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Chloroplast engineering of the green microalgae *Chlamydomonas reinhardtii* for the production of HAA, the lipid moiety of rhamnolipid biosurfactants

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ABSTRACT

Hydroxyalkanoyloxyalkanoates (HAA) are lipidic surfactants with a number of potential applications, but more remarkably, they are the biosynthetic precursors of rhamnolipids (RL), which are preferred biosurfactants thanks to their excellent physicochemical properties, biological activities, and environmental biodegradability. Because the natural highest producer of RLs is the pathogenic bacterium *Pseudomonas aeruginosa*, important efforts have been dedicated to transfer production to heterologous non-pathogenic microorganisms. Unicellular photosynthetic microalgae are emerging as important hosts for sustainable industrial biotechnology due to their ability to transform CO_2 efficiently into biomass and bioproducts of interest. Here, we have explored the potential of the eukaryotic green microalgae *Chlamydomonas reinhardtii* as a chassis to produce RLs. Chloroplast genome engineering allowed the stable functional expression of the gene encoding RhlA acyltransferase from *P. aeruginosa*, an enzyme catalyzing the condensation of two 3-hydroxyacyl acid intermediaries in the fatty acid synthase cycle, to produce HAA. Four congeners of varying chain lengths were identified and quantified by UHPLC-QTOF mass spectrometry and gas chromatography, including C_{10}-C_{10} and C_{10}-C_8, and the less abundant C_{10}-C_{12} and C_{10}-C_6 congeners. HAA was present in the intracellular fraction, but also showed increased accumulation in the extracellular medium. Moreover, HAA production was also observed under photoautotrophic conditions based on atmospheric CO_2. These results establish that RhlA is active in the chloroplast and is able to produce a new pool of HAA in a eukaryotic host. Subsequent engineering of microalgal strains should contribute to the development of an alternative clean, safe and cost-effective platform for the sustainable production of RLs.

Abbreviations: RL, Rhamnolipids; 3-HA, 3-hydroxylated fatty acid or 3-hydroxyacyl acid; 3-HA(C_{10}), 3-hydroxydecanoic acid; HAA, hydroxyalkanoyloxyalkanoates or 3-(3-hydroxyalkanoyloxy)alkanoic acid; HAA(C_{10}-C_{10}), 3-hydroxydecanoyl-3-hydroxydecanoic acid; 3-HA(C_{10})-ME, 3-hydroxydecanoic acid methyl ester; ACP, Acyl Carrier Protein; Rha, rhamnose; RhlA, acyltransferase; RhlB, RhlC, rhamnosyltransferase B or C; TAP, Tris-Acetate-Phosphate; HSM, high salt medium; GC-FID, gas chromatography-flame ionization detector; UHPLC-QTOF, ultra-high performance liquid chromatography-quadrupole time-of-flight; ESI, electrospray ionization.

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1. Introduction

A circular bioeconomy demands the use of biological platforms and renewable resources to enable the sustainable production of high-value chemicals and pharmaceuticals. Surfactants are amphiphilic compounds widely used in industry owing to their tendency to accumulate between polar and non-polar interfaces, thus reducing their surface and interfacial tension. In contrast to synthetic surfactants, that are mostly derived from petrochemicals, biosurfactants are obtained from biological sources and renewable feedstocks, and show enhanced biodegradability and reduced ecotoxicity [1]. Because of their higher surface activity and emulsifying power and lower critical micelle concentrations compared to synthetic surfactants, biosurfactants are gaining attention in industry, and their global market is increasing [1,2].

Among the different biosurfactants, rhamnolipids (RLs) are a glycolipid class that contains rhamnose (Rha) as a sugar moiety linked to 3-hydroxylated fatty acid chains (Fig. 1) [3]. RLs comprise a family of more than 60 compounds or congeners that were first isolated from the bacterium *Pseudomonas aeruginosa*. They differ from each other in the number of rhamnose moieties (mono-RL with one Rha or di-RLs with two Rha), in the number of 3-hydroxyacyl (3-HA) chains (one or two), and in their chain length and number of unsaturated bonds [4,5]. In *P. aeruginosa*, RLs are secreted into the media and regulate multicellular group behavior such as quorum sensing, biofilm formation, and swarming motility [4]. They have antimicrobial activities to dominate the ecological niche and virulence capacity to modulate host immune responses in animals and plants [6,7]. With their outstanding physicochemical and biological activities, RLs are being exploited in multiple biotechnological and industrial applications such as bioremediation and enhanced oil recovery, pharmaceuticals, cosmetics, food, detergents and cleaners, and agriculture [2,8,9]. Moreover, the lipidic hydroxyalkanoyloxyalkanoate (HAA) precursor of RLs also has interesting bioactivities, such as an immunostimulant of plant defense responses [10].

