a Poroshell 120 EC-C18 column (4.6 × 75 mm, 2.7 µm; Agilent Technologies, Mississauga, ON, Canada) that was set at 30 °C during analysis. The injection volume was 10 µL. Chromatographic separation of targeted metabolites was performed using mobile phase A (Milli-Q water containing 0.1% formic acid *v/v*) and mobile phase B (methanol containing 0.1% formic acid *v/v*) and a flow rate of 1 mL/min. The gradient elution program used was as follows: 70% B from 0 to 1 min, 23:70 for 5 min, 10:90 for 9 min and 30:70 for 3 min. Compounds were analyzed at 220 nm. Compound identification on HPLC-DAD was performed by comparison of retention time and UV spectrum of signals obtained in samples with those of authentic standards.

Confirmation of the identification of the molecule of interest was done using an HPLC system coupled with a tandem mass spectrometer (MS/MS) (Agilent, QC, Canada) equipped with an Agilent Jet Stream ionization source, a binary pump, an autosampler and a column compartment. Compound separation was achieved using an InfinityLab Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 µm; Agilent Technologies, QC, Canada). Prior to their injection, samples were vortexed, centrifuged for 10 min at 12,000 g and diluted 10-fold in mobile phase (formic acid 0.1% in Milli-Q water and formic acid 0.1% in methanol (30:70)). Five microliters of each sample were injected onto the column that was set at 50 °C. A gradient method made of (A) formic acid 0.1% *v/v* in Milli-Q water and (B) formic acid 0.1% *v/v* in methanol with a flow rate of 0.5 mL/min was used to achieve chromatographic separation. The HPLC elution program used was as follows: 0 min, 70% B; 7.0 min, 100% B; 10.0 min, 100% B; 12.0 min, 70% B. The total run time of each sample was 14 min to allow the reconditioning of the column prior to the next injection. Mass spectrometry was performed in positive ion mode and the parameters used in the MS/MS source were as follows: gas flow rate 8 L/min, gas temperature 220 °C, nebulizer 55 psi, sheath gas flow 12 L/min at a temperature of 380 °C, capillary voltage 4500 V and nozzle voltage 0V. Agilent MassHunter Data Acquisition version 1.2 and MassHunter Qualitative Analysis version 10.0 software were used for data acquisition and processing, respectively. Sample analyses were carried out in triggered multiple reaction monitoring (tMRM) acquisition mode, allowing compound identification using authentic standards. Table 4 shows MRM transitions and MS/MS parameters used for targeted compound identification.

Table 4. Optimized instrumental parameters used for LC-MS/MS analyses in ESI⁺ mode. Retention Time (RT) window: 0.8 min. See Supplementary File S1—Data S1 for abbreviations, list of standards, acronyms used, CAS number, and solvent used.

Compound	RT (min)	Parent Ion (m/z)	Fragmentor (V)	CE (V)	Product Ion (m/z)
OA	3.960	225	75	10	207
				18	123
				18	189
OL	3.797	181	75	10	111
				10	71
				26	93
THC	8.890	315	80	22	193
				34	123
				18	135
CBD	7.423	315	75	22	193
				34	123
				18	135
THCA	9.985	359	80	14	341
				34	219
				26	285
CBDA	7.620	359	80	10	341
				30	219
				26	261
CBGA	7.955	361	80	26	219
				10	343
				10	237
CBG	7.337	317	80	14	193
				34	123
				10	207

4.10. Untargeted Metabolomics

An untargeted metabolomic analysis was conducted to compare global metabolic profiles between engineered strains and controls and identify differentially abundant metabolic features.

Extraction—Metabolite extraction was done according to Lee and Fiehn [44] with minor modifications. A volume of 30 mL of cell culture was centrifuged at 3500 g for 10 min at 4 °C; the supernatant was discarded, and conical tubes were inverted to dry. A volume of 1 mL of methanol:chloroform:water 5:2:2 was added to 100 mg of wet biomass (ratio 1 mL/100 mg), the sample was vortexed for 10 s and incubated overnight at −20 °C. The total metabolite extract (supernatant) was separated from the pellet by centrifugation at 3500 g for 10 min at 4 °C, transferred to a clean tube, then dried with nitrogen. Dried extract samples were then weighed and kept at −80 °C for further analysis. All culture samples and extraction were prepared in triplicate.

Data Acquisition—Dried metabolite extracts were sent to PhenoSwitch Bioscience for untargeted metabolomics. The dried samples were resuspended in 100 µL of pure water for reversed-phase (RP) analysis and the others identified with (B) were resuspended in 100 µL of reconstitution solvent (ACN: H₂O (95:5) + 10 mM NH₄ NH₄HCO₂, pH 3.8) for HILIC analysis. Samples were vortexed and sonicated for 2 min, then centrifuged at maximum speed for 5 min. The supernatants were then collected and diluted 1:100 with the same solvents. All diluted samples were filtered using 0.45 µm AcroPrep filters under 5

mm Hg vacuum. The acquisition was performed with an Eksigent μ U HPLC (Eksigent, Redwood City, CA, USA) coupled to an ABSciex TripleTOF 6600 (ABSciex, Foster City, CA, USA) system equipped with an electrospray interface. The software Analyst TF version 1.8 was used to control the instrument and for data processing and acquisition. The acquisition was performed in SWATH mode and Data Independent Acquisition (DIA) for positive ionization mode. The source voltage was set to 5.5 kV and maintained at 220 °C, with curtain gas at 50 psi, ion source gas 1 at 40 psi, and ion source gas 2 at 50 psi. For both RP and HILIC columns, the mobile phases used consisted of the following solvents: A ($\text{H}_2\text{O} + 0.1\%\text{Formic acid (FA)}$ and $\text{H}_2\text{O} + 10 \text{ mM NH}_4\text{HCO}_2$, respectively) and solvent B ($\text{ACN } 0.1\%\text{FA}$ and $\text{ACN: H}_2\text{O (95:5)} + 10 \text{ mM NH}_4 \text{ HCO}_2$, respectively). Both columns, Luna Omega Polar 100x2.1 1.6 μm (RP) and BEH Z-HILIC 100x2.1 Particle 1.7 μm HILIC from Waters, were maintained at 50 °C. The gradient started at 0% B for RP runs and 100% for HILIC runs.

Eleven standards (cannabinoids and their precursors) were given at 100 mg/L (Supplementary File S1—Data S1) to PhenoSwitch Bioscience to run them under the same metabolite detection conditions. All standards were diluted to a 0.5 ppm final concentration in water/MeOH (50:50) prior to their injections. Eight out of 11 standards were detected in ESI⁺ (HILIC and reversed-phase). Also, the standard mix was spiked at different concentrations in a wild-type matrix sample and diluted with water:MeOH (50:50); 8 out of 11 standards were detected at 0.05, 0.1, and 0.25 ppm final concentration in the RP mode acquisition. Data was analyzed with PeakView version 2.2. The same result was obtained from HILIC data, except that spiked olivetol was not detected under these experimental conditions. Metabolite extract from the WT strain was used as a negative control since no cannabinoid standards used in this study were detected in WT. Also, filtered and unfiltered standard mixtures were tested, and there was not much difference between the filtered and unfiltered standard mixture signals (Supplementary File S1—Data S2). Bioinformatics Analysis—The raw mass spectrum data (MetaboLights accession MTBLS9692 (www.ebi.ac.uk/metabolights/MTBLS9692, last accessed on 28 July 2026)), obtained in profile mode, were processed with the software MS-Dial version 4.80 [45–47] with the following parameter settings: retention time range of 0–20 min; mass range between 50 and 1250 Da; MS1 and MS2 tolerances of 0.01 Da and 0.025 Da, respectively; minimum peak height and width at 100 amplitude and 5 Da, respectively; linear-weighted moving average as the smoothing method, at smoothing level of 3 scans and mass slice width of 0.1 Da; sigma window value of 0.5. For identification, we used accurate mass tolerance for MS1 and MS2 of 0.01 Da and 0.05 Da and retention time tolerance of 0.1 min; the adduct ions selected were $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{NH}_4]^+$; the parameters for the alignment of our data to the MS-Dial[®] ESI⁺ spectral library (were the default of MS-Dial, except for the tolerances for retention time and MS1 and MS2 masses, which were set to 0.5 min, 0.015 Da and 0.05 Da, respectively. MS-dial analysis result tables for both RP and HILIC are available in Supplementary File S1—Data S2 (Reversed-phase, HILIC). Subsequent analyses were performed with R software version 4.3.1 [48]. Annotations with MS/MS matches and total annotation score > 70% were maintained; otherwise, the analyte was considered as “unknown”.

We used the MAFFIN algorithm [49] for sample normalization, with intensity threshold = 1000. Then, for each analyte, the abundance estimate was defined as $peak_area_{sample} - (3 * peak_area_{blank})$. If this estimate ≤ 0 , the analyte was considered absent. For each strain, analytes present in a single replicate were considered absent. We selected for further analysis the analytes that were considered present in at least one strain. We then verified the homoscedasticity across the measurements in each strain and performed either ANOVA followed by Tukey[®] test or Kruskal–Wallis[®] test followed by Dunn[®] test to check for differential abundance of the analyte. Fold change was calculated

as $\log_2 \left(\frac{\text{mean}_{\text{sample1}} + 1}{\text{mean}_{\text{sample2}} + 1} \right)$. Analytes were considered different between strains if $|\text{fold change}| > 1.5$ and $p\text{-value} < 0.05$. These statistical analyses were performed with the packages multcomp version 1.4 [50] and FSA version 0.9.4 [51].

