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Heterologous production of β-pinene in the chloroplast of the marine diatom *Phaeodactylum tricornutum*

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ABSTRACT

Microalgae are considered promising hosts for the sustainable production of plant-derived secondary metabolites, such as terpenoids. In particular, the model marine diatom *Phaeodactylum tricornutum* is a promising candidate for the heterologous production of such chemicals due to its robustness, relatively fast growth, and high natural accumulation of terpenoids. In addition, *P. tricornutum* possesses two separate pathways for the synthesis of terpenoid precursors, the MEP pathway in the chloroplast and the MVA pathway in the cytosol. In this study, we explored the two pathways for the heterologous β-pinene production in *P. tricornutum* by episomally expressing pinene synthase in either the chloroplast or cytosol for the first time. Our results show that the chloroplast expression from episomes led to β-pinene titers of up to $10.27 \pm 1.45\ \mu g \cdot L^{-1}$. No β-pinene synthesized from the cytosolic MVA pathway precursors was detected, however the expression and functionality of the pinene synthase was confirmed both in the cytosol and in the chloroplast. Furthermore, to enhance production in the chloroplast, we developed more stable transgenic lines with random chromosomal integration of two different pinene synthase genes. We observed higher titers compared to the episomal mutants, up to $19.35 \pm 1.42\ \mu g \cdot L^{-1}$ with the pinene synthase from *Abies grandis* and $20.07 \pm 0.51\ \mu g \cdot L^{-1}$ with the pinene synthase from *Citrus limon*. All the β-pinene producing strains used in this study also produced α-pinene as a side product, which accounted for 20–25 % of total monoterpenoid production. Overall, this study represents a fundamental step in microalgal engineering towards the synthesis of monoterpenoids.

Introduction

Terpenoids are a broad class of chemicals that are abundant in nature and have several commercial applications. The ever-expanding chemical space of terpenoids makes them ubiquitous across several industries, from low-value food and cosmetic ingredients (*e.g.* limonene, geraniol) [1] to high-value pharmaceuticals (*e.g.* taxol, artemisin) [2,3]. Terpenoids are mostly synthesized from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) and are classified by the number of carbons in their chemical structure. Monoterpenoids are distinguished by their skeleton of 10 carbons and are among the simplest terpenoids [4]. They are predominantly synthesized by higher plants through enzymatic reactions localized in the chloroplast [5] and they mainly serve as signaling molecules and defense mechanisms against pests [6].

The monoterpenoid β-pinene has gained attention in recent years for its chemical properties and applications, with an estimated market size of 102 million USD per year [1,7]. In addition to its established use in the fragrance and resin industries, β-pinene is emerging as a precursor for food ingredients, high density jet fuel substitutes, and biomaterials [8–10]. Furthermore, several medicinal effects have recently been reported, allowing for potential pharmaceutical applications of β-pinene [11,12]. β-pinene is rarer and more valuable than its structural isomer α-pinene, which is the most abundant terpenoid found in nature [13]. Pinenes are produced industrially by fractional distillation of turpentine. Turpentine is a natural resource with variable chemical composition, obtained either by tapping live conifers or as a by-product of wood pulping in the paper production process. While precise global data are limited, turpentine, a by-product of the wood pulp and paper industry,

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represents the main industrial source of α- and β-pinene, with estimated production on the order of hundreds of thousands of tons per year [14]. Turpentine contains complex mixtures of terpenoids, with α-pinene usually being the most abundant. The concentration of β-pinene in turpentine is highly dependent on the plant species it is extracted from, the time of harvest, and the geographical origin of the trees [15,16]. The dependency on the plant biomass, and the low yields of extraction make the current production process for β-pinene suboptimal [17]. Consequentially, attention has been focused on the microbial synthesis of β-pinene to attain a more sustainable and reliable process that results in higher chemical purities of the final product [8]. The microbial synthesis of pinene has been achieved in several biotechnologically relevant chassis', both prokaryotic and eukaryotic [8,18], with promising results but not yet economically viable. The highest success has been achieved in the bacterium *Escherichia coli*, in which the structural isomer α-pinene was produced at 1035 $mg{\cdot}L^{-1}$ [20].

Eukaryotic microalgae are promising and underexplored microbial platforms to produce chemicals such as terpenoids, because they can be cultivated photosynthetically in a controlled environment with the requirement of minimal resources [19]. Several species are commercially exploited because they accumulate extremely high levels of terpenoids, for example astaxanthin by *Haematococcus pluvialis* and β-carotene by *Dunaliella salina* [20,21]. However, the synthetic biology toolbox required to turn these non-model species into biomanufacturing chassis' for heterologous terpenoids is still limited. Some success has been achieved with the production of heterologous terpenoids in eukaryotic microalgae, such as the freshwater species *Chlamydomonas reinhardtii* and *Cyanidioschyzon merolae* [22,23], and the marine diatom *Phaeodactylum tricornutum* [24]. *P. tricornutum* is an established microalgal species that is exploited commercially for its naturally high content of poly-unsaturated fatty acids and high value terpenoids, in particular the antioxidant fucoxanthin [25]. In the last decade, *P. tricornutum* has been proposed as a promising chassis for the heterologous synthesis of a wide range of terpenoids, including the monoterpenoid geraniol [24,26]. Additionally, some strains of *P. tricornutum* were reported to naturally produce monoterpenoids [27,28].

One peculiarity of this microalgal species is that *P. tricornutum* naturally synthesizes the precursors of all terpenoids, DMAPP and IPP through two separate pathways: the methylerythritol phosphate (MEP) pathway and the mevalonate (MVA) pathway [29] (Fig. 1). The MEP pathway is localized in the chloroplast and mostly provides the building blocks for carotenoids, chlorophylls, and plastoquinones. Conversely, the MVA pathway is present in the cytosol and mostly fuels the synthesis of sterols, which are essential structural components of cell membranes [30]. The presence of both pathways simultaneously is mostly a feature of organisms that natively synthesize several terpenoids, such as terrestrial plants [31]. *In silico* predictions suggest that genetically engineering industrial microorganisms to harbor both pathways can increase the maximum theoretical yield for terpenoids precursors [32]. Experimentally, introducing a second pathway (MVA or MEP) often results in an increased production of terpenoids, compared to the strains with only the native pathway [8,33,34].

P. tricornutum naturally harbors both the MVA and MEP pathways, providing a significant advantage by eliminating the need for complex and time-consuming genetic engineering to introduce an additional non-native pathway. Additionally, the presence of both pathways may provide metabolic flexibility in case of depletion of substrates that are necessary for the healthy proliferation of cells. For example, a recent study proposed that an outflow of GPP from the chloroplast to the cytosol helps supplementing precursors for the synthesis of heterologous geraniol in *Nicotiana benthamiana* [6]. Experiments in *Arabidopsis thaliana* suggest that common metabolites from MEP and MVA pathway such as IPP or DMAPP can flow into the cytosol to maintain a steady production of sterols if the MVA pathway was impaired [35]. The mechanisms and regulation of the crosstalk between the MEP and MVA pathway are still unknown, but it is speculated that they also occur in diatoms such as *P. tricornutum* [26,30,36].

Previous studies have focused on the production of monoterpenoids in *P. tricornutum*. A geraniol synthase from *Catharanthus roseus* (CrGES) was expressed in the cytosol of *P. tricornutum* and up to 0.89 $mg{\cdot}L^{-1}$ geraniol was synthesized from cytosolic MVA precursors [37]. However, the potential of monoterpenoid production from MEP precursors in the chloroplast remains unexplored. The chloroplast is the organelle where monoterpenoids are natively produced in plants from MEP pathway precursors. Moreover, due to the electron transport chain driven by photosynthesis in the chloroplast, the MEP pathway in *P. tricornutum* chloroplast should have an abundance of reduced redox cofactors to exploit [38].

In this study we aimed to establish for the first time the production of β-pinene in *P. tricornutum* CCAP 1055/1, which does not produce any monoterpenoid naturally. We initially investigated which pathway (MVA or MEP) would supply most precursors for the synthesis of β-pinene, by expressing and localizing a heterologous pinene synthase (PinS) either in the cytosol or in the chloroplast. Moreover, we explored whether transgenic lines with a pinene synthase-encoding gene randomly integrated in the genome would outperform strains that express the pinene synthase from an episome. Finally, we generated transgenic lines of *P. tricornutum* that heterologously express pinene synthases from different plants, to evaluate the effect of their performance in subcellular environments such as the chloroplast. We present insights on the influence of subcellular localization on the heterologous synthesis of β-pinene, and a valuable strategy to further increase the titers of heterologous terpenoids in *P. tricorntutum*.