Despite their ecological, industrial, and biomedical interest, the use of RLs is limited by current production costs and market price [8,9]. Their complex structure and stereochemistry make RL production by chemical synthesis very challenging [1]. Consequently, extraction from natural producer organisms, such as the highest producer *P. aeruginosa*, a biosafety level 2 opportunistic human pathogen, has been exploited. However, it is an expensive procedure associated with biosafety containment measures and strict purification processes [1,8]. To circumvent these limitations, non-pathogenic strains and other bacterial producers have been explored to produce RLs [8,11,12], resulting in lower RL yields compared to *P. aeruginosa*, probably due to tight control of RL biosynthesis. Genetic and metabolic engineering coupled with novel high-throughput screening methodologies are being developed to identify novel or improved producer strains [8,11,13,14].

Several species of bacteria such as *Escherichia coli, Pseudomonas putida,* or *Burkholderia kururiensis* have been engineered [1,8,15–21] to produce RLs by introducing the *P. aeruginosa* mono- and/or di-RLs biosynthetic pathway (Fig. 1). RL biosynthesis requires RhlA acyltransferase, which condenses two 3-hydroxyacyl-ACP or 3-hydroxyacyl-CoA units to generate the lipid moiety 3-(3-hydroxyalkanoyloxy)

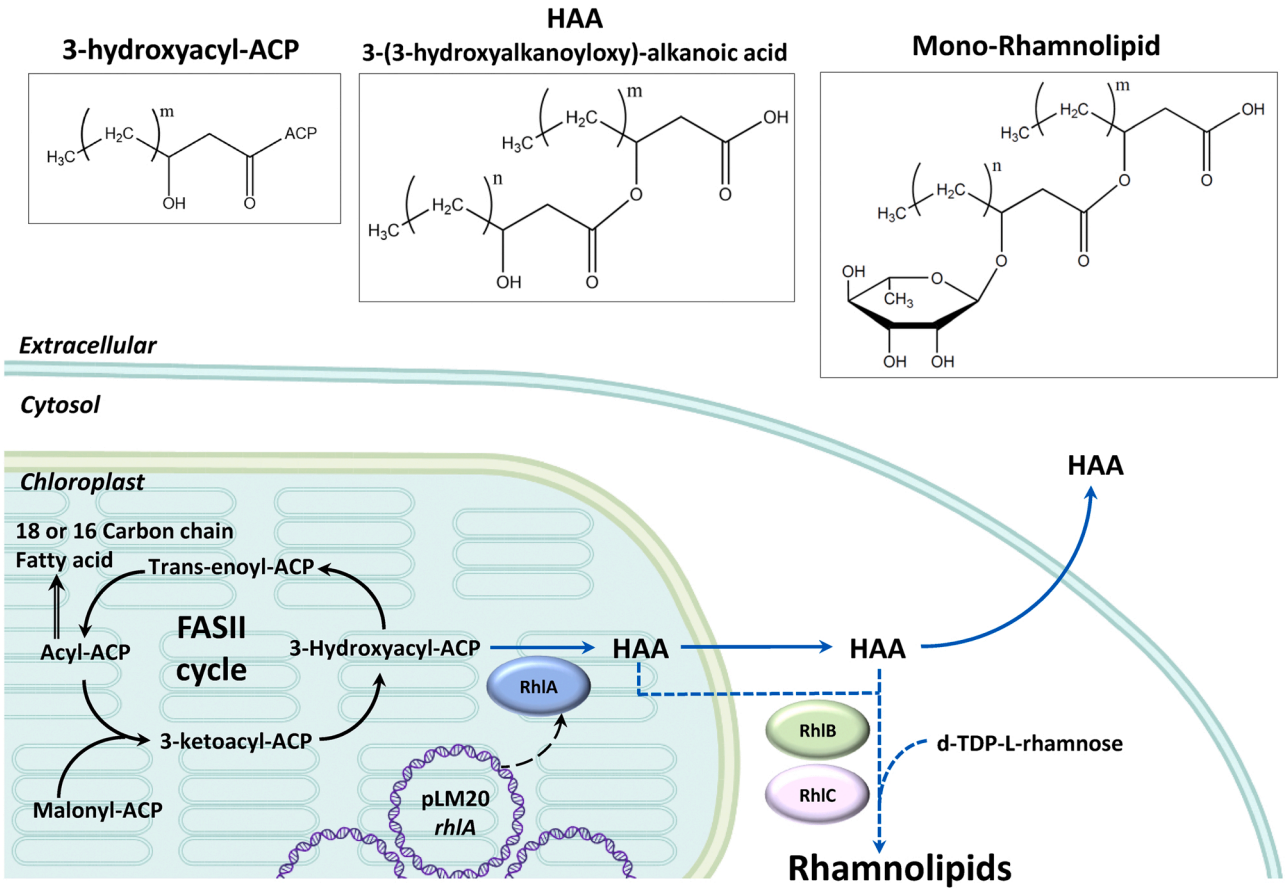

Fig. 1. Engineering rhamnolipid biosynthetic *de novo* pathway in *Chlamydomonas reinhardtii*. The acyltransferase RhlA catalyzes the condensation of two 3-hydroxyacyl-ACPs of varying carbon length (m,n = 2–14) derived from the Fatty Acid Synthase II (FASII) cycle, which results in the formation of different congeners of the lipid moiety 3-(3-hydroxyalkanoyloxy)-alkanoic acid (HAA). In this study, chloroplast genome transformation of *C. reinhardtii* was performed to express RhlA enzyme in the chloroplast for the production of HAA lipidic precursor (solid blue arrows). Subsequent addition of rhamnosyltransferases RhlB and RhlC (discontinuous blue arrows) will enable the synthesis of Rhamnolipids in *C. reinhardtii* cells.

alkanoic acid (HAA) [22], followed by the sequential action of rhamnosyl transferases RhlB and RhlC to generate mono- and di-RL, respectively [8,15,19,23]. Engineered strains produce recombinant RLs although at lower levels than the natural producer *P. aeruginosa*. In several cases, the availability of the sugar nucleotide precursor dTDP-Rha used by RhlB and RhlC limits the production capacity [8,15], indicating that precursor pathway optimization must also be addressed in engineered strains. In addition, tailor-made production of HAA and RL congeners has been explored by combining RhlA/B/C enzymes from different natural producer organisms [5,12,15,22]. Nevertheless, some companies are already producing HAA and/or RL at industrial scale [11], such as AGAE technologies Ltd. (Corvallis, OR, USA) or Evonik (Essen, Germany), the latter using genetically-engineered *Pseudomonas putida*.