4.11. Use of Generative Artificial Intelligence (GenAI)

During the preparation of this manuscript, the authors used ChatGPT (OpenAI, GPT-5.3) to assist with refining the clarity and structure of the text, specifically for enhancing readability and improving sentence flow. The tool was not used to generate scientific content, data, or graphics, nor did it contribute to study design, data collection, analysis, or interpretation. All content was carefully reviewed, edited, and approved by the authors, who take full responsibility for the final version of the manuscript.

5. Conclusions

This study demonstrates that *Cannabis sativa* polyketide biosynthetic enzymes can be stably expressed and remain functionally active in the green microalga *C. reinhardtii*. In vitro enzymatic assays confirmed OA synthesis catalyzed by heterologously expressed CsTKS and CsOAC. However, the absence of detectable intracellular accumulation of OA and downstream pathway products underscores intrinsic metabolic constraints that limit productive flux in a photosynthetic microalgal host. Untargeted metabolomic analyses revealed extensive metabolic rewiring associated with pathway introduction, indicating that expression of plant polyketide enzymes induces broad cellular responses even in the absence of product accumulation. Together, these findings highlight a clear distinction between functional enzyme expression and effective metabolic integration, emphasizing the importance of systems-level considerations when transferring plant specialized metabolic pathways into microalgae. By identifying biological constraints rather than production outcomes, this work provides experimental insight into the challenges of implementing plant polyketide metabolism in photosynthetic microorganisms. These results establish a foundation for future efforts aimed at overcoming pathway bottlenecks through coordinated metabolic and cellular engineering strategies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal16080713/s1>. Supplementary File S1: Data S1 and Data S2. The Supplementary File S1 contains details and data related to the metabolomic analysis, including Data S1. Standard list: list of standards, acronyms used, CAS number, and solvent used. Standard detection: optimization of the concentration of standards for detection with HPLC-MS. Data S2. Summary-Annotated analytes: putative annotation of analytes from untargeted metabolomic analysis of reversed-phase (RP) and HILIC in ESI⁺. Reversed-phase: MS-Dial raw abundance file for untargeted metabolomic analysis of reversed-phase in ESI⁺. HILIC: MS-Dial raw abundance file for untargeted metabolomic analysis of HILIC in ESI⁺.

Author Contributions: B.B.M.: Conceptualization, data curation, formal analysis, investigation, methodology, Writing—original draft, Writing—review and editing; K.C.G.d.S.: data curation, formal analysis, visualization, Writing—original draft, Writing—review and editing; R.B.: Conceptualization, investigation, methodology, resources, Writing—review and editing; S.B.N., D.P.S., M.G. and A.C.: Investigation, methodology, Writing—review and editing; S.-E.G.: data curation, investigation, Writing—review and editing; F.M.-M.: Conceptualization, Project administration, Validation, Writing—original draft, Writing—review and editing; I.D.-P.: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing—original draft, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All the data pertaining to the present study have been included in table and/or figure form in the present manuscript and authors are pleased to share analyzed/raw data and plant materials upon reasonable request. Raw data from untargeted metabolomic analysis were deposited in MetaboLights [52] under the accession MTBLS9692 (www.ebi.ac.uk/metabolights/MTBLS9692, last accessed on 28 July 2026).

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

Abbreviations

The following abbreviations are used in this manuscript:

CBDA	Cannabidiolic acid
CBD	Cannabidiol
GPP	Geranyl pyrophosphate
LC-MS	Liquid chromatography–Mass-spectrometry
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
OAC	Olivetolic acid cyclase
TKS	Tetraketide synthase

Appendix A

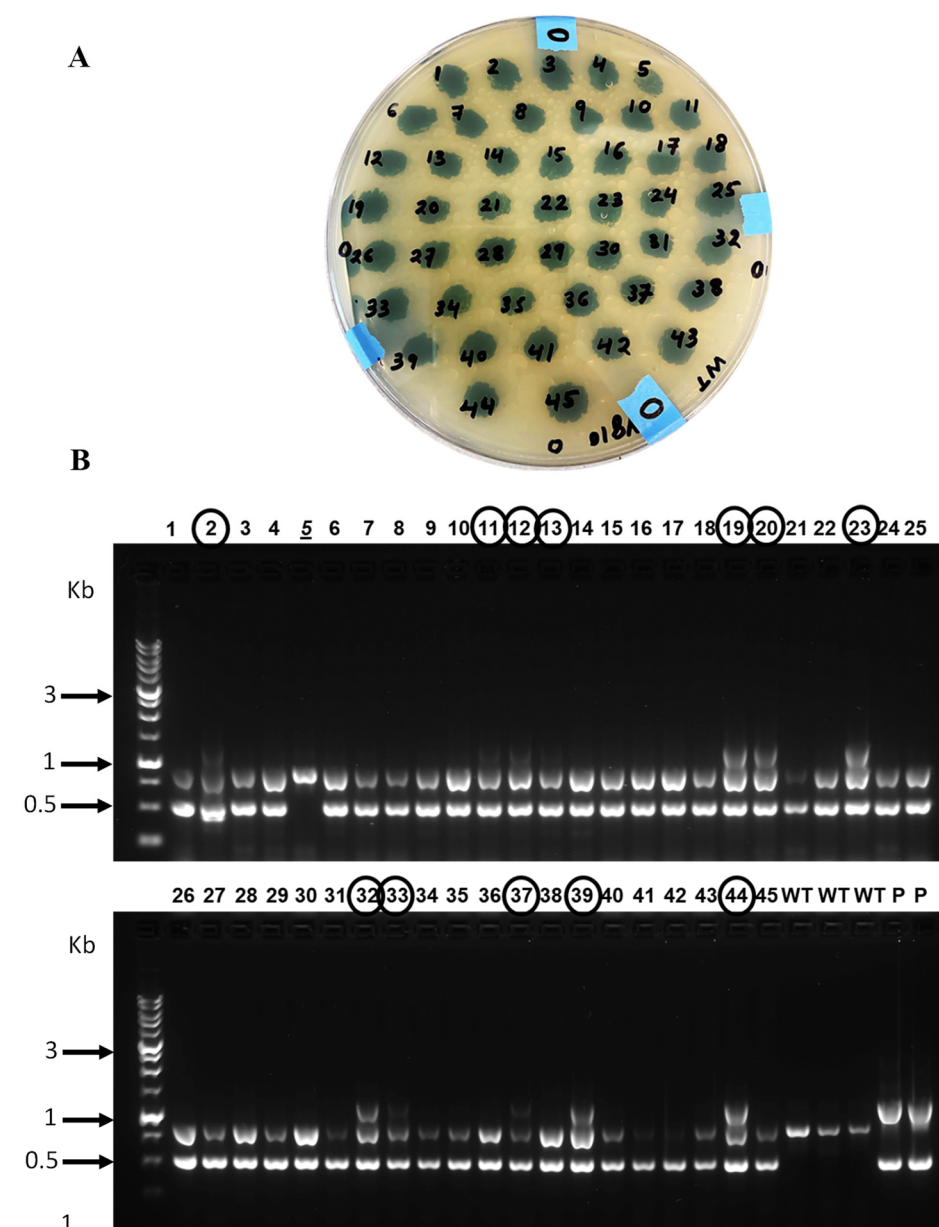


Figure A1. Selection of genetically transformed *C. reinhardtii*. (A) Example of transformed *C. reinhardtii* growing on an antibiotic-selective TAP agar plate. (B) stained two SYBR safe agarose gel (1%) showed multiplex amplification of amplicons of 45 colony samples: *phosphoglycerate kinase* (PGK) gene PCR amplification at 855 bp; *TKS-OAC* gene of interest PCR amplification at 1212 bp and *aminoglycoside phosphotransferase* (*HYG^R*): 506 bp. WT *C. reinhardtii* was used as a negative control; P: recombinant plasmid used as a positive control. PCR samples were migrated at 100 volt/cm for 60 min. Colony numbers are shown on top of the wells. M: 1kb DNA ladder, FroggaBio cat #DM010-R500.