Results

Generation of transgenic lines and subcellular localization of heterologous AgPinS enzymes

In this study we challenged the hypothesis that β-pinene production could be influenced by the subcellular localization of the pinene synthase (PinS), using a conventional continuous extraction with isopropyl myristate (IM) [26]. Thus, we generated transgenic lines capable of episomal expression (EE) of a PinS, a mNeonGreen (mNG) fluorescent protein, or their protein fusion (Table 1). Each of these proteins were either retained in the cytosol or enabled to be shuttled to the chloroplast, by fusing the encoding gene with a chloroplast localization signal, native to *P. tricornutum* (Fig. 1, Fig. 2). We selected the PinS from the grand fir *Abies grandis* (AgPinS) because this enzyme has been successfully heterologously expressed in multiple different species [8,39,40]. This demonstrated versatility suggests its robust functionality and compatibility with various expression systems, making it an ideal starting point for our study. Plants generally synthesize monoterpenoids in the chloroplast, therefore the biosynthetic enzymes possess a hloroplast transit peptide (CTP). Such peptide often causes the formation of inclusion bodies in bacteria or has negative effects on the enzyme function, therefore it is often removed [26,41]. Compared to chloroplasts in higher plants, the single chloroplast of *P. tricornutum* originated from a secondary endosymbiosis event, thus CTPs from plants are considered incompatible with the chloroplast import mechanisms of *P. tricornutum*. In this study, we adapted PinS gene sequences based on previous studies that truncated the predicted encoding sequence for the native CTP [8,42]. For AgPinS-encoding gene, the first 114 nucleotides (corresponding to 38 amino acids in the wild-type enzyme) were removed, while for ClPinS, the first 159 nucleotides (53 amino acids) were truncated. Both truncations were designed to retain the conserved tandem arginine motif (RRX8W), which is important for proper enzyme function.

To target the enzymes to the chloroplast, we fused the truncated PinS gene sequences to a bipartite CTP-encoding sequence derived from the γ-subunit of chloroplast ATP synthase (AtpC) in *P. tricornutum*, which had been characterized in a previous study [43]. We assembled the plasmids modularly following the uLoop synthax (Fig. 2a) and

introduced them into *P. tricornutum* cells via conjugation with *E. coli* (Fig. 3a). The subcellular localization of the AgPinS fused with mNG was then confirmed with confocal fluorescence microscopy. We observed that transgenic lines expressing proteins fused to the CTP always localized in the chloroplast, as confirmed by the overlap with the chlorophyll autofluorescence (Fig. 2b).

Chloroplast localization determines β-pinene synthesis in P. tricornutum transgenic lines

Transgenic lines EE_AgPinS and EE_CTP_AgPinS were generated by bacterial conjugation, generally regarded as a suitable method to ensure that the episomes are maintained extrachromosomally without genomic integration [37]. Extrachromosomal expression was necessary to allow comparability in gene expression between all the different transgenic strains tested. Three colonies for each transformation event were subjected to *in vivo* β-pinene production experiments. One transgenic line expressing a fluorescent marker (mNG) and a zeocin resistance protein was included as a control to evaluate if the protein expression from a plasmid affects growth. The WT strain was also included as a control. Every *P. tricornutum* strain except the WT was cultivated with zeocin to ensure the propagation of the episomes for the whole duration of the experiment. The overlayer was harvested on the fifth day of cultivation, ~11 h after the onset of the diurnal cycle. Remarkably, β-pinene was detected in the IM overlayer exclusively for the EE_CTP_AgPinS strains, while no pinene was detected from EE_AgPinS lines (Fig. 2a).

We investigated whether the transgenic strains generated in this study were impaired in growth by analyzing the areas under the curve (AUC) of the growth curves (Figure S1). The analysis of variance (ANOVA) between all the samples gave a significant result ($p = 2.11 \cdot 10^{-5}$), indicating that at least one group differs from the

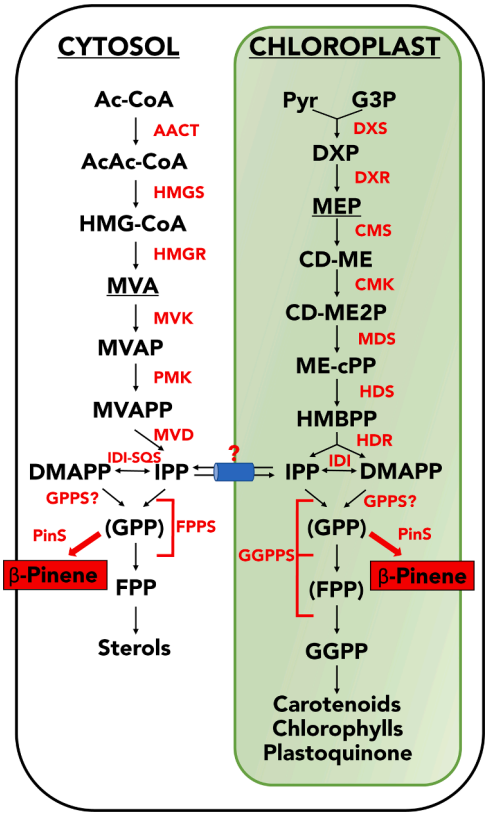

Fig. 1. Heterologous biosynthesis of β-pinene via the cytosolic MVA pathway or the chloroplastic MEP pathway. β-pinene is synthesized by cyclization of the precursor GPP by a pinene synthase (PinS) which is heterologously introduced into *P. tricornutum* cells (this study). GPP is synthesized from the condensation of one molecule of IPP and one of DMAPP, which are the end products of the MEP and MVA pathways. The MVA pathway is localized in the cytosol and it starts from the product of primary metabolism Ac-CoA, while the MEP pathway is chloroplastic and starts from Pyruvate and G3P. IPP and/or DMAPP could be exchanged between cytosol and chloroplast by a yet unknown mechanism, and this exchange could extend to other pathway intermediates and downstream products. GPP could exist as a synthesis product of a dedicated GPPS or as an intermediate in the synthesis of GGPP by GGPPS or FPP by FPPS. The main downstream product of the MVA pathway is sterols while the MEP pathway supplies the precursors for photosynthesis-related molecules such as carotenoids, chlorophylls, and quinones. Ac-CoA: acetyl coenzyme A; AcAc-CoA: aceto-acetyl coenzyme A; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; MVA: mevalonate; MVAP: mevalonate-5-phosphate; MVAPP: mevalonate-5-diphosphate; IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; Pyr: pyruvate; G3P: glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; MEP: 2-C-methylerythritol 4-phosphate; CDP-ME: 4-diphosphocytidyl-2-C-methylerythritol; CDP-ME2P: 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; MEcPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP: (*E*)-4-Hydroxy-3-methylbut-2-enyl pyrophosphate; GGPP: geranyl geranyl diphosphate; AACT: acetoacetyl-CoA thiolase (EC 2.3.1.9); HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase (EC 2.3.3.10); HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.17.1.2); MVK: mevalonate kinase (EC 2.7.1.185); PMK: phosphomevalonate kinase (EC 2.7.4.2); MDD: diphospho-mevalonate decarboxylase (EC 4.1.1.33); IDI: isopentenyl diphosphate Δ-isomerase (EC 5.3.3.2); SQS: Squalene synthase (EC 2.5.1.21); GPPS: geranyl diphosphate synthase (EC 2.5.1.29); FPPS: farnesyl diphosphate synthase (EC 2.5.1.10); PinS: Pinene synthase (EC 4.2.3.120); DXS: 1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7); DXR: 1-deoxy-D-xylulose 5-phosphate reducto-isomerase (EC 1.1.1.267); CMS: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (EC 2.7.7.60); CMK: 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (EC 2.7.1.148); MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (EC 4.6.1.12); HDS: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (EC 1.17.1.2); HDR: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; GGPPS: geranylgeranyl diphosphate synthase (EC 2.5.1.29). Parentheses indicate that the compound is an intermediate in the synthesis of another compound with a longer prenyl chain. Question mark (?) indicates that it is unknown whether that enzyme or transporter is present is *P. tricornutum*.

Table 1
List of transgenic strains used in this study.