Microalgae are a diverse group of unicellular photosynthetic organisms found in freshwater and marine habitats, which are emerging as sustainable hosts for industrial biotechnology as they use photosynthetic light to efficiently transform CO_2 into biomass and added value bioproducts [24,25]. Microalgae possess some potential advantages such as low production costs and reduced carbon footprint if no organic carbon source is added to the media, frequent GRAS (Generally Regarded As Safe) status, no requirement for arable land, and rapid growth in both large-scale outdoor systems and in fully contained photobioreactors [25]. *Chlamydomonas reinhardtii* is a eukaryotic green microalga that has been developed not only as a model organism [26], but also as a biotechnological platform, empowered by a large portfolio of available genetic and molecular tools [27–29] together with novel synthetic biology and high throughput screening technologies [25,30,31]. *C. reinhardtti* has been successfully used for the production of recombinant proteins [28,32,33] and fine chemicals such as terpenoids, ketocarotenoids, lipids or bioplastics [34–39], or for bioplastic degradation [40]. Moreover, recent US Food and Drug Administration (FDA) approval of *C. reinhardtii* as a food additive and GRAS status has expanded the applications of this microalga to the food industry.

Given that microalgae produce and accumulate glycolipid products [39,41,42], the aim of the present study was to develop *C. reinhardtii* as a novel cellular platform for the orthogonal production of RLs. As proof of principle, *C. reinhardtii* strains were constructed to produce HAA, a lipidic biosurfactant with reported bioactive properties [10,12] and building block of RLs. Because 3-HA-ACP precursors are intermediaries of the Fatty Acid Synthase (FAS) cycle and are located in the chloroplast (Fig. 1), the *C. reinhardtii* chloroplast genome was transformed with a construct allowing the expression of RhlA acyltransferase from *P. aeruginosa* PAO1 strain. Intracellular and secreted production of HAA and congener profiles were determined and quantified in stable transplastomic lines by GC-FID and UHPLC-QTOF. As far as we are aware, this is the first report demonstrating HAA production capability in a eukaryotic, photosynthetic, and GRAS host, providing proof of principle that microalgae can be explored as an alternative clean, safe, and cost-effective host for RL production.

2. Materials and methods

2.1. Strains and growth conditions

Wild type (WT) 137c strain of *Chlamydomonas reinhardtii* (CC-125 wild type mt^+, Chlamydomonas Stock Center) was used. Cells were grown in Tris-Acetate-Phosphate (TAP) liquid medium [43] under constant illumination (50 μmol m^{-2} s^{-1}) at 25 ºC and 125 rpm in an orbital shaker Innova 42 (Eppendorf, Hamburg, Germany), and monitored by measuring the optical density (OD) at 750 nm. TAP solid medium was prepared with 1.5% agar and supplemented with spectinomycin (100 mg/L). For photoautotrophic growth conditions, a minimal high salt medium (HSM) was used as described [44].