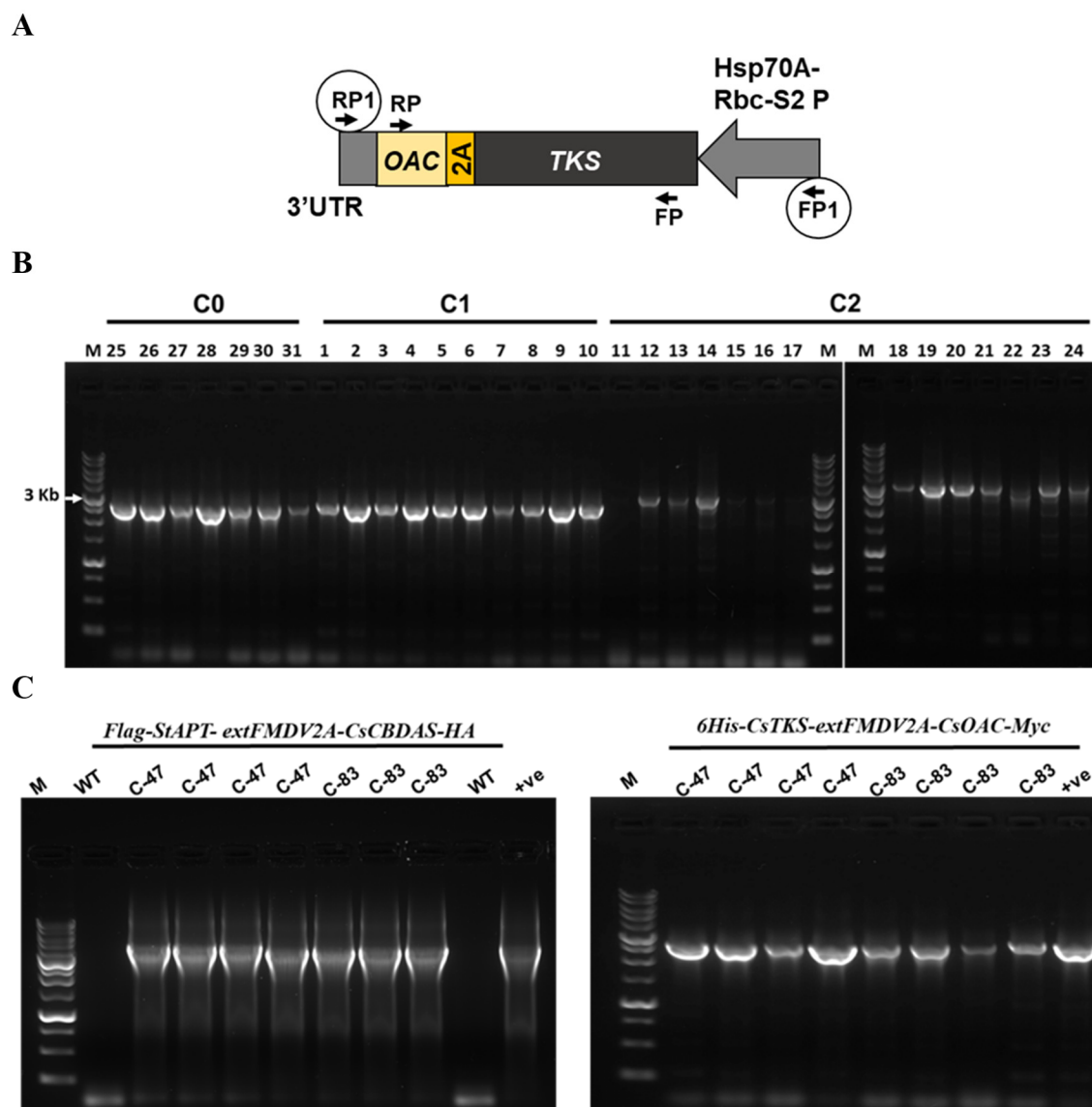


Figure A2. Stained SYBR Safe agarose gel (1%) showing the amplicons of genetically transformed *C. reinhardtii* clones. **(A)** Scheme of the full cassette (*Pro::TKS-OAC:3'UTR*) showing FP1 and RP1 primers, from promoter *Hsp70A-Rbc-S2* to terminator *3'UTR*. **(B)** Shows the amplification of full cassette amplicons of 31 colony samples: 7 clones for cassette C0 with a size of 2488 bp, 10 clones for cassette C1 with a size of 2584 bp and 14 clones for cassette C2 with a size of 3019 bp. **(C)** showed the amplification of the two citrons of construct C3 (left gel, expected size of 4701 pb and right gel 2064 pb) from two positive clones. Colony numbers are shown on top of the wells. WT: untransformed *C. reinhardtii* was used as a negative control, +ve: DNA plasmid used as a positive control, M: 1kb DNA ladder, FroggaBio cat #DM010-R500.

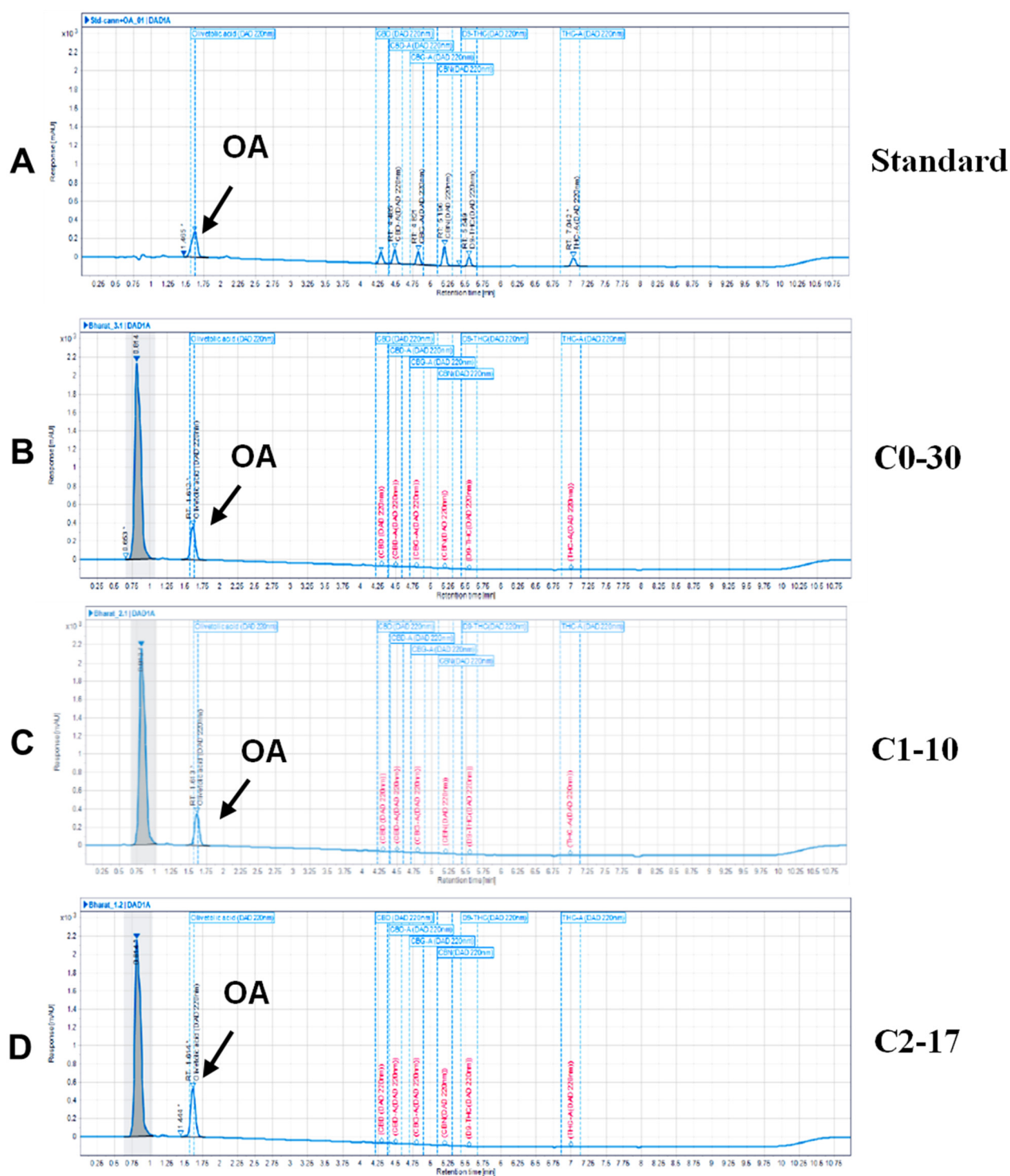
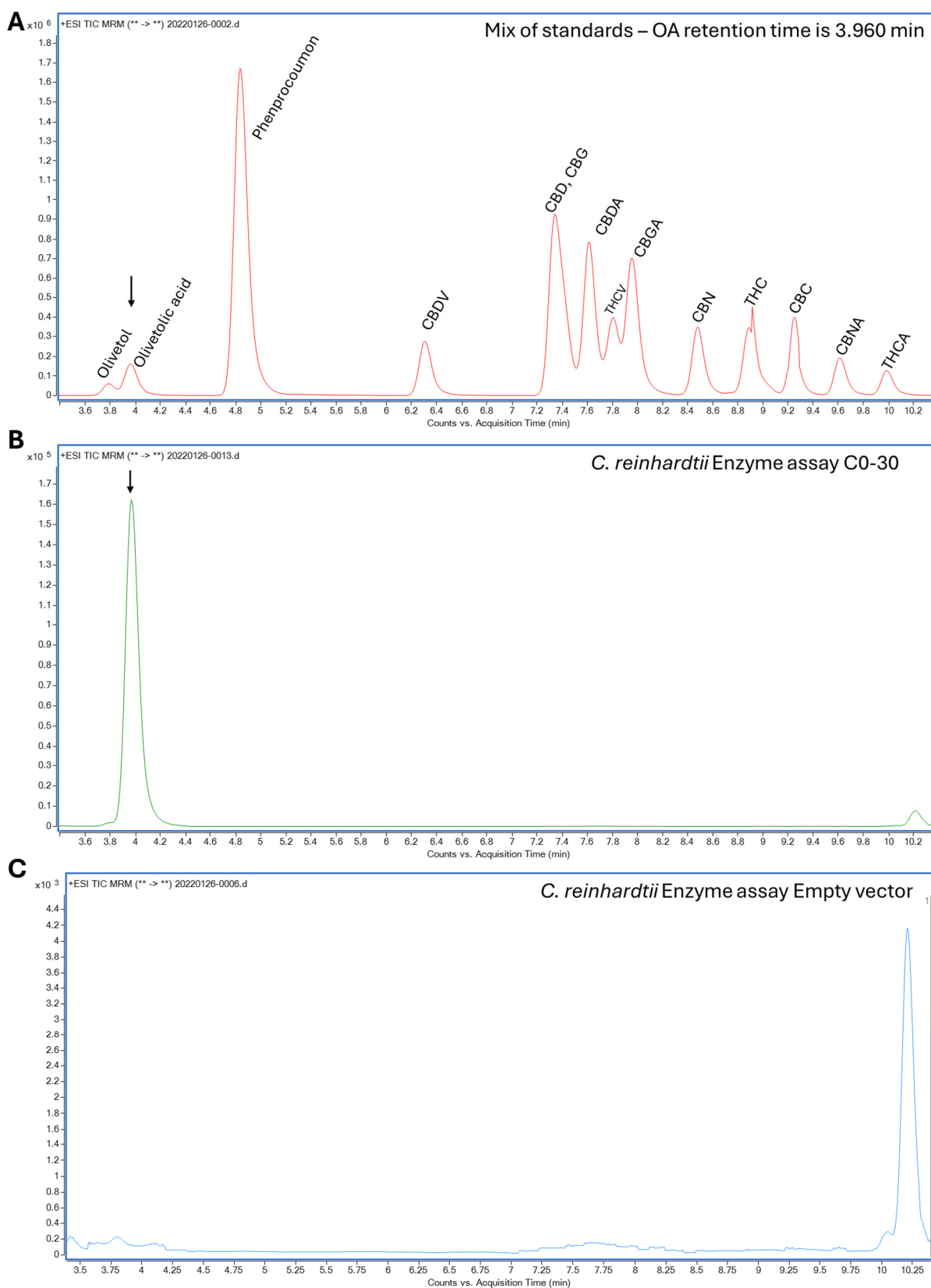