Strain used	Gene of interest	Expression mode	Purpose in this study
EE_AgPinS	*PinS* from *A. grandis*	EE	Pinene production in the cytosol
EE_CTP_AgPinS	*PinS* from *A. grandis* coupled to *atpC* CTP	EE	Pinene production in the chloroplast
EE_AgPinS_mNG	*PinS* from *A. grandis* fused to mNG	EE	Microscopy study to verify localization in the cytosol
EE_CTP_AgPinS_mNG	*PinS* from *A. grandis* fused to *mNG*, coupled to *atpC* CTP	EE	Microscopy study to verify localization in the chloroplast
EE_mNG	*PinS* from *A. grandis* fused to *mNG*, coupled to *atpC* CTP	EE	Microscopy study to verify cytosolic localization / Control for pinene production experiments
EE_CTP_mNG	*mNG* coupled to *atpC* CTP	EE	Microscopy study to verify localization in the chloroplast
RICE_CTP_AgPinS	*PinS* from *A. grandis* coupled to *atpC* CTP	RICE	Pinene production in the chloroplast
RICE_CTP_ClPinS	*PinS* from *C. limon* coupled to *atpC* CTP	RICE	Pinene production in the chloroplast

AgPinS: Pinene synthase*; mNG:* mNeongreen fluorescent protein; atpC CTP: Chloroplast-targeting peptide from the γ subunit of the ATP synthase (atpC) from *P. tricornutum*; EE: Episomal expression. RICE: Randomly integrated chromosomal expression.

others. However, all transgenic strains and the WT exhibited similar growth profiles. The WT control did not differ significantly from transgenic lines EE_CTP_AgPinS 2 and EE_CTP_AgPinS 3 (Tukey HSD test, $p > 0.65$), but was slightly higher than the other transgenic strains in terms of AUC. This indicates that in general the maintenance of the plasmid, the production of pinene, and the presence of the antibiotic zeocin in the medium are not a strong metabolic burden to the cells. The β-pinene titers were similar across all three EE_CTP_AgPinS transgenic lines, with the highest being EE_CTP_AgPinS 3 at 0.98 ± 0.13 ng·10^{-7}cells, leading to a volumetric yield of 3.27 ± 0.64 μg·L^{-1}. As expected, the structural isomer α-pinene was also detected as a secondary product (23:77 mixture of α-pinene to β-pinene).

Pinene synthases are expressed and functional regardless of the subcellular localization

The absence of detectable amounts of pinene from transgenic strains with cytosolic AgPinS in *in vivo* production experiments could be caused by inactive or non-functional enzymes in the cytosol. While the expression of the enzymes was confirmed by Western blot at an early stage of this study (data not shown), such assay does not provide information about the correct activity of the AgPinS. We tackled this limitation by performing an *in vitro* assay, where the AgPinS synthesized in *P. tricornutum* was probed for its activity by providing the substrate GPP and the cofactor Mn^{2+}. We used the whole crude lysate from the microalgal cells mixed with GPP and Mn^{2+} to produce β-pinene, as we do not expect any activity from endogenous proteins of *P. tricornutum*. The same amount of total protein was used for each *in vitro* reaction. Strikingly, β-pinene was produced from the crude lysate of all transgenic lines expressing AgPinS, regardless of the subcellular localization (Fig. 3d). As expected, the WT and EE_mNG controls do not show any production (Fig. 3d). The lysate from EE_AgPinS 1 produced a markedly lower amount of β-pinene compared to all the other transgenic lines expressing AgPinS. EE_AgPinS 1 showed a slightly but statistically significant lower growth compared to EE_AgPinS 2 (Tukey HSD test, $p = 0.043$), while its growth was not significantly different from EE_AgPinS 3 (Figure S1). Therefore, the reduced pinene production observed in EE_AgPinS 1 cannot be attributed to growth defects. We speculate that genetic instability has caused the levels of gene expression or protein expression in EE_AgPinS 1 to be lower compared to other transgenic strains. This could have been caused, for example, by a point mutation in the genome or in the episome but we did not investigate this in detail, because out of scope. When the *in vitro* assay was performed without the addition of GPP, no β-pinene synthesis was observed (data not shown).

Genomic integration enhances β-pinene production compared to extrachromosomal expression

We generated transgenic lines that express PinS genes via randomly integrated chromosomal expression (RICE), as an alternative to EE to achieve more stable strains that produce higher pinene titers. This was done by amplifying the expression cassette containing the genes encoding the zeocin resistance protein and the CTP_AgPinS, and electroporating them into *P. tricornutum* cells. 20 transgenic lines isolated from single colonies were pre-screened before choosing candidate mutants to systematically test for β-pinene productivities. This approach aimed to exclude any transgenic line where the transformed DNA integrated in an epigenetically silenced locus or in a genomic region that would cause growth defects when disrupted. The 20 strains were tested for cell density at the end of the cultivation and for β-pinene titers in a miniaturized experiment (Figure S2). The five transgenic lines with the highest β-pinene titers without hampered growth were assessed in comparable conditions to EE transgenic lines. Ultimately, we obtained a strain (RICE_CTP_AgPinS 10) that could produce 10.95 ± 1.14 ng·10^{-7}cells pinene (β-pinene + α-pinene), with a volumetric yield of 26.91 ± 1.92 μg·L^{-1} and a cell density on the day of harvest of 2.46·10^7 cells·mL^{-1}. Specifically, the β-pinene titers of this strain were 7.88 ± 0.85 ng·10^{-7}cells, leading to a volumetric yield of 19.35 ± 1.42 μg·L^{-1}.This titer is 3-fold higher than the episomal control EE_AgPinS 2 cultivated in the same experiment (2.62 ± 0.11 ng·10^{-7} cells of β-pinene, with a volumetric yield of 6.00 ± 1.07 μg·L^{-1}). RICE_CTP_AgPinS 10 did not display growth defects compared to the other transgenic lines (Fig. 4b). Similarly to what we observed for EE_CTP_AgPinS transgenic lines, RICE_CTP_AgPinS transgenic lines produce both β-pinene and α-pinene in a ~25:75 mixture on average.

We then used the same approach to generate RICE transgenic lines expressing a pinene synthase from the plant *Citrus limon* (ClPinS). This enzyme was reported to have a higher substrate affinity (K_m=0.0031 mM, pH=7, T = 30°C) [42] compared to AgPinS (K_m=0.006, pH=7.8, T = not provided) [44], according to *in vitro* studies. We speculated that expressing a ClPinS in the chloroplast of *P. tricornutum* could improve pinene production titers or the specificity for β-pinene. Following a prescreening of 17 independent transgenic lines that assessed the strain viability and pinene production (Figure S3), we selected five with the highest pinene titers. The transgenic strain with the highest β-pinene titers was RICE_CTP_ClPinS 6, which produced 7.49 ± 0.48 ng·10^{-7} cells, leading to a volumetric yield of 20.07 ± 0.51 μg·L^{-1} β-pinene (Fig. 4d). These values are similar to what we measured from RICE_CTP_AgPinS 10, suggesting that the availability of free GPP might be a limiting factor in the heterologous synthesis of pinene from MEP-derived precursors in the chloroplast of *P. tricornutum*. More experiments and statistical power are required to solidify this hypothesis.