2.2. Plasmid construction and generation of transplastomic lines

The *P. aeruginosa* (PAO1) *rhlA* gene sequence (Pseudomonas Genome DataBase code PA3479) fused to a hemagglutinin (HA) epitope YPYDVPDYA was codon optimized according to the codon usage of *C. reinhardtii* chloroplast genome, and a synthetic gene was obtained from GeneArt (Thermo Fisher, Waltham, USA). The *psaA* promoter fragment was amplified from pSRSapI vector [45], obtained from the Chlamydomonas Stock Center. The *rbcL* terminator fragment was amplified from the pLM20 vector [46], kindly provided by G. Allorent (CNRS-CEA Grenoble). Primers listed in Suppl. Table S1 were used to amplify *psaA* promoter, *rhlA-HA* gene fusion, and *rbcL* terminator fragments by PCR. pLM20 vector was digested with *Eco*RV and *Sma*I restriction enzymes, and the four pieces were assembled by Gibson Assembly (New England BioLabs, Ipswich, MA, USA) and transformed into *E. coli* DH5α cells (Thermo Fisher). The resulting construct was confirmed by sequencing (Stab Vida, Caparica, Portugal).

C. reinhardtii 137c chloroplast were transformed by microbombardment, either with the Bio-Rad Biolistic PDS-1000/He Particle Delivery System (Hercules, USA) at the Center for Research in Agricultural Genomics, CRAG (Barcelona) as described [47] (lines RhlA#1–2) or with the in house built helium-driven particle gene gun at the Institute of Physico-Chemical Biology IBPC (Paris) as described [48] (lines RhlA#3–5). After the initial selection of transformants growing on spectinomycin-supplemented TAP medium (100 mg/L), several rounds of subcloning were performed on TAP-spectinomycin 500 mg/L until reaching homoplasmy, i.e., a state where all copies of the chloroplast genome were shown to harbor the gene of interest. Homoplasmy was confirmed by PCR genotyping of the resulting transplastomic lines using the primers listed in Suppl. Table S1. Genomic DNA was extracted by resuspending cells from colonies in a 50-μL solution of 10 mM EDTA at pH 8 and boiling for 5 min

2.3. Protein extraction and immunoblot analysis of transplastomic lines

Wild-type 137c and RhlA-HA expressing lines were grown in 15 mL of TAP medium to a 0.26 $OD_{750\ nm}$, corresponding to 1.6×10^6 cells/mL. Cells were harvested by centrifugation for 20 min at 900 xg, and total protein extracts were obtained by acetone precipitation upon incubation in 1 mL cold acetone followed by 20 min centrifugation at 4 ºC and 16000 xg. Protein pellets were resuspended into a final volume of 100 μL of Lysis Buffer (50 mM Tris-HCl pH 6.8, 4% SDS, 20 mM EDTA, Protease Inhibitor Complete according to manufacturer instructions (Merck, Darmstadt, Germany)), and boiled for 10 min at 95 ºC. Protein concentration was determined from clarified extracts by Pierce™ BCA Protein Assay Kit (Thermo Fisher).

Total protein extracts were separated on a 12% polyacrylamide gel electrophoresis (Bio-Rad, Hercules, USA) and transferred onto a PVDF membrane (Millipore, Burlington, MA, USA). Immunodetection was performed with a primary rat anti-HA high-affinity antibody (1:1000) (Merck) followed with secondary mouse anti-Rat-HRP antibody (1:25000) (Merck). Chemiluminescence detection was performed using SuperSignal™ West Pico PLUS (Thermo Fisher) in a ImageQuant LAS4000 system (GE Healthcare, Chicago, USA).

2.4. Detection and identification of congeners of HAA by UHPLC-QTOF

WT 137c and RhlA cell cultures were grown to saturation in TAP medium under continuous light. To obtain the extracellular fraction, 50-mL cultures were centrifuged at 900 xg for 20 min. The resulting supernatant was acidified to pH 2.0 and extracted three times by liquid-liquid extraction with 50 mL of chloroform/methanol (2:1). Chloroform phase was evaporated with N_2 and resuspended in 500 μL of methanol. 3-hydroxydecanoic acid (Merck,) and mono-rhamnolipids with a purity of 95% (Merck) were used as standards. The same procedure was used for cultures grown under photoautotrophic conditions

in HSM medium, except that a larger culture volume was used (150 mL) at a lower OD 750 nm of 0.65.

An UHPLC Exion LC™ AD System (AB Sciex, Framingham, USA) coupled with an X500B QTOF System (MS/MS) (AB Sciex) was used, with electrospray ionization (ESI) in negative mode for instrumental determination, and a Kinetex C18 Column (150 × 2.1 mm id, 2.6 μm particle size, 100 Å) from Phenomenex (Torrance, USA) for chromatographic separation. Further experimental details can be found in the Supplementary Text.

A non-targeted UHPLC-QTOF analysis was run to identify HAA congeners, following three criteria: (i) an area ratio threshold (RhlA line / WT 137c) of at least 100; (ii) a proposed chemical formula that do not exceed $C_{50}H_{100}N_{10}O_{10}$; and (iii) a mass error lower than 2 ppm between the proposed chemical formula and the obtained isotopic profile from experimental MS spectra.