Figure A3. HPLC analysis of in vitro enzyme assay from total protein extracted from *C. reinhardtii* transformants. OA detection by HPLC-DAD at 220 nm. (A) 10 ppm of commercial standards, (B,C and D) in vitro enzymatic assays of clones C0-30, C1-10, and C2-17, respectively.



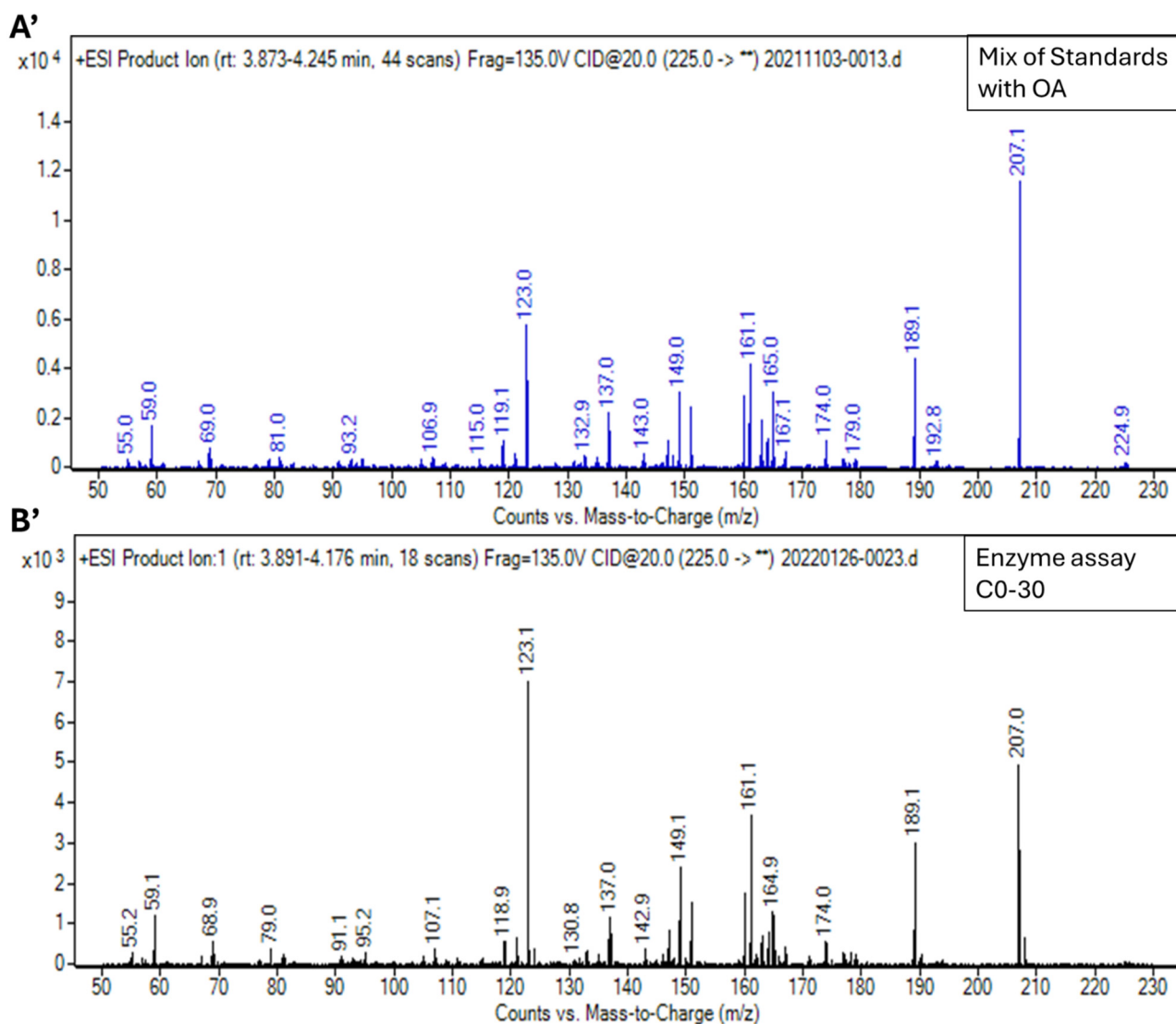
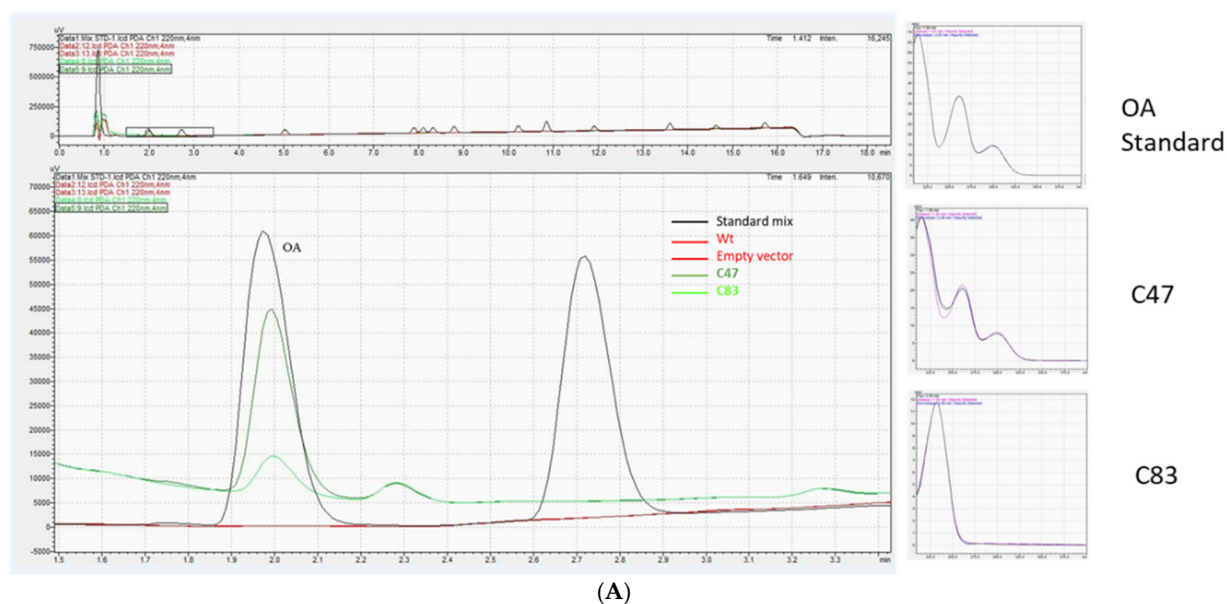


Figure A4. HPLC-MS/MS analysis of in vitro enzyme assay from total protein extracted from *C. reinhardtii* transformants. OA production confirmed by HPLC-MS/MS. Chromatograms (A–C) and Fragmentation patterns (A',B') of OA (standard, upper panels (A,A')) and sample from enzymatic assay of clone C0-30 (lower panels (B,B')) and negative control empty vector.