Discussion

Similar to terrestrial plants, the diatom *P. tricornutum* synthesizes the terpenoid precursors IPP and DMAPP via two distinct pathways: the

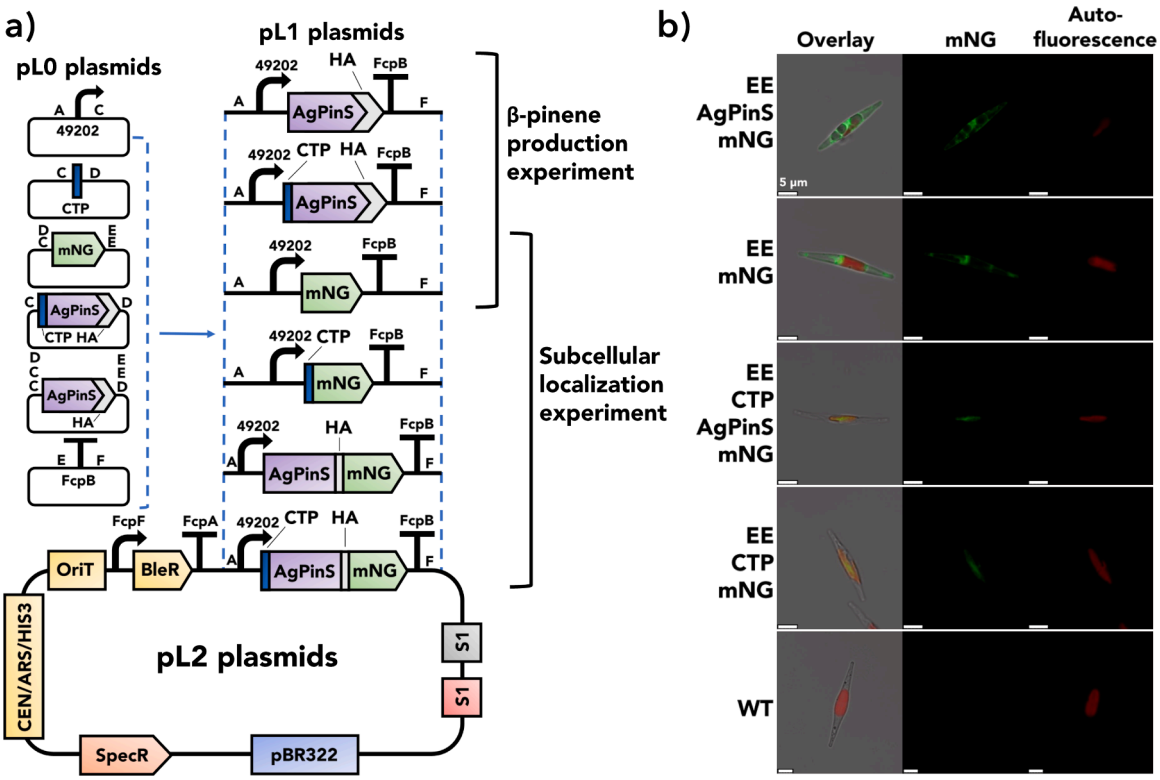

Fig. 2. Generation of transgenic lines and subcellular localization of heterologous AgPinS enzymes. a) Assembly of plasmids used to generate episomal transgenic lines for β-pinene production and for visualizing the subcellular localization of the enzymes.. Level 0 plasmids (pL0) were either sourced from the uloop repository or generated for this study. Letters from A to F represent connectors used to control the order of assembly of pL0 parts. Level 1 plasmids (pL1) occupying position 2 of the final level 2 plasmid (pL2) were customized for this study while the others were part of the uLoop repository. 49202: putative protein promoter; CTP: chloroplast targeting peptide from ATP synthase γ subunit. mNG: mNeonGreen fluorescent protein; AgPinS: Pinene synthase from *A. grandis*; HA: human influenza hemagglutinin tag; FcpB: Fucoxanthin-chlorophyll a-c binding protein B terminator; CEN/ARS/HIS3: centromeric region/autonomous replicating sequence/imidazole glycerol-phosphate dehydratase gene; OriT: origin of transfer sequence; FcpF: Fucoxanthin-chlorophyll a-c binding protein F promoter; BleR: Bleomycin resistance gene; FcpA: Fucoxanthin-chlorophyll a-c binding protein A terminator; S1: uLoop Spacer sequence 1; pBR322: origin of replication for *E. coli*; SpecR: spectinomycin resistance cassette. B) Representative confocal microscopy images showing the subcellular localization of the expressed proteins. EE_AgPinS_mNG and EE_mNG: transgenic lines expressing cytosol-retained AgPinS fused to mNG or mNG, respectively. EE_CTP_AgPinS_mNG and EE_CTP_mNG: transgenic lines expressing chloroplast-targeted AgPinS fused to mNG or mNG, respectively. The wild type (WT) strain was included as a control. The first column shows the overlay of the mNG fluorescence signal (in green) and the chlorophyll autofluorescence signal (in red). The second and third column show the individual mNG and chlorophyll autofluorescence signal, respectively. Scale bars correspond to 5 μm.

MVA pathway in the cytosol and the MEP pathway in the chloroplast. Our objective was to test which subcellular route was the most suitable to heterologously synthesize the monoterpenoid β-pinene, considering that plants natively produce monoterpenoids in the chloroplast. We found that targeting the AgPinS to the chloroplast enabled the production of pinene in *P. tricornutum*, whereas no pinene was detected when AgPinS was retained in the cytosol. Subcellular pathway localization is a metabolic engineering strategy applied to a variety of different products, including terpenoids [45–47]. Some advantages of using a specific subcellular environment are the higher local concentration of enzymes, higher availability of substrates, reduced toxicity of the desired product to the cell, and avoidance of competing pathways or degradation pathways that affect the substrate(s) or product(s) of interest [45,48]. Localization of terpenoids biosynthesis has been successful in eukaryotic cells, where the subcellular architecture is more complex, and many biochemical reactions are already compartmentalized (*e.g.* in organelles).

The chloroplast has been successfully exploited for the heterologous synthesis of terpenoids, with promising results in both microalgae and plants. The most relevant microalgal chassis to produce terpenoids is *C. reinhardtii*, a model freshwater species valued for its fast growth and genetic accessibility. It can only synthesize terpenoid precursors DMAPP and IPP in the chloroplast via the MEP pathway, therefore chloroplast localization of terpenoid biosynthetic pathways has been successfully implemented in *C. reinhardtii*. A variety of terpenoids have been heterologously produced in the chloroplasts, including monoterpenoids, sesquiterpenoids (C15), diterpenoids (C20) and carotenoids (C40) [49–52]. When the same terpene synthase was localized in the chloroplast or in the cytosol, the final titers were either equal or higher in the chloroplast [22,49]. In plants, MEP pathway-derived precursors in the chloroplast have been shown to yield higher monoterpenoid titers compared to MVA pathway-derived precursors, both for native and heterologous production [53]. Furthermore, new techniques for chloroplast engineering are emerging, such as the expression of synthetic droplets to increase the stability and storage of terpenoids [54]. This strategy has not yet been combined with the production of monoterpenoids.

We speculate that the availability of precursors is the biggest contributor to the higher β-pinene production of transgenic lines with chloroplast-targeted PinS in *P. tricornutum*. The chloroplast supply of terpenoid precursors is maintained by the MEP pathway, which functions efficiently in the chloroplast due to its reducing environment and the high availability of glyceraldehyde 3-phosphate and pyruvate, key metabolic substrates of the pathway. *P. tricornutum* uses the IPP and DMAPP from the MEP pathway for the biosynthesis of photosynthetic pigments and carotenoids, which are abundant in the chloroplast [50]. GPP can be synthesized by dedicated GPP synthases (GPPSs), and it can exist as an intermediate product of the reactions of other