2.5. Detection, identification, and quantification of 3-HA (C_{10}) derived from HAA by GC-FID and GC-MS

Extracellular and intracellular fractions were prepared from 50-mL cultures of WT 137 and RhlA lines as for UHPLC-QTOF, except that samples were resuspended in 200 μL of chloroform after chloroform phase evaporation. The esterification reaction was performed by adding 100 μL of methanol and 450 μL of HCl/Methanol 0.6 M (Merck,) for 1 h at 85 °C. After 15 min at room temperature, samples were extracted with 400 μL of n-hexane, and 200 μL of the upper n-hexane phase were saved, of which 2 μL were injected into a TRB-Wax-Omega Column 30 m x 0.25 mm × 0.25 μm (Teknokroma, Sant Cugat, Spain) and analyzed in a 7890 A GC System (Agilent Technologies, Santa Clara, USA). Peak identification was performed on a 6890 N Network GC System and 5973 inert Mass Selective Detector (Agilent Technologies). Further experimental details can be found in the Supplementary Text.

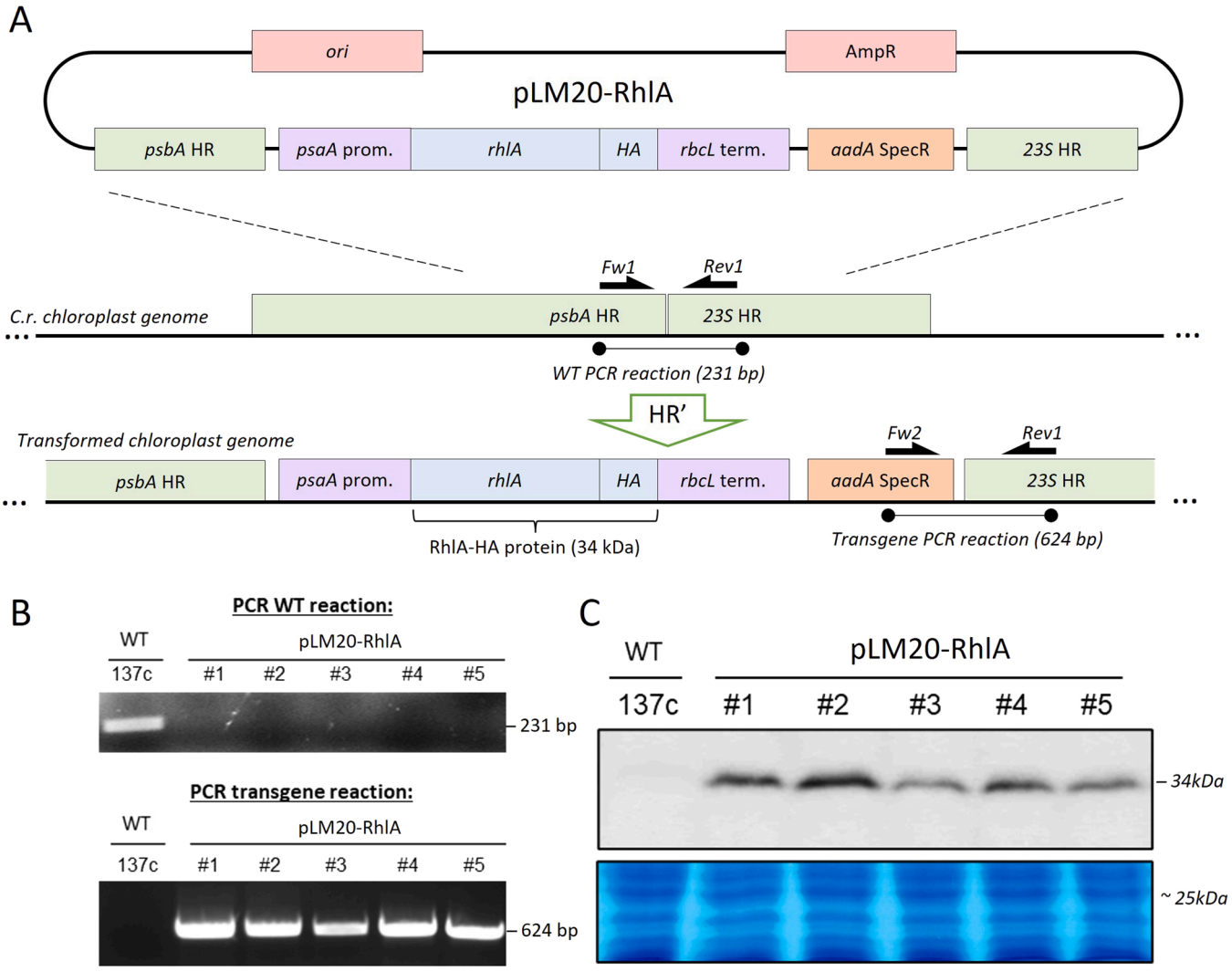

Fig. 2. Construction of transplastomic lines expressing RhlA-HA. (A) Generation of transplastomic lines by homologous recombination (HR') within the psbA and the 23 S homologous region (HR) sites from the constructed pLM20-RhlA vector and the chloroplast genome of *Chlamydomonas reinhardtii* (*C.r.*) 137c strain. In pLM20-RhlA, the *psaA* promoter drives the expression of the *rhlA* gene fused to an HA epitope for immunoblot detection, and the *rbcL* is used as terminator. The Spectinomycin resistance gene (*aadA*) is also inserted and used as a selection marker. For transgene detection and homoplasmy confirmation by PCR, Fw1 and Rev1 primers were used to detect the wild type (WT) copies generating a 231 bp fragment, and Fw2 and Rev1 primers were used to detect the chloroplast copies having integrated the transgene, yielding a 624 bp fragment. (B) Transgene detection and homoplasmy confirmation by PCR. 1ul of extracted genomic DNA was used as template in the PCR reactions detecting either the WT or transgene containing chloroplast copies. The amplified products were analyzed in a 1% agarose gel. (C) Immunoblot detection of RhlA-HA protein in transplastomic lines. RhlA-HA protein (34 kDa) was detected with rat anti-HA and anti-rat-HRP antibodies (upper image). A Coomassie blue staining of a replicated gel was analyzed as a visual control for equivalent sample loading (lower image). Five independent pLM20-RhlA transplastomic lines (#1 to #5) were analyzed together with a wild type 137c (WT) line used as a negative control. *ori: E. coli* origin of replication. *AmpR:* Ampicillin Resistance.

3. Results

3.1. Sequence optimization and cloning of Pseudomonas aeruginosa RhlA acyltransferase for recombinant expression in the chloroplast of Chlamydomonas reinhardtii