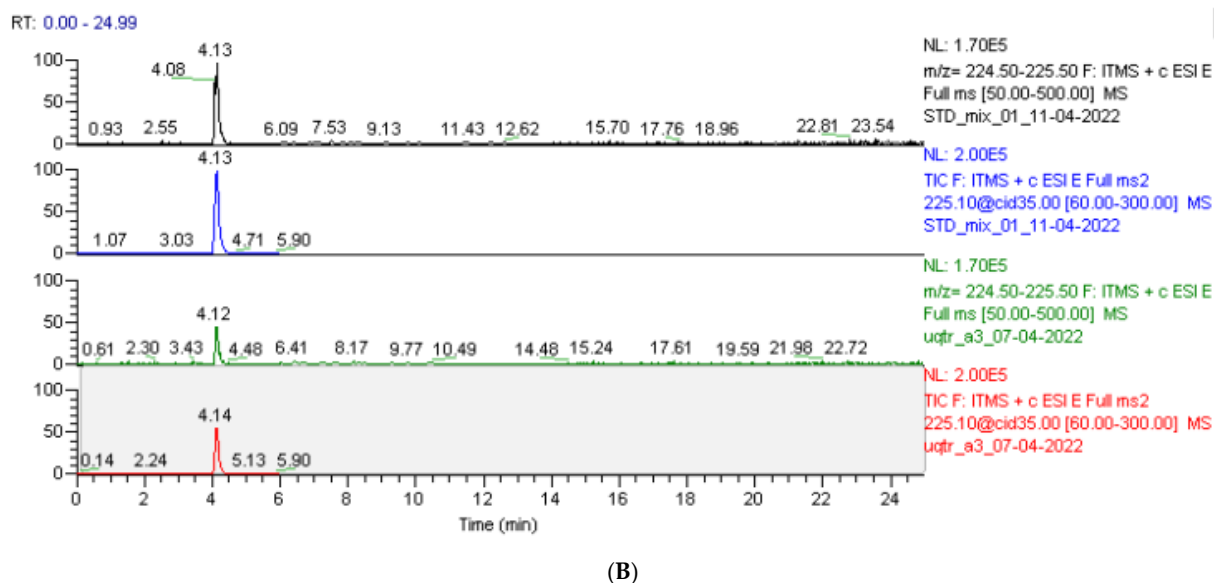


Figure A5. OA detection and identification in samples from in vitro assays. (A) HPLC-DAD at 220 nm from 10 mg/mL of commercial standard and (C3-47, C3-83, Empty Vector (EV)) and Wild-Type (WT). (B) OA production confirmed by HPLC-MS. Full MS and MS2 of OA standard and from in vitro assay of clone C3-47.

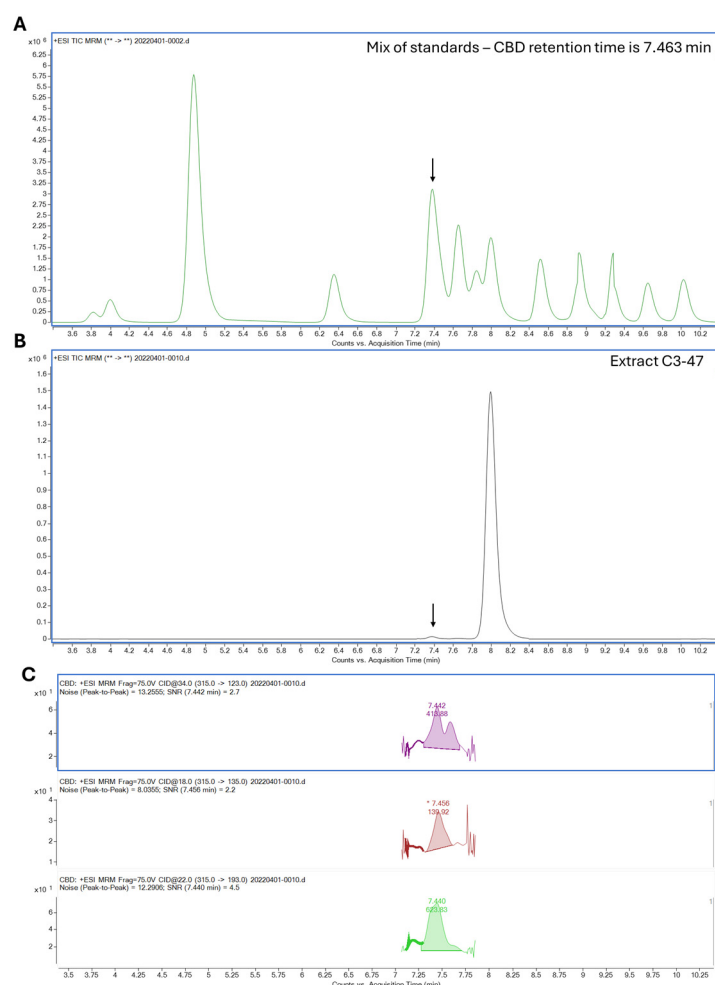


Figure A6. HPLC-MS/MS analysis of C3-47 metabolite extracts. Total Ion Chromatogram (TIC) in positive electrospray ionization (ESI⁺) analyzed in multiple reaction monitoring (MRM) detection

mode of (A) mix of standards including black arrow indicating CBD (RT 7.4 min; $[M+H]^+ = 375$; mass spectra for CBD published in the literature [53]) and (B) C3-47 extracts. (C) Collision-induced dissociation (CID) of RT 7.4 min; $[M+H]^+ = 375$ for ion transitions $315.0 \rightarrow 123$ ((C), top panel), $315.0 \rightarrow 135$ ((C), middle panel) $315.0 \rightarrow 193$ ((C), bottom panel) to confirm CBD.

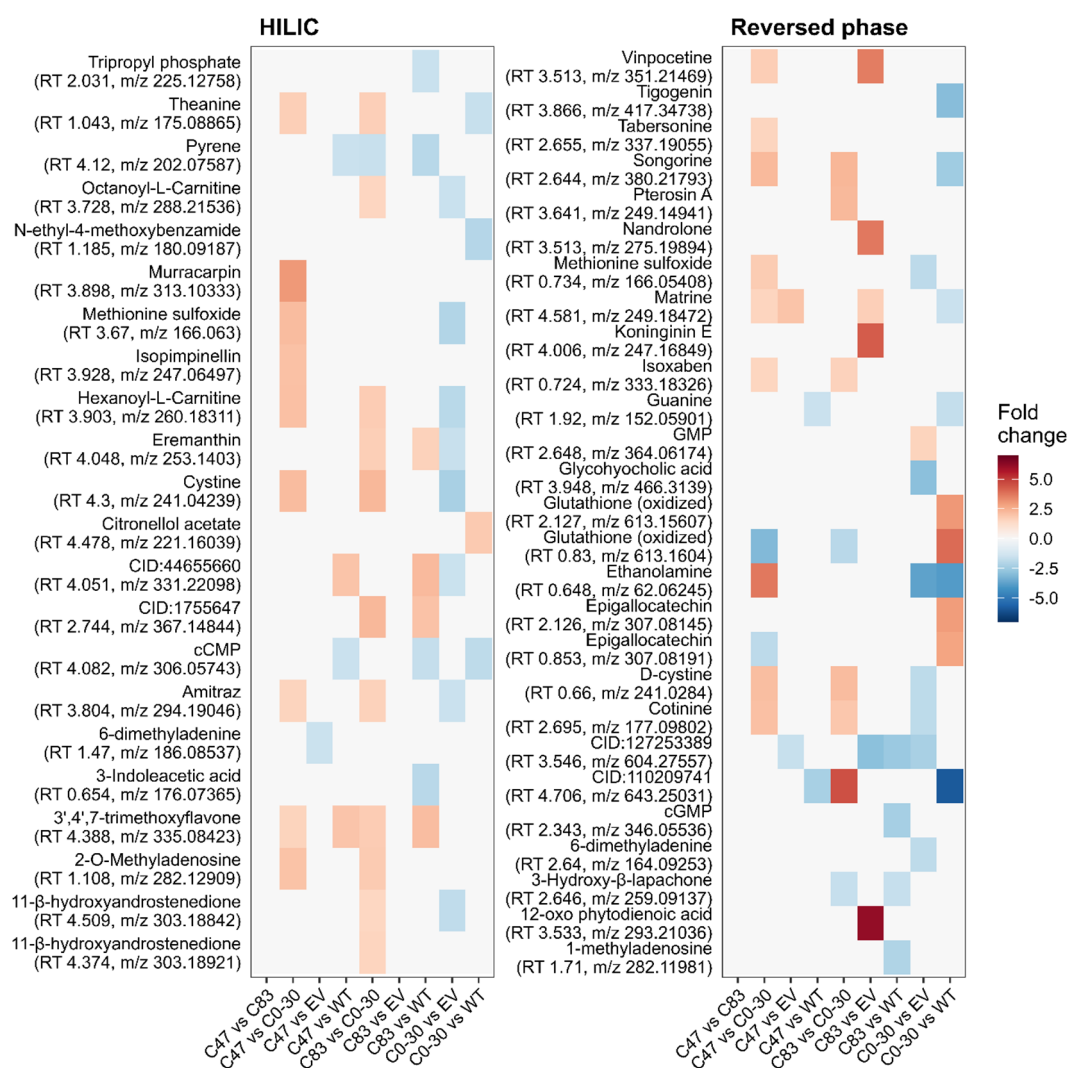


Figure A7. Differentially abundant analytes in engineered strains (C0-30, C3-47, and C3-83) putatively annotated using the MS-Dial[®] ESI⁺ spectral library. Analytes were detected in ESI⁺ mode using HILIC (**left panel**) and reversed-phase chromatography (**right panel**). Metabolites with long names are labeled by their PubChem Compound ID (CID). Average retention time and mass/charge ratios (m/z) are also shown.