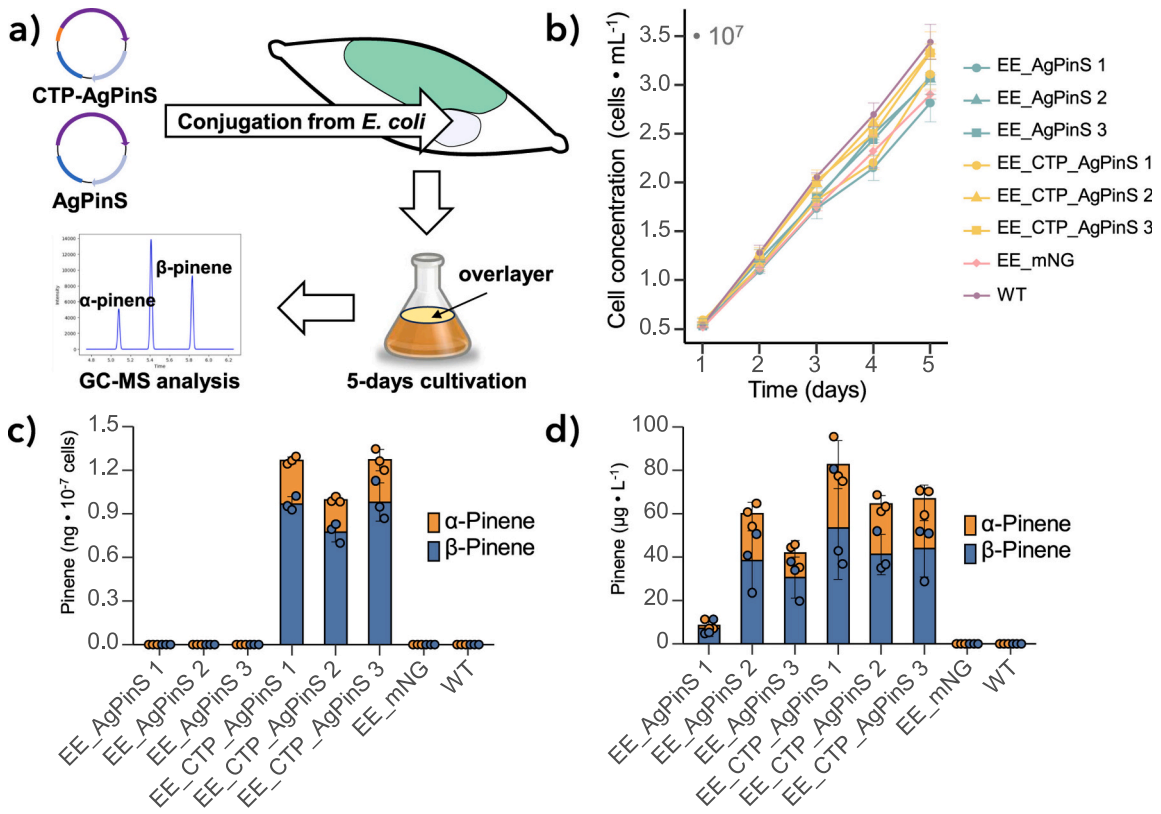

Fig. 3. Biosynthesis of pinene in P. tricornutum transgenic lines expressing AgPinS episomally (EE). a) Experimental workflow for the generation and screening of pinene-producing transgenic lines. Plasmids expressing chloroplast-targeted or cytosol-retained AgPinS were transformed in P. tricornutum via bacterial conjugation. Three independent colonies for each transformation were precultured in liquid media and then cultivated in biological triplicate overlayed with 1 % isopropyl myristate (IM). Finally, the overlayer was recovered and probed for the content of pinene by gas chromatography coupled with mass spectrometry (GC-MS) using commercial standards as reference. A transgenic line expressing the fluorescent protein mNeonGreen (mNG) b) Growth profile of transgenic strains EE_AgPinS, and EE_CTP_AgPinS. EE_mNG and the wild type (WT) strain were used as controls (n = 3, error bar indicates standard deviation from the mean). c) Final pinene titers in EE_AgPinS, EE_CTP_AgPinS transgenic strains after 5 days of cultivation, compared to EE_mNG and WT controls. Mean, standard deviation and geometric points of each individual replicate are depicted (n = 3). Colorized histogram bars and geometric points represent the two structural isomers of pinene β-pinene (teal) and α-pinene (orange). d) Conversion of GPP into pinene with crude cellular lysate obtained at the end of the 5-days cultivation from EE_AgPinS and EE_CTP_AgPinS strains. Crude lysates from EE_mNG and WT strains were included as controls. Mean, standard deviation and geometric points of each individual replicate are depicted (n = 3) Colorized histogram bars and geometric points represent the two structural isomers of pinene β-pinene (teal) and α-pinene (orange).

prenyl-transferases, such as farnesyl diphosphate synthases (FPPSs) or geranylgeranyl diphosphate synthases (GGPPS) (Fig. 1). Dedicated GPPSs have been reported to exist in the chloroplast of monoterpenoids-producing plants [6], and this could also apply to *P. tricornutum*. A recent computational analysis revealed the presence of 5 prenyl-transferases in the genome of *P. tricornutum* [26]. Among the 5 identified, only one is predicted to be targeted to the chloroplast (Phatr3_J19000), although no experimental validation has been performed yet. If the prediction is correct, Phatr3_J19000 should be a GGPPS to supply GGPP for the synthesis of carotenoids and chlorophylls, among others. Consequentially, GPP would only be available transiently in the chloroplast during the chain elongation in the synthesis of GGPP.

The expression of the *AgPinS* gene from an episome offered us a reproducible tool to establish that the chloroplast is superior to the cytosol to produce the monoterpenoid β-pinene in the experimental conditions used in this study. However, episomal expression has some limitations that might prevent the full exploitation of *P. tricornutum* for the high and stable production of monoterpenoids. It has been determined that subpopulations with lower productivities can originate from EE strains over time, and this can be due to structural or segregational instability of plasmids [55]. We have limited knowledge about the segregation mechanisms of episomes in *P. tricornutum*, but heterogenous subpopulations have been observed especially with high transgene expression from the episomes [37]. Structural instability of plasmids due to genetic rearrangements has also been observed in *P. tricornutum* transgenic lines, for example with an episome encoding a biosynthetic pathway for vanillin [56]. We overcame the limitations of EE strains by generating RICE strains expressing AgPinS (RICE_CTP_AgPinS) which could give additional insights on the potential of *P. tricornutum* for the production of monoterpenoids. RICE strains reached up to 3-fold higher β-pinene titers compared to the EE strains. RICE strains that outperform EE strains in terms of production titers can be obtained when enough transformants are screened. This has been shown for multiple eukaryotic species including *P. tricornutum* [37,57], although it does not always apply especially compared to EE strains with high-copy plasmids [58]. RICE can benefit from DNA landscapes with high transcription profiles, high-copy integration, and potentially less burden on the DNA replication machinery. At population level among the same RICE transgenic line the expression levels are more consistent and stable [55,57]. Regarding the analytical method for pinene recovery, in this study we have used a standard *in situ* liquid-liquid biphasic extraction during cultivation [26,37,45]. Pinene is one of the most volatile monoterpenes in nature and therefore it is possible that it may escape the isopropyl myristate (IM) overlayer over several days of cultivation [59]. Similar concerns have been raised in a previous study using such experimental setup to produce the volatile monoterpenoid geraniol in *P. tricornutum* [26]. Another study confirmed that *in situ* extraction with biphasic cultivation is less effective than direct headspace capture, when evaluating the production titers of yeast strains that produce the volatile monoterpenoid limonene [60]. Taken together, these findings suggest