To generate a synthetic *C. reinhardtii* strain capable of producing the RL precursor 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) (Fig. 1) in the chloroplast, first the amino acid sequence of the acyltransferase RhlA encoded by the natural producer strain *P. aeruginosa* PAO1 was identified [22]. The sequence coding for RhlA (295 amino acids, 32.9 kDa) fused to a C-terminal HA tag for immunoblot detection was optimized according to the codon usage of the *C. reinhardti* chloroplast genome. The resulting synthetic gene encoded a 34 kDa fusion protein (RhlA-HA) that was cloned in the chloroplast expression vector pLM20 [46], under the control of the strong psaA promoter and the rbcL terminator (Fig. 2A). Upon transformation, the resulting pLM20-RhlA construct drives the insertion of the sequence encompassing the gene of interest and the spectinomycin resistance selection marker *aadA* in the chloroplast genome, by homologous recombination between the plasmid and chloroplast genomic loci *psbA* and *23 S* (Fig. 2A).

C. reinhardtii 137c strain was transformed with the pLM20-RhlA vector by microparticle bombardment, and the transformed spectinomycin resistant colonies were selected, isolated, and propagated. Five independent transplastomic RhlA lines were assessed for homoplasmy by PCR. As shown in Fig. 2B, primers designed to detect the transgene (Fig. 2A) were able to amplify a 624 bp product in the RhlA lines as expected, but not in the parental wild type (WT) 137c line used as a control. In contrast, primers designed to detect the wild type locus with no insertion (Fig. 2A) were able to amplify a 231 bp product in the WT line, but not in the transgenic lines, indicating that all chloroplast genome copies carry the transgene.

The expression of the RhlA-HA transgene in the selected transplastomic lines was investigated by immunoblot analysis using anti-HA antibody (Fig. 2 C). Importantly, the presence of the 34 kDa recombinant RhlA-HA protein was detected in all five selected lines, but not in the WT 137c. Together, these results indicate that all five transplastomic lines have reached homoplasmy and stably express the RhlA-HA fusion.

3.2. 3-hydroxydecanoyl-3-hydroxydecanoic acid (C_{10}-C_{10}) congener of HAA is detected in RhlA-expressing transplastomic lines by UHPLC-QTOF

The next aim was to detect HAA in the RhlA transplastomic lines, which is the product of the acyltransferase reaction catalyzed by RhlA as a result of the condensation of two 3-hydroxylated fatty acids (Fig. 1). Although RhlA from *P. aeruginosa* PAO1 has some substrate promiscuity as it accepts 3-hydroxylated fatty acids of varying hydrocarbon lengths (mainly 8, 10, 12, 14 carbon chains) to generate different HAA congeners [12], it has been reported to show some preference for the ten carbon 3-hydroxydecanoic acid (3-HA(C_{10})) [22,49].