Figure A8. Principal component analysis of the normalized abundances of analytes with the (A) exclusion or (B) inclusion of the Standard and Wild-type (WT)-spiked samples from untargeted metabolomic analysis.

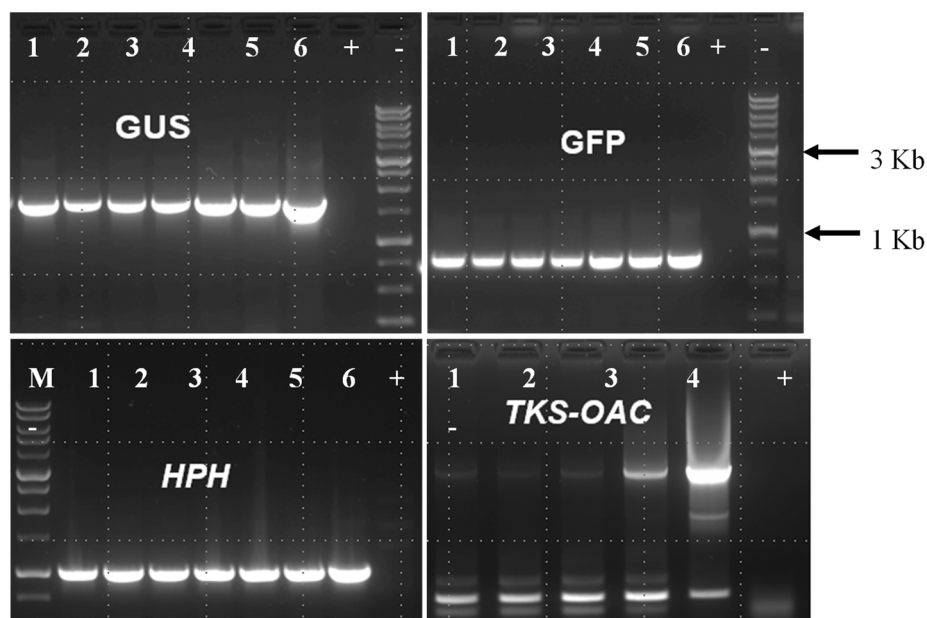


Figure A9. Agarose gel (1%) showing gene (*GUS*, *GFP*, *HPH* and *TKS-OAC*) amplification by colony PCR from *Agrobacterium* GV3101 transformants. M: 1 kb ladder, 1 to 6: colonies of *Agrobacterium* GV3101 transformants, +: positive control plasmid pCambia1304, -: PCR negative control mix without DNA.

Table A1. Sequence information of all the genetic components used in this study for plasmid constructs with corresponding references and accession numbers. Three distinct expression cassettes (C0, C1, and C2) were designed for nuclear integration into the *C. reinhardtii* C137 strain. These cassettes harbored the coding sequences of the *CsTKS* and *CsOAC* genes, interconnected by the extended Foot-and-mouth disease virus 2A peptide (FMDV2A). The FMDV2A peptide enables ribosome skipping at the cleavage site, facilitating polycistronic expression and ensuring the simultaneous production of both enzymes from a single transcript [41]. To enhance transgene expression, the constructs were designed with (C2) or without (C0 and C1) introns derived from *C. reinhardtii* *Rubisco small subunit 2* (*RBCS2*) gene (NCBI Accession: X04472.1) [54]. Specifically, C2 incorporated three introns, including the entire 145 bp intron 1 (i1) from *RBCS2*, which has been shown to enhance nuclear transgene expression in *C. reinhardtii* [54]. The details of each construct, including codon optimization strategies and regulatory elements, are provided in Tables A1 and A2. The C0 construct represents a minimal expression cassette without additional tags and was subjected to simple codon optimization for *C. reinhardtii*, whereas C1 and C2 were optimized according to the nuclear codon bias of *C. reinhardtii* and incorporated two affinity tags, Myc at the 3' end and 6xHis at the 5' end, to facilitate downstream protein detection. To further extend the cannabinoid biosynthetic pathway beyond OA and facilitate the production of CBGA, CBD, and CBDA, we designed construct C3 (Figure 2). This construct contained two cistrons: the first cistron for OA production mirrored that used in C2 (*6His-CsTKS-extFMDV2A-CsOAC-Myc* with intron), while the second cistron comprised two additional genes with two tags, Flag and HA, at 3' and 5' ends, respectively (*Flag-StAPT-extFMDV2A-CsCBDAS-HA*). All expression cassettes were driven by the *C. reinhardtii* endogenous *Heat Shock 70A-Rubisco* small subunit two fusion promoter with *Rubisco* small subunit intron 1 (*HSP70A-RBCS2-i1*) and *RBCS2* 3' untranslated region (3' UTR) as regulatory elements, ensuring strong transcriptional regulation [42]. Recombinant plasmids pCAMBIA1304 carrying one cistron (C0, C1, and C2) were introduced by *Agrobacterium*-mediated transformation, while those containing two cistrons (C3) were introduced by electroporation. *Cannabis sativa* tetraketide synthase gene *CsTKS* (accession number: AB164375), *C. sativa* olivetolic acid cyclase gene *CsOAC* (accession number: JN679224), aromatic prenyltransferase gene *StAPT* (NphBY^{288A/G286S}) from *Streptomyces* sp. strain CL190, *C. sativa* cannabidiolic acid synthase gene *CsCBDAS*, self-cleavable sequence from the foot and-mouth-disease-virus *extFMDV2A* is in black and italic, intron *RbcS2-1* is in gray (accession number: X04472.1); *c-Myc*, *6His* tag, *Flag* and *HA* tags are in black and underlined. Colored nucleotides are codons optimized for best *C. reinhardtii* usage.

Name	Nucleotide Sequence	Accession Number
<i>CsTKS-extFMDV2A-CsOAC</i>	ATGAACACCTGCGGGCGGAGGGCCCCGCCTCCGTGCTGGCGATCGGCACTGCCAACCCCGA- GAACATCCTGCTGCAGGACGAGTTTCCTGACTACTACTTTTCGCGTGACGAAGTCGGAGCACATGACCCAGCTCAAGGAGAAGTTT CGCAAGATTTGCGACAAGAGCATGATCCGCAAGCGCAACTGCTTCCTGAACGAGGAGCAC- CTGAAGCAGAACCCCCGCCTGGTGGAGCACGAGATGCAGACCCTCGACGCTCGGCAGGACATGCTCGTGGTGGAGGTGCCCAAG CTGGGTAAGGACGCCTGCGCGAAGGCCATCAAGGAGTGGGGCCAACCCAAGTCGAA- GATCACCCACCTGATCTTCACGAGCGCCTCGACGACCGACATGCCCCGCGCCGATTACCACTGCGCCAAGCTGCTGGGCCTGTCTCG CCGTCGGTGAAGCGCGTGATGATGTACCAGCTGGGTTGCTACGGCGGCGGCACTGTCTGCG- GATCGCTAAGGACATCGCGGAGAAACAAGGGCGCGCGCTCCTGGCGGTCTGCTGCGACATCATGGCCTGCCTGTTCCGGGG CCCCTCCGAGAGCGACCTGGAGCTGCTGGTGGGCCAGGCGATTTTCGGCGAC- GGCGCGGCCGCGGTGATCGTCGGCGCCGAGCCGACGAGAGCGTGGGGGAGCGCCCCATCTTCGAGCTGGTGTCCACCGGCCAG ACCATCCTCCCTAACAGCGAGGGACCATCGGCGGCCACATCCGGGAGGCCGCGCTGAT- TTTCGACCTGCACAAGGACGTCCCCATGCTGATCTCGAACACATCGAGAAGTGCCTGATCGAGGCTTTCACTCCCATCGGCATCT	<i>CsTKS</i> (AB164375) <i>CsOAC</i> (JN679224)