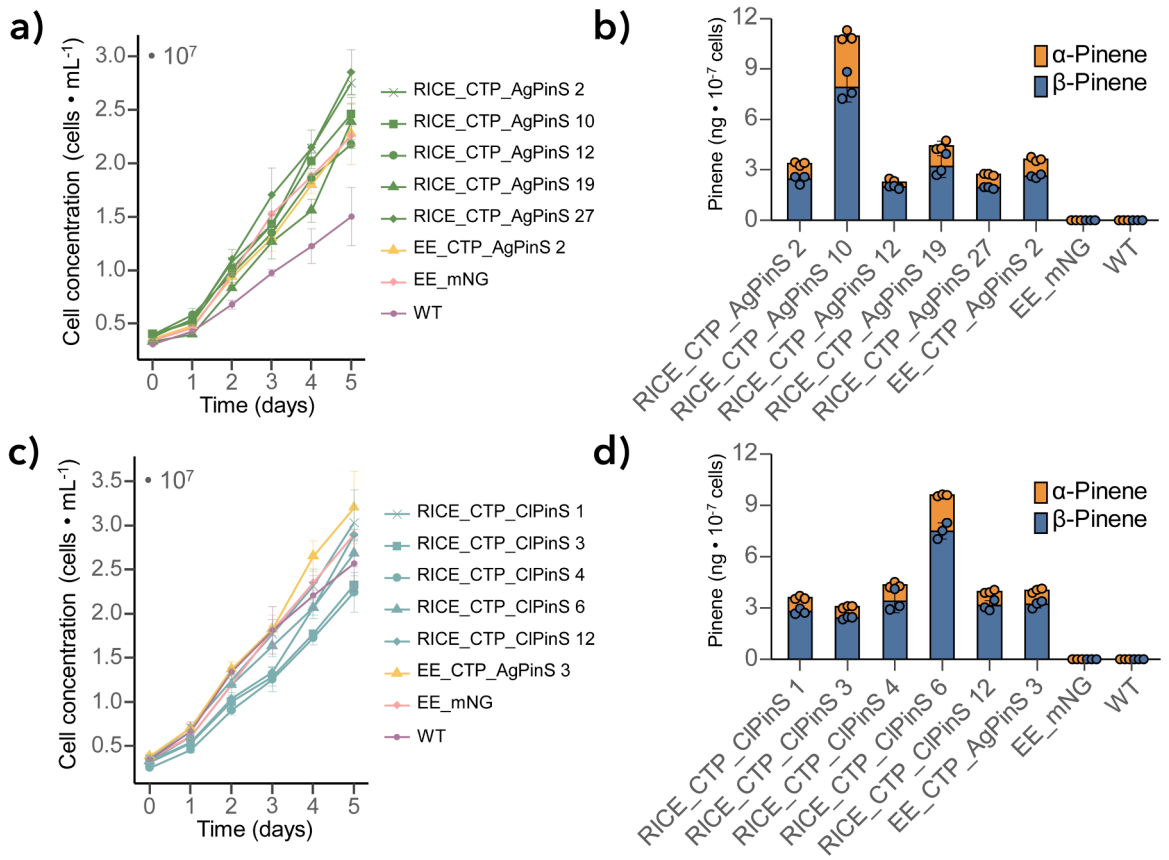

Fig. 4. Biosynthesis of pinene in *P. tricornutum* transgenic lines expressing AgPinS or ClPinS genes randomly integrated in chromosomes (RICE) of the nuclear genome. a) Growth profile of transgenic strains RICE_CTP_AgPinS. The episomal transgenic strains EE_mNG and EE_CTP_AgPinS 2, and the wild type (WT) strain were used as controls (n = 3, error bar indicates standard deviation from the mean). b) Final pinene titers in five independent RICE_CTP_AgPinS transgenic strains after 5 days of cultivation, compared to EE_mNG, EE_CTP_AgPinS 2 and WT controls. Mean, standard deviation and geometric points of each individual replicate are depicted (n = 3). Colorized histogram bars and geometric points represent the two structural isomers of pinene β-pinene (teal) and α-pinene (orange). c) Growth profile of transgenic strains RICE_CTP_ClPinS. The episomal transgenic strains EE_mNG and EE_CTP_AgPinS 3, and the WT strain were used as controls (n = 3, error bar indicates standard deviation from the mean). d) Final pinene titers in five independent RICE_CTP_ClPinS transgenic strains after 5 days of cultivation, compared to EE_mNG, EE_CTP_AgPinS 3 and WT controls. Mean, standard deviation and geometric points of each individual replicate are depicted (n = 3). Colorized histogram bars and geometric points represent the two structural isomers of pinene β-pinene (teal) and α-pinene (orange).

that the standard method adopted may underestimate actual pinene production, potentially due to volatilization during cultivation. Future studies may address this technical concern and establish a laboratory analytical method tailored for high volatile molecules. Monoterpenoids such as β-pinene are notoriously challenging to synthesize heterologously in model microorganism such as *E. coli* and *S. cerevisiae* due to their toxicity and to the low pool of GPP in these organisms [61]. The native low pool of GPP is usually compensated by metabolic engineering strategies, which can be complicated and time-consuming to implement. Such metabolic engineering efforts often involve the overexpression of entire pathways (MEP or MVA), either to increase the amount of precursors or to involve rerouting metabolism to produce more GPP. Metabolic engineering performed in *E. coli* and *S. cerevisiae* advanced these strains from producing low or undetectable amounts of monoterpenoids to titers above 1 $g{\cdot}L^{-1}$ [62,60,63–65]. Although comparing experiments involving different organisms with varying monoterpenoid synthases expressed at disparate levels is difficult, these results demonstrate that the pinene titers achieved in *P. tricornutum* are within the same order of magnitude as the initial titers reported for monoterpenoid production in other robust industrial microorganisms [60,65,66]. Notably, *P. tricornutum* cells were grown entirely photoautotrophically, unlike the other studies referenced. Currently, extensive metabolic interventions in *E. coli* and *S. cerevisiae* have enabled pinene titers of up to 1035 $mg{\cdot}L^{-1}$ and 10.2 $mg{\cdot}L^{-1}$, respectively [18,62].

Previous research demonstrated that *P. tricornutum* cells have a considerable pool of intracellular GPP to fuel the production of monoterpenoids. Previous studies generated EE strains capable of producing up to 210 $ng{\cdot}10^{-7}$ cells of the monoterpenoid geraniol from cytosolic GPP [26], which is higher than the pinene titers obtained in this study from chloroplastic GPP. In another experiment, George and colleagues generated RICE strains that reached geraniol titers of 304.4 $ng{\cdot}10^{-7}$ cells (leading to a volumetric yield of 0.89 $mg{\cdot}L^{-1}$), without negative effects on cellular fitness [37]. These results suggest that the availability of GPP in the cytosol may not limit the production of monoterpenoids at the titers indicated. In our study, we could only detect pinene when AgPinS was localized in the chloroplast in our tested conditions, which is intriguing. However, our study differs in type of molecule produced, enzyme used, and also the cultivation parameters, which could have also affected the abundance of terpenoid precursors from the MVA pathway or the MEP pathway. For example, while both geraniol and pinene strongly partition into the IM overlayer [67,68], pinene has a higher vapor pressure (mmHg at 20°C, geraniol: 0.00954; α-pinene: 2.93; β-pinene: 2.2) and therefore could volatilize in the headspace at a higher rate, negatively affecting its recovery and quantification [69–71]. Regarding cultivation conditions, we enriched CO_2 concentration in the headspace to 2.5 % and applied a 16:8-h day:night photoperiod, while geraniol production was achieved growing the cells in continuous light [26,37]. Additionally, the AgPinS used in this study and the CrGES used for geraniol production in *P. tricornutum* might differ in catalytic efficiency k_{cat}/K_m, and these differences could explain why our

EE_CTP_PinS strains did not generate enough pinene in the cytosol to be recovered. However, there is not enough data on the enzymes' functional parameters to support this hypothesis yet. A recent study on monoterpenoid biosynthesis in the yeast peroxisome also highlighted that high product titers can be attributed to high catalytic efficiency of the respective monoterpenoid synthase [45]. The chemical (bio)conversion or degradation of pinene molecules could have contributed to the lower titers of pinene obtained in this study compared to the previously reported titers for the monoterpenoid geraniol produced in *P. tricornutum* [26,37]. While biotransformation of α-pinene and β-pinene has been reported for bacterial and fungal strains, there is no evidence that this could also occur in *P. tricornutum* [9]. Further research could focus on profiling the metabolome of these transgenic lines to identify potential degradation or conversion products of pinene.

Pinene production has been achieved in other photosynthetic microorganisms besides *P. tricornutum*, but never in other eukaryotic microalgae. A pinene synthase from *Pinus taeda* was introduced in the cyanobacterium *Synechocystis sp.* PCC 6803, and the authors measured 40 $\mu g \cdot L^{-1}$ pinene captured with gas stripping and cold-trapping [72]. The pinene synthase from *P. taeda* has a higher affinity for GPP than most pinene synthases (K_m=0.0014 mM), however it has α- to β-pinene mixture more shifted to the least valuable structural isomer α-pinene (85.7:14.3) [72]. Other studies in photosynthetic organisms expressed AgPinS, which is considered more efficient than PinSs from *P. taeda* and *P. abies* in vivo experiments in *E. coli* [8]. AgPinS produces similar α - to β-pinene mixtures in *E. coli* (~46:54) and *in vitro* (42:58), slightly in favor of the more valuable β-pinene [8,73]. When AgPinS was expressed in *Synechococcus* sp. PCC 7002, 1.5 $mg \cdot L^{-1}$ of pinene were recovered from the biphasic overlayer. The authors reported a ~33:67 mixture of α- to β-pinene, remarkably shifted towards β-pinene [39]. AgPinS expressed in *R. sphaeroides* as a fusion protein with a GPPS from *A. grandis* enabled the biosynthesis of 97.51 $\mu g \cdot L^{-1}$ pinene, with a ~33:67 mixture of α- to β-pinene [74]. Our results show an even bigger shift towards β-pinene (~23:77 mixture of α- to β-pinene for EE_CTP_AgPinS strains), suggesting that the chloroplast environment in *P. tricornutum* favors the production of β-pinene. Previous studies associated the shift in product specificity to the local availability of GPP, the local concentration of the cofactors Mg^{2+} and Mn^{2+}, and the degree of reduction of certain amino acids in the catalytic site (Cys364 residue in AgPinS) [8,75]. However, other factors impacting product specificity may still need to be identified. In our study, transgenic *P. tricornutum* strains expressing AgPinS or ClPinS in the chloroplast produced similar mixtures of α- to β-pinene despite the reported differences in product specificities of the two enzymes *in vitro* [42,73].