In order to detect the 3-hydroxydecanoyl-3-hydroxydecanoic acid (C_{10}-C_{10}) congener of HAA (designated HAA(C_{10}-C_{10})), an UHPLC-QTOF method was set up. In the absence of HAA standard, MS identification was based on the fragmentation spectra of the precursor 3-HA and the final RL products. These standards (3-hydroxydecanoic acid (3-HA(C_{10})) and mono-rhamnolipid (mono-RL)) were prepared in methanol at a concentration of 0.8 mg/mL and 1.5 mg/mL, respectively, and analyzed by UHPLC-QTOF in ESI negative mode. The 3-HA(C_{10}) standard eluted at 5.70 min and, consistent with the calculated monoisotopic mass of 188.1412 Da, the MS spectra showed a peak at *m/z* 187.1333 ($[M-H]^-$) (Suppl. Fig. S1). The MS/MS fragmentation of 3-HA (C_{10}) resulted in a major fragment of *m/z* 59.0135 (Suppl. Fig. S1). In the case of the mono-RL standard, the compound eluted at 8.11 min with *m/z* 503.3225 in the MS spectra, which corresponds to Rha-C_{10}-C_{10} containing the 3-HA(C_{10}) congener of HAA (calculated monoisotopic mass of 504.3298 Da) (Fig. S1). Importantly, MS/MS fragments of Rha-C_{10}-C_{10} from the mono-RL standard were observed at *m/z* 59.0132 and 187.1338 (Suppl. Fig. S1) that were indeed coincident with the 3-HA (C_{10}) $[M-H]^-$ ion (*m/z* 187.1333) and with its main MS/MS fragment (*m/z* 59.0135).

Production of HAA(C_{10}-C_{10}) was evaluated in the RhlA transplastomic line #1 (and wild type strain as negative control) (Fig. 3 and Suppl. Fig. S2). Because HAA and RLs are reported to be secreted metabolites [50], the organic extracts were analyzed from extracellular media of WT 137c and RhlA-HA-expressing cultures. The UHPLC-QTOF total ion chromatograms in ESI negative acquisition mode are shown in Suppl. Fig. S2A. Consistent with the calculated monoisotopic mass of HAA(C_{10}-C_{10}) (358.2728 Da), a peak ($[M-H]^-$) of *m/z* 357.2645 was detected. This value was selected to extract ion chromatograms of WT and the transgenic line with *m/z* 357.265 ± 0.010 (Suppl. Fig. S2B). The results (Fig. 3A and B) show a main elution peak at 11.36 min that is present in the RhlA-expressing line, but not in the WT (Fig. S2B). MS/MS analysis of this parental ion *m/z* 357.2645 confirms the identification of HAA(C_{10}-C_{10}). At a collision energy of − 35 ± 15 V, fragments at *m/z* 187.1338 and 59.0140 were observed (Fig. 3 C), which were coincident with the parent ion of 3-HA(C_{10}) ($[M-H]^-$ *m/z* 187.1333) and its main MS/MS fragment (*m/z* 59.0135) as identified in the 3-HA standard (Suppl. Fig. S1). The fragment profile under different collision energies (from −10 to −50 V) provided additional support to the assignment (Suppl. Fig. S3). At low energy, the parental ion at *m/z* 357.2649 is still observed while it disappears at higher collision energies with the concomitant increase of the fragmentation peaks. Further confirmation was obtained from the analysis of the adducts with chlorine, with a characteristic isotopic profile with a main peak at *m/z* 393.2415 corresponding to $[M+Cl]^-$ (Suppl. Fig. S4). Altogether it was concluded that the 3-hydroxydecanoyl-3-hydroxydecanoic acid (C_{10}-C_{10}) congener of HAA was produced and detected in the extracellular medium of RhlA-HA-expressing lines but not in the WT 137c.

3.3. HAA congeners other than C_{10}-C_{10} are detected in RhlA-expressing transplastomic lines by UHPLC-QTOF

Because RhlA from *P. aeruginosa* PAO1 also shows specificity for 3-hydroxylated fatty acids of chain length other than 10 carbons, and because HAA congeners other than C_{10}-C_{10} are produced when RhlA is heterologously expressed in bacteria such as *E. coli* [12,15,49,51], the next aim was to detect additional HAA congeners among the compounds produced in RhlA lines. Therefore, a non-targeted UHPLC-QTOF analysis was run to identify other congeners among the compounds found. A list of 9 compounds was obtained that differentially accumulated in the extracellular medium of RhlA-expressing lines compared to WT 137c (Suppl. Table S2). Interestingly, in addition to the identified C_{10}-C_{10} congener of HAA (elution at 11.36 min) (Fig. 3 and Suppl. Table S2), the compound that eluted at 10.51 min showed a prominent peak with *m/z* 329.2334 that was consistent with the monoisotopic mass of the C_{10}-C_8 congener (330.2408 Da) (Suppl. Table S2 and Fig. S5). Two other HAA congeners were also detected with lower peak areas, with MS spectra ($[M-H]^-$) that were consistent with C_{10}-C_6 (monoisotopic mass 302.2098 Da, retention time 9.97 min), and with C_{10}-C_{12} (monoisotopic mass 386.3038 Da, retention time 12.67 min) (Suppl. Table S2 and Fig. S5). The MS/MS fragmentation provided support to the identity of these compounds (Fig. 4). Of the unidentified peaks, only one compound (#7 in Suppl. Table S2B) appeared to contain 3-HA(C_{10}) according to the MS/MS fragmentation and was disregarded for the rest of the analysis because of the minor relative abundance (< 2%) compared to the identified HAA congeners.