	CCGACTGGAAGTCCATCTTCTGGATCACGCACCCGGGCGGCAAGGCCATTCTGGACAAGGTG- GAGGAGAAGCTGCACCTGAAGAGCGACAAGTTTGTGGACAGCCGCCACGTCTGAGCGAGCACGGGAACATGTCCAGCTCGAC GGTGTCTTTGTCATGGACGAGCTGCGCAAGCGGAGCCTGGAGGAGGGCAAGTCGACTAC- CGGTGACGGCTTCGAGTGGGGGGTGTCTGTTCCGGCTTCGGCCCCGGCCTGACGGTTCGAGCGGGTGGTGGTCCGCAGCGTGCCGATC AAGTACGCGCCCCGTGAAGCAGACCCTGAACCTCGACCTGCTGAAGCTGGCGGGCGATGTG- GAGTCCAACCCCGGCCCCATGGCCGTGAAGCACCTCATCGTGTCTGAAGTTCAAGGACGAGATCACGGAGGCCAGAAGGAGGAG TTTTTCAAGACTTACGTGAACCTGGTCAACATCATCCCTGCGATGAAGGACGTCTACTGGGG- CAAGGACGTACCCAGAAGAACAAGGAGGAGGGCTACACCCATATTGTGGAGGTGACCTTTGAGTCGGTTCGAGACCATCCAGGA CTACATCATCCACCTGCCCCACGTGGGCTTTGGCGACGTGTACCGCTCGTTTTGGGAGAA- GCTGCTGATTTTCGACTACACCCCCCGCAAGTAA	
6His- CsTKS-extFMDV2A- CsOAC-Myc 6His-CsTKS-extFMDV2A- CsOAC-Myc	ATGCACCACCACCACCACCATAACCACTGCGCGCG- GAGGGCCCCGCGTCGGTGCTGGCCATTGGCACCGCGAAACCCGAGAAACATCCTGCTGCAGGACGAGTTCCCCGACTACTACTTTA GGGTGACCAAGTCCGAGCACATGACCACTGAAGGAGAAGTTCCGCAA- GATCTGTGACAAGTCTATGATCCGCAAGCGCAACTGCTTCCTGAACGAGGAGCATCTCAAGCAGAACCCCGGCTGGTTCGAGCA CGAGATGCAGACGCTAGACCAACGCCAGGACATGCTGTTGTGGAGGTGCCAAGCTGGG- CAAGGACGCCTGCGCCAAGGCCATCAAGGAGTGGGGCCAGCCGAAGAGCAAGATCACCCACCTGATCTTCACCTCCGCGTCCAC CACCGACATGCCCCGGCGCGGACTACCACTGCGCCAA- GCTGCTGGGCCTGTCCCCCTCCGTGAAGCGCGTGATGATGTACCAGCTGGGCTGCTACGGCGGCGGCACGGTGCTGCGCATCGCC AAGGACATCGCCGA- GAACAACAAGGGCGCCCCGCTCTGCGCGTGCTGCGACATCATGGCCTGCCTGTTCCGCGGCCCGTCGGAGTCCGACCTCGAG CTGCTGGTGCGGAGGCCATCTTCGGCGACGGCGCCGCGGCCGTGATCGTGGGCGCG- GAGCCCCGACGAGAGCGTGGGCGAGCGCCGATCTTCGAGCTGCTCTCCACCGGCCAGACCATCCTGCCGAACCTCGGAGGGCACC ATCGGCGGCCACATCCGGGAGGCGGGCCTGATCTTCGACCTCCACAAGGAC- GTCCCCATGCTGATCTCCAACAACATCGAGAAGTGCCTGATCGAGGCGTTCACCCCGATCGGCATCTCCGACTGGAACCTCATCTT CTGGATCACGCACCCCGGCGGCAAGGCGATCCTGGACAAGGTGGAGGAGAAGCTGCAC- CTGAAGAGCGACAAGTTCTCGACAGCCGCCACGTGCTGTCCGAGCACGGCAACATGAGCAGCAGCACCGTGCTGTTTCGTGATG GACGAGCTGCGGAAGCGAGCCTGGAGGAGGGCAAGTCGACCACCGGCGACGGCTTCGAG- TGGGGCGTGCTGTTCCGGCTTCGGCTCAGGCCTGACGGTCGAGCGGTGCTGTGCGCTCCGTGCCGATCAAGTACCTGCTGGCCAT CCACCCACCGAGGCGCGGCACAAGCAGAAGATCGTGGCGCCCGTGAA- GCAGACCCTCAACTTCGACCTCCTGAAGCTGGCCGGGGACGTGGAGAGCAACCCCGGCCCCATGGCCGTGAAGCACCTGATCTCC TGAAGTTCAAGGACGAGATCACGGAGGCCAGAAGGAGGAGTTCTTCAAGACCTACGTGAAC- CTGGTGAACATCATCCCCGCCATGAAGGACGTGTAAGGGGCAAGGACGTGACCCAGAAGAACAAGGAGGAGGGCTACACGCA CATCTCGAGGTGACCTTCGAGTCGGTGGAGACGATCCAGGAC- TACATCATCCACCCCGCCACGTGGGCTTCGGCGACGTGTACCGCAGCTTCTGGGAGAAGCTGCTGATCTTCGACTACACCCCGC GCAAGGAGCAGAAGCTGATCAGCGAGGAGGACCTGTAA	This study
6His-CsTKS-extFMDV2A- CsOAC-Myc with introns	ATGCACCACCACCACCACCATAACCACTGCGCGCG- GAGGGCCCCGCGTCGGTGCTGGCCATTGGCACCGCGAAACCCGAGAAACATCCTGCTGCAGGACGAGTTCCCCGACTACTACTTTA	This study

Flag-StAPT-extFMDV2A-
CsCBDAS-HA

GGGTGACCAAGTCCGAGCACATGACCCAACTGAAGGAGAAGTTCCGCAA-
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GCGGCCATGGAGGAGGCCGCCGGCTGCTGGGCGTGCCCTGCGCCCGGACAAAGATCTACCCGCTGCTGAGCACCTTCCAGGAC
ACCCTGGTGGAAGGCGGCAGCGTGGTGGTTCAGCATGGCCAGCGGCCGCCACAGCAC-
CGAGCTGGACTTCAGCATCAGCGTGCCACCTCCACGGCGACCCCTACGCCACGGTGGTGGAGAAGGGCCTGTTCCCCGCGACC
GGCCACCCCGTGGACGACCTGCTGGCGGACACGCAGAAGCACCTGCCGTGAG-
CATGTTGCCATCGACGGCGAGGTGACGGCGGCTTCAAGAAGACCTACGCGTTCTTCCCCACCGACAACATGCCCGCGTGCC
GAGCTCTCGGCGATCCCCTCGATGCCCCCGCCGTGGCCGAGAACGCG-
GAGCTGTTGCGCGGTACGGCTGGACAAGGTGCAGATGACGTCATGGACTACAAGAAGCGCCAGGTGAACCTGTACTTCTCC
GAGCTGTGCGCGCAGACCCTGGAGGCCGAGAGCGTGCTGGCCCTGGTGCGG-
GAGCTGGGCTGCACGTGCCAACGAGCTGGGCCTGAAGTTCTGCAAGCGCTCCTTCTCCGTGTACCCACCTGAACTGGGAGA