While this study focused on understanding which cellular pathway of terpenoid precursors was the most promising for β-pinene production in *P. tricornutum*, further genetic and metabolic engineering approaches can fully uncover its potential in the biosynthesis of β-pinene and possibly other monoterpenoids. To boost the production of heterologous terpenoids, a simple strategy is to introduce several copies of terpenoid synthase genes, which was deemed successful in bacteria, yeast and microalgae [41,49,76]. Alternatively, stronger gene expression could be attained either by using stronger promoters or by targeting specific genomic *loci* [77]. For example, genomic safe harbors associated with high production titers of the monoterpenoid geraniol have been identified [37] and could be exploited for the insertion of a PinS gene. Another promising approach to achieve higher titers of monoterpenoids is boosting the availability of GPP and improving the channeling of reaction intermediates towards the desired product. For example, expressing a GPP synthase (GPPS) highly specific to GPP, without triggering further prenylation reactions (*e.g.* to FPP or GGPP), has been achieved in many microorganisms [61]. In some cases, fusing a GPPS with a monoterpene synthase has resulted in an even greater increase in final product titers [8]. Furthermore, overexpressing rate limiting enzymes in the MEP pathway can improve the production of terpenoids [78] (Fig. 1). The overexpression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS) and 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK)in *P. tricornutum* successfully enhanced the synthesis of terpenoids such as fucoxanthin and β-carotene [79–81]. Lastly, targeting a PinS to the cytosol and to the chloroplast simultaneously could be an approach to fully capture monoterpenoid precursor from both MVA and MEP pathway, and compensate for potential pathway cross-talks. This approach was effective in tomato fruits, where it is suggested that chloroplastic GPP is exported into the cytosol [82]. Taken together, these genetic interventions and metabolic engineering approaches can be considered suitable for improving the synthesis of monoterpenoids in *P. tricornutum*, and possibly, with minor modifications, for the production of even more complex and higher value terpenoids.

Concluding remarks

This study lays the fundamentals for the photosynthetic production of monoterpenoids in the diatom *P. tricornutum* by localizing the biosynthesis enzymes in the chloroplast. This strategy was here implemented to produce β-pinene, a monoterpenoid with applications in the flavour and fragrance industries and with potential uses as a pharmaceutical and biofuel. Moreover, the volatile nature of β-pinene facilitates its extraction from microalgal cultures without disrupting the high-value biomass. This allows for the retention of biomass for further cultivation or co-product extraction, such as omega-3 fatty acids and fucoxanthin. Such an approach enhances the overall economics and sustainability of the microalgal bioprocess, aligning with principles of a circular bioeconomy. Microalgae can be cultivated at large scales in photobioreactors or open ponds, utilizing CO_2 from various sources, including direct air capture systems, to support growth and product formation [83]. Condenser technologies, such as steam distillation, can be used to efficiently capture and separate volatile compounds like β-pinene from the culture medium [84]. These methods are scalable and compatible with continuous processing, making them suitable for integration into industrial-scale microalgal biorefineries, which represents a promising revenue stream yet to be fully established in microalgal research. The combination of efficient volatile compound extraction and high-value biomass retention contributes to a more sustainable and economically viable microalgal bioprocess, promoting the development of a bio-based economy.

In this study, we implemented different gene expression strategies and different pinene synthases to reach high titers, achieving a volumetric yield of up to 19.35 ± 1.42 $\mu g \cdot L^{-1}$ β-pinene with AgPinS and up to 20.07 ± 0.51 $\mu g \cdot L^{-1}$ β-pinene with ClPinS. While promising, these titers are not yet sufficient to commercially compete with current less sustainable β-pinene production practices. Future experiments should focus on improving the availability of precursors (*e.g.* GPP) by metabolic engineering and by further increasing the gene expression level for the pinene synthase. Furthermore, engineering the *PinS* gene to improve both catalytic activity and product specificity toward β-pinene could be considered in the future. It has been reported that the product specificity of a pinene synthase can be influenced by the local concentration of its substrates and cofactors, and by the redox state of certain amino acids in the catalytic site (*e.g.* Cys364 residue in AgPinS) [8,75]. Increased specificity for β-pinene would reduce the formation of unwanted monoterpene byproducts, easing downstream purification and enhancing the efficiency and predictability of the production process. This would contribute to a more streamlined and scalable biotechnological production, specifically tailored to β-pinene isomer. Lastly, more research is needed to optimize the recovery method of volatiles to obtain higher titers and to allow recovery at higher scales.

Experimental procedures

Cultivation of P. tricornutum cells

P. tricornutum strain CCAP 1055/1 was grown in artificial seawater (ASW; 419.23 mM NaCl, 22.53 mM Na_2SO_4, 5.42 mM $CaCl_2$, 4.88 mM K_2SO_4, 48.21 mM $MgCl_2$, 372 µM H_3BO_3, and 20 mM HEPES at pH 8) supplemented with 2 mL·L^{-1} of commercial nutribloom plus (Necton, Portugal). Cells were cultivated in liquid media inside an HT Multitron Pro (Infors Benelux, Netherlands) orbital shaker unit operated at 20°C, 85 rpm, 2.5 % CO_2 enriched air and illuminated with warm-white, fluorescent light bulbs with an intensity of 120 µmol photons ·m^{-2}·s^{-1} with a 16:8-h diurnal cycle. Cell concentration of liquid cultures was measured with a Multisizer 3 Coulter Counter (Beckman Coulter, Netherlands). For growth curves, cells were sampled daily approximately six hours after the start of the diurnal cycle. For growth assessment analysis, the area under the curve (AUC) was calculated using the *trapz* function from the *pracma* package in RStudio version 2024.09.0 + 375 "Cranberry Hibiscus". Statistical tests on biological triplicates were performed with the same software. When cells were grown on solid media, ASW was supplemented with 2 mL·L^{-1} nutribloom plus and 1.2 % agar. *P. tricornutum* was maintained on solid media inside a climate room at 20°C and environmental CO_2 and was illuminated with warm-white, fluorescent light bulbs with an intensity of 100 µmol photons·m^{-2}·s^{-1} with a 16:8-h diurnal cycle.

Selection of PinS genes and construction of plasmids

The plasmids used in this study were assembled by recursive golden gate using the uLoop assembly synthax [85]. Unless stated otherwise, DNA sequences were kindly provided by Christopher Dupont (J. Craig Venter Institute, San Diego, USA) and are retrievable online from the uLoop catalogue [86]. Full information on DNA sequences used in this study are reported in Table S1. The protein sequences of AgPinS (GenBank U87909.1) and ClPinS (GenBank AF514288.1) were retrieved from the literature. The 38 aa putative plant chloroplast targeting peptide (CTP) of AgPinS was removed as done by Sarria and colleagues [8]. Likewise, the putative 55 aa CTP of ClPinS was removed as performed by Lücker *et al.* [42], with the only exception of retaining the Isoleucine amino acid in position 55. Additionally, a Serine Glycine dipeptide linker (to avoid protein destabilization), and a human influenza hemagglutinin (HA)-detection tag (for further immunoblotting analyses) were added to the PinS protein at the N-terminus and C-terminus, respectively. The resulting *AgPinS* and *ClPinS* genes were codon optimized for *P. tricornutum* by GeneWiz (South Plainfield, USA) and domesticated for uLoop assembly, by removing internal *Bsa*I and SapI restriction sites. The genes were synthesized as plasmids by GenScript (Rijswijk, Netherlands) using a pBluescript II KS(-) backbone. In case of PinS chloroplast localization, a *P. tricornutum* AtpC CTP sequence [43] was added to the N-terminus of the PinS. The *atpC* CTP DNA sequence was amplified from the nuclear genome of *P. tricornutum* by PCR with a Q5® High-Fidelity 2X Master Mix (New England Biolabs, USA). Primers used in this study are listed in Table S2. The amplicon was purified from a 0.8 % agarose gel with a GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermofisher Scientific, USA) and ligated into a uLoop-compatible receiver plasmid. Genetic sequences that were present in the uLoop catalogue but required different uLoop connectors were adapted from the original plasmid using the same procedure described above for the AtpC CTP. All the final plasmids used for conjugation contained a OriT, CEN/ARS/HIS3, and the *bleR* gene regulated by the FcpF promoter and the FcpA terminator. The gene of interest for each plasmid was regulated by the 49202 promoter and the FcpB terminator. The plasmid containing *ClPinS* fused to *atpC* CTP, that was linearized and later electroporated, was also generated as described above. The plasmid linearized and used for electroporation, containing *AgPinS* fused to *atpC* CTP, was constructed differently. It contained CEN/ARS sequence previously used in *Nannochloropsis oceanica* [87], and the *bleR* gene regulated by the FcpF promoter and the FcpA terminator. Plasmids were transformed by heat shock and multiplied in chemically competent *E. coli* DH5α and isolated with GeneJET Plasmid Miniprep Kit (Thermofisher Scientific, USA).