It was concluded that 4 HAA identified congeners are produced and secreted in *C. reinhardtii* 137c as a result of RhlA expression in the chloroplast, with C_{10}-C_{10} and C_8-C_{10} apparently showing higher abundance according to the observed peak area (Fig. 4, Suppl. Table S2).

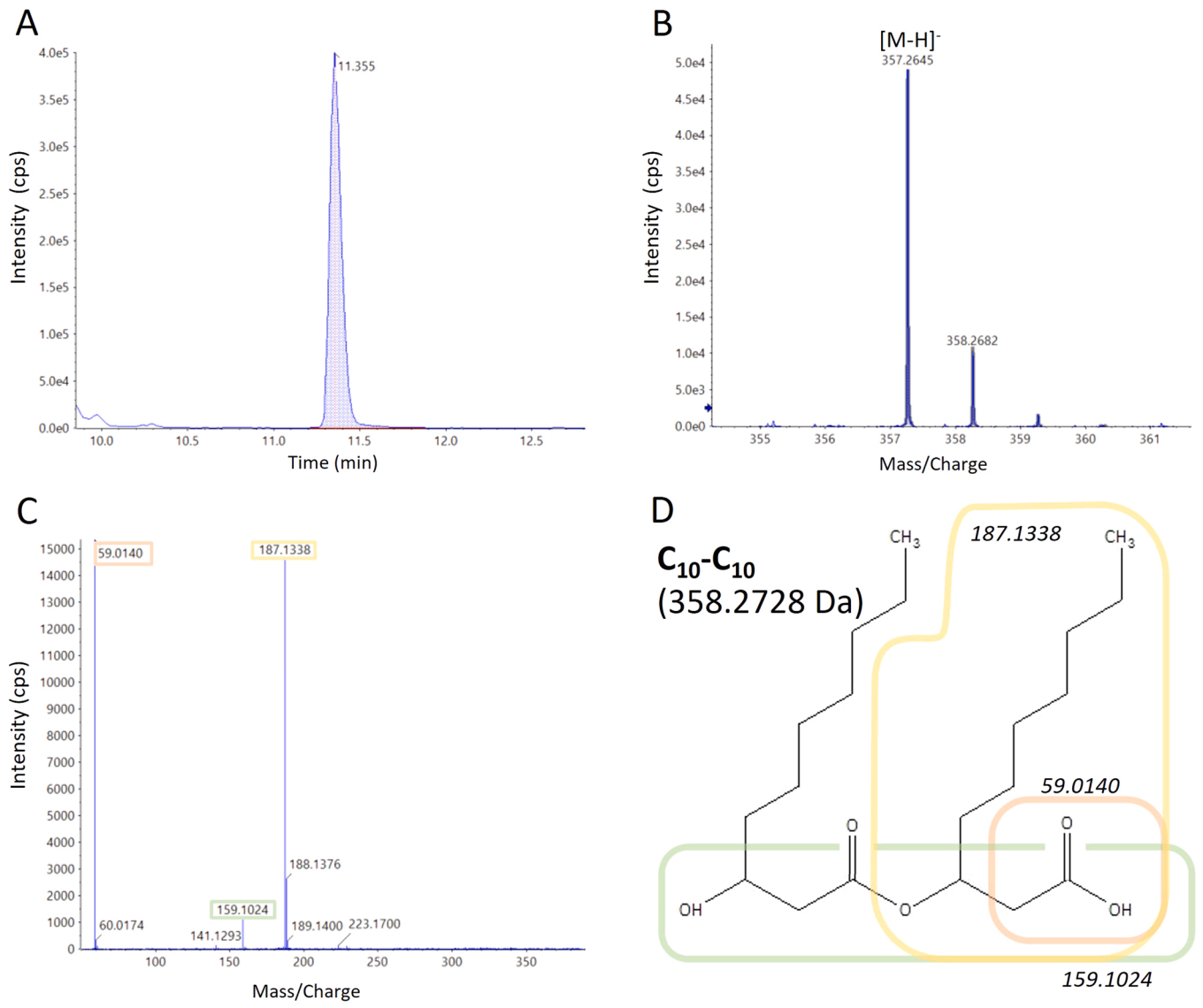

Fig. 3. Identification of C_{10}-C_{10} congener of HAA in RhlA-HA expressing lines by UHPLC-QTOF. The extracellular fraction of RhlA#1 strain culture, grown to saturation, was extracted with chloroform/methanol and analyzed by UHPLC-QTOF. (A) Extracted ion chromatogram (XIC) for a