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	CCGGCAAGATTGACCGCCTGTGCTTCGCCGTGATTAGCAAC- GACCCCAACCCTGGTGCCCTTCAGCGACGAGGGCGACATCGAGAAGTTCCACAACCTACGCCACCAAGGCGCCGTACGCCTACGTG GGCGAGAAGCGCACCCCTGGTGACGGCCTTACCCTGAGCCCCAAGGAGGAGTATTACAA- GCTGTCTGGCCGCCCTATCACATCACCGACGTCCAGCGCGGCTGTCTAAGGCGTTTCGACAGCCTGGAGGACCTGCTGGCCATCCAC CCCACCGAGGCCCGGCACAAGCAGAAGATCGTGGCCCCCGTCAAGCAGAC- GCTGAACTTCGACCTGCTGAAGCTGGCCGGCGACGTGGAGTCGAACCCCGGCCCAATGAAACCCACGCGAGAAGTTCTGAAGTGC TTCAGCCAGTACATCCCGAACAACGCCACCAACCTGAAGCTGTCT- TATACCCAGAACAAACCCCTGTACATGTCCGTCTCTGAACAGCACCATCCACAACCTGCGCTTACCAGCGACACGACCCCGAAG CCCCCTGTGATCGTGACCCCAAGCACGTGTCCACATCCAGGGCACCATCCTGTGCTCGAA- GAAGGTGGGCTGCAGATCCGCACCCCGACGCGCGGCCACGACTCTGAGGGCATGTCTGACATCAGCCAGGTGCCGTTCGTGAT CGTCGACCTGCGCAACATGCGCTCCATCAAGATCGACGTGCACTCCGACACCGCCTGGGTG- GAGGCGGGGGCCACCCTCGGCGAGGTCTACTACTGGGTGAACGAGAAGAACGAGAACCTGAGCCTGGCCGCCGGCTACTGCCC GACCGTCTGCGCGGGCGGCCACTTCGGCGGGCGGGCTACGGCCCCCTGATGCGCAACTAC- GGCCTGCGGGCCGACAACATCATCGACCGGCACCTCGTGAACGTGCACGGCAAGGTGCTGGACCGCAAGTCGATGGGGAGGA CCTGTTCTGGGCCCTGCGCGGGCGGGCGCGCCGAGAGCTTCGGCATCATCTCGCCTGGAA- GATCCGCCTGGTGGCGGTGCCCCAAGTCCACCATGTTTCAGCGTGAAGAAGATCATGGAGATCCACGAGCTCGTGAAGCTGTCAA CAAGTGGCAGAACATCGCGTACAAGTACGACAAGGACCTGCTGCTGATGACCCAC- TTCATCACGCGAACATCACGACAACCAGGGCAAGAACAAGACCGCCATCCACACCTACTTCAGCAGCGTGTTCCTGGGCGGC GTGGACAGCCTGGTCGACCTGATGAACAAGAGCTTCCCCGAGCTGGGCATCAAGAAGAC- CGACTGCCGCCAGCTGTCTGGATCGACACCATCATCTTCTACTCCGGCTCGTGAAGTACGACACCGACAACCTCAACAAGGAG ATCCTGCTGGACCGTCCGCGGGCCAGAACGGCGCGTTCAAGATCAAGCTGGACTACGTGAA- GAAGCCCATCCCGGAGAGCGTGTTCGTGCAGATCCTGGAGAAGCTGTACGAGGAGGACATTGGCGCCGGCATGTACGCGCTGTA CCCCTACGGCGGCATCATGGACGAGATCTCCGAGTCCGCCATCCCGTTCCCCAC- CGGGCGGGCATCTGTACGAGCTGTGGTACATCTGCAGCTGGGAGAAGCAGGAGGACAACGAGAAGCACCTGAAGTGGATCCG CAACATCTACAACCTCATGACGCCCTACGTGAGCAAGAACCCCCGCCTGGCCTACCTGAAC- TACCGGACCTGGACATCGGCATCAACGACCCCAAGAACCCGAACAACCTACACGAGGCCCGCATTTGGGGCGAGAAGTATTTTC GGCAAGAAGTTCGACCGATTGGTTAAGGTGAAGACTCTGGTGATCCTAATAACTTCTTCG- TAATGAGCAGTCCATCCCCCCCCTGCCCGGCACCGCCACTACCCCTATGACGTGCCTGACTACGCCTAG	
<i>Hsp70A Rbc-S2p</i>	GCTGAGGCTTGACATGATTGGTGCGTATGTTTGTATGAAGCTACAGGACTGATTTGGCGGGC- TATGAGGGCGGGGAAGCTCTGGAAGGGCCGCGATGGGGCGCGGGCTCCAGAAGGCGCCATACGGCCCCGTGGCGGCACCC ATCCGGTATAAAAGCCCGGACCCCGAACGGTGACCTCCACTTTCAGCGACAAACGAGCAC- TTATACATACGCGACTATTCTGCCGTATACATAAACCCTCAGCTAGCTTAAGATCCCATCAAGCTTGCATGCCGGGCGCGCCAG AAGGAGCGCAGCCAAACCAGGATGATGTTTGTATGGGGTATTTGAGCACTT- GCAACCCCTATCCGGAAGCCCCCTGGCCCAAAAGGCTAGGCGCAATGCAAGCAGTTCGCATGCAGCCCCTGGAGCGGTGCC TCCTGATAAACCGGCCAGGGGGCCTATGTTCTTTACTTTTTTACAA	[16]
3' UTR	AATGCCCCGAATGTTGGGTATCTAGCTCACACGGCAGTTTGTAAAGGTGCTGAGGCGTT	[16]
<i>Aph7</i>	ATGACACAAGAATCCCTGTTACTTCTCGACCGTATTGATTCCGATGATTCCTAC- GCGAGCCTGCGGAACGACCAGGAGTTCTGGGAGCCGCTGGCCCGCCGAGCCCTGGAGGAGCTCGGGCTGCCGGTGCCGCCGGTG	[16]

CTGCGGGTGCCCGGCGAGAGCACCAACCCCGTACTGGTCGGCGAGCCCGGCCCGGTGATCAA-
 GCTGTTCCGGCGAGCACTGGTGCGGTCCGGAGAGCCTCGCGTCGGAGTCGGAGGCCTACGCGGTCTGGCGGACGCCCCGGTGCC
 GGTGCCCCGCCTCCTCGGCCGCGGCGAGCTGCGGCCCGGCACCG-
 GAGCCTGGCCGTGGCCCTACCTGGTGATGAGCCGGATGACCGGCACCACTGGCGGTCCGCGATGGACGGCACGACCGACCGGA
 ACGCGCTGCTCGCCCTGGCCCGGAACTCGGCCGGGTGCTCGGCCGGCTG-
 CACAGGGTGCCGCTGACCGGGAACACCGTGCTACCCCCATTCCGAGGTCTTCCCGGAACTGCTGCGGGAACGCCGCGCGGCG
 ACCGTCGAGGACCACCGCGGGTGGGGTACCTCTCGCCCCGGCTGCTGGACCGCCTGGAG-
 GACTGGCTGCCGGACGTGGACACGCTGCTGGCCGGCCGGAACCCCGGTTTCGTCCACGGCGACCTGCACGGGACCAACATCTTC
 GTGGACCTGGCCGCGACCGAGGTACCGGGATCGTCGACTTCACCGACGTGTATGCGGGA-
 GACTCCCGCTACAGCCTGGTGCAACTGCATCTCAACGCCTTCCGGGGCGACCGCGAGATCCTGGCCGCGCTGCTCGACGGGGCGC
 AGTGAAGCGGACCGAGGACTTCGCCCCGGAAGTCTCGCCTTACCTTCTGAC-
 GACTTCGAGGTGTTGAGGAGACCCCGCTGGATCTCTCCGGCTTACCGATCCGGAGGAACTGGCGCAGTTCCTCTGGGGGCCGC
 CGGACACCGCCCCCGGCGCCTGA

Table A2. Amino acid sequences of CsTKS, CsOAC, *StNphB*, CsCBDAS and extended FMDV-2A used in this study to build the expression cassettes.

Sequence Name	Amino Acid Sequence	Length (a.a.)	Accession Number/ Reference
<i>C. sativa</i> type 3 tetraketide synthase (TKS)	MNHLRAEGPASVLAIGTANPENILLQDEFDPDYFRVTKSEHMTQLKEKFRKICDK- SMIRKRNCFLNNEHLKQNPRLVEHEMQTLRDARQDMLVVEVPKLGKDACAKAIKEWGQPKSKITHLIFTSASTTDMPGA DYHCAKLLGLSPSVKRVMMYQLGCGGGTTLRIAKDIAENNKARVLAVCCDI- MACLFRGPSESDLELLVGQAIFGDGAAAVIVGAEPDESVERPIFELVSTGQTILPNSEGTIGGHIREAGLIFDLHKDVPMLI SNNIEKCLIEAFTPIGISDWSIFWITHPGGKAILDKVEEKLHLKSDKFVDSRHVLSEHGN- MSSSTVLFVMDLKRKRSLEEGKSTTGDGFEGVLFVFGPGLTVERVVVRSVPIKY	385	ACD76855.1
<i>C. sativa</i> olivetolic acid cyclase (OAC)	MAVKHLIVLKFDEITEAQKEEFFKTYVNLVNIIPAMKDVYWGKDVTQKNKEEGYTHIVEV- TFESVETIQDYIIHPAHVGFVDVYRSFWEKLLIFDYTPRK	101	NP_001384868
<i>C. sativa</i> Cannabidiolic acid synthase (CBDAS)	MNPRENFLKCFSQYIPNNATNLKL VYTQNNPLYMSVLNSTIHNLRFTSDTTPK- PLVIVTPSHVSHIQGTILCSKKVGLQIRTRSGGHDSEGMSYISQVPFVIVDLRNMRSIKIDVHSQTAWVEAGATLGEVYYW VNEKNENLSLAAGYCPTVCAGGHFGGGGYGPLMRNYGLAADNI- IDAHLNVNHGKVLDRKSMGEDLFWALRGGGAESFGIIVAWKIRLVAVPKSTMFSVKKIMEIHEL VKLVNWKQNIAYKY DKDLLLMTHTFTRNITDNQGNKTAIHTYFSSVFLGGVDSLVDLMNKSFPGLIKKTD- CRQLSWIDTIIFYSGVVNYDTDNFNKEILLDRSAGQNGAFKIKLDYVKKPIPESVFVQILEKLYEEDIGAGMYALYPYGGIM DEISEAIPFPHRAGILYELWYICSWEKQEDNEKHLNWIRNIYNFMTPTYVSKNPRLAYL- NYRDLDIGINDPKNPNNYTQARIWGEKYFGKNFDRLVKVKTLVDPNNFFRNEQSIPPLPRHRH	517	NP_001384865
<i>Streptomyces</i> sp. CL190 NphB ^{Y288A/G286S} Aromatic prenyl-transferase	MSEAADVERVYAAMEEAAGLLGVACARDKIYPLLSTFQDTLVEGGSVVVFSMASGRHSTELD- FSISVPTSHGDPYATVVEKGLFPATGHPVDDLLADTQKHLPLVSMFAIDGEVTGGFKKTYAFFPTDNMPGVAELSAIPSM	307	1ZB6_A [55]

	PAVAENAELFARYGLDKVQMTSMDYKKRQVNLYFSELSAQTLE- AESVLALVRELGLHVPNELGLKFCRFSVYPTLNWETGKIDRLCFAVISNDPTLVPSSDEGDIEKFHNYATKAPYAYVGE KRTLVIYGLTSPKEEYKLSAAYHITDVQRGLLKAFDSLED		
Extented FMDV-2A	LLAIHPTEARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP	40	[41]

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