Transformation of P. tricornutum cells to obtain transgenic lines

For episomic expression, *P. tricornutum* was transformed by conjugation as described by Moosburner and colleagues [88]. Colonies were selected on ASW agar plates with 2 mL·L^{-1} nutribloom plus and 75 mg·L^{-1} Zeocin (Invitrogen, USA). Prior to the conjugation to *P. tricornutum*, the plasmids were transformed into *E. coli* TransforMax EPI300 harboring the conjugative plasmid pTA-MOB as described previously [89]. To generate *E. coli* electrocompetent cells, a preculture was inoculated overnight at 250 rpm, 37°C in Lysogeny broth (LB) supplemented with 20 mg·L^{-1} gentamycin (LB+Gm20). The next day, 500 µL of preculture were diluted with 49.5 mL of LB+Gm20 (1:100 dilution) and grown until the optical density at 600 nm was between 0.4 and 0.6. The *E. coli* cells were incubated at 4°C for 30 min and then pelleted at 3000 rpm for 10 min at 4°C. The pellet was then washed with 50 mL of ice-cold milliQ water and pelleted again as described above. After two more washes with 25 mL of ice-cold 10 % glycerol, cells were resuspended in 500 µL of ice-cold 10 % glycerol and 50 µL aliquots were mixed with 100 ng of plasmid DNA. To electroporate *E. coli*, the mixture of DNA and cells was transferred in a 2 mm electroporation cuvette and pulsed with a GenePulser Xcell (Biorad, USA) at 2,5 kV, 200 Ω, 50 µF. After the pulse, cells were resuspended in 1 mL LB, incubated 1 h at 37°C, and plated in LB agar+Gm20 supplemented with 100 mg·L^{-1} of spectinomycin antibiotic. For stable genome integration, *P. tricornutum* cells were electroporated with linear DNA. The linear gene expression cassettes were amplified by PCR with a Q5® High-Fidelity 2X Master Mix (New England Biolabs, USA). The PCR amplicons were excised from a 0.8 % agarose gel and purified with a GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermofisher Scientific, USA). 4 µg of each DNA fragment were used for electroporation following the procedure described by Hu and Pan [90]. All the transgenic strains used in this study can be found in Table 1.

Verification of subcellular localization by fluorescence microscopy

One colony from each transgenic *P. tricornutum* strain EE_mNG, EE_CTP_mNG, EE_CTP_AgPinS_mNG, EE_ AgPinS_mNG was inoculated at a concentration of 2·10^6 cells·mL^{-1} in ASW with 2 mL·L^{-1} nutribloom plus and 75 mg·L^{-1} Zeocin, and grown until mid-exponential phase (~ 3 days). The WT strain was included as a control with the only difference that no antibiotic was added to the medium. 3 µL of cells were mounted on a microscope slide and imaged with 38X water immersion lens on a Leica SP8-SMD multimode confocal microscope (Leica, Germany). mNG and chlorophyll were excited with a 488 nm laser and their respective fluorescence and autofluorescence were detected with a 525 nm filter and 680 nm filter, respectively.

Recovery and quantification of pinene from P. tricornutum cultures

After conjugation, three colonies of transgenic strain EE_AgPinS and three colonies of transgenic strain EE_CTP_AgPinS were randomly selected. One colony of transgenic strain EE_mNG and the WT strain were selected as controls. The selected colonies were precultured for at least two generations in liquid ASW supplemented with 2 mL·L^{-1} nutribloom plus and 75 mg·L^{-1} Zeocin, except for the WT, for which no antibiotic was used. Precultures in late-exponential growth stage were inoculated at a final concentration of 4·10^6 cells·mL^{-1} into 140 mL ASW, supplemented with 4 mL·L^{-1} nutribloom plus and 75 mg·L^{-1} Zeocin. 1 % vol·vol^{-1} isopropyl myristate (IM; Sigma- Aldrich, USA) was added to 140 mL of microalgal culture at the time of inoculation, forming a

biphasic overlay to allow the capture of pinenes. The IM layer was harvested by centrifugation for 30 min at 4500 x *g* at the end of the cultivation (day 5, ~11 h after illumination) and spiked with 3 $mg{\cdot}L^{-1}$ camphene (Sigma-Aldrich, USA) as internal standard. Subsequently, the IM layer was diluted with ethyl acetate (1:5) and directly analyzed by gas chromatography couple with mass spectrometry (GC-MS). 10^9 cells were pelleted at 2000 x *g* for 15 min and stored at −20°C until further processing. The overlayers samples were run on a Trace 1300 GC (Thermofisher scientific, USA) equipped with a Triplus RSH autosampler (Thermofisher Scientific, USA). The column used was a DB-WAX Ultra Inert fused silica capillary column (Agilent Technologies, USA; 30.0 m × 0.25 mm × 0.5 μm). Hydrogen was used as carrier gas at constant flow of 1.0 $mL{\cdot}min^{-1}$, with a 1:20 split-flow. Each sample was measured in technical duplicate with injection volume of 1 μL. The temperature gradient of the oven was 50°C for 3 min, then sequentially increased at the rate of 35°C min^{-1} to 200°C and then held at 200°C for 4 min. A ISQ7000 single quadrupole mass spectrometer (Thermofisher Scientific, USA) was used for mass spectrometry. The injector temperature was maintained at 220°C, the MS transfer line temperature was maintained at 250°C, and the ion source temperature at 290 °C. Quantitative analysis of pinene was run in Selective Ion Monitoring mode, where ions in the range 92.5 – 93.5 $m{\cdot}z^{-1}$ were measured with a dwell time of 0.1 s. The MS detector was set to start measuring after 4.2 min and to stop after 6.5 min, to avoid the detection of the solvents ethyl acetate and IM. The calibration curves used to determine the sample concentration were generated by measuring a range of known concentrations of (-)-α-pinene and (-)-β-pinene standards (Sigma-Aldrich, USA) in triplicate. The same procedure was repeated for transgenic strains RICE_CTP_AgPinS and RICE_CTP_ClPinS. However, before selecting the 5 most performant strains, a pre-screening was conducted on a larger number of transformant lines. For such pre-screening, 20 colonies of RICE_CTP_AgPinS were cultured without biological replicates in 20 mL medium with 1 % IM. 17 colonies of RICE_CTP_ClPinS were cultured without biological replicates in 20 mL medium with 1.5 % IM. Processing of the IM overlayer and GC-MS analyses was performed in the same way as for the transgenic lines with episomal expression.

Production of pinene from cellular crude lysate

The 10^9 cells frozen at the end of the pinene production experiment (day 5) were resuspended in 3 mL of 0.1 mM HEPES and disrupted by sonication (Braun-Sonic 2000 with microprobe at maximum power for 15 s on ice) and clarified by centrifugation (4000 x *g*, 30 min, 4°C). The total protein content of the supernatant was determined with the Pierce Bradford Plus Protein Assay Kit (Thermofisher Scientific, USA) according to manufacturer's instructions. A volume of crude lysate containing 700 ng of protein was added to a 2 mL glass vial and spiked with 1X protease inhibitor cocktail (MedChemExpress, USA), 10 mM $MgCl_2$, and 50 μM geranyl diphosphate ammonium salt (GPP; Sigma Aldrich, USA) and adjusted to 1 mL with 0.1 mM HEPES. The mixtures were overlayed with 200 μL IM and incubated at 30°C in the dark for 16 h. The IM layer was harvested, clarified by centrifugation in a miniG spinner (IKA, Germany) for 20 min at maximum speed, and diluted 1:5 with ethyl acetate. Pinene content was analyzed by GC-MS as described in the previous section.

CRediT authorship contribution statement

Nicola Trevisan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Michele Fabris:** Writing – review & editing, Supervision. **John van der Oost:** Writing – review & editing, Supervision, Resources, Project administration. **Maria Barbosa:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Sarah D'Adamo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2025.10.003.

Data availability

Data will be made available on request.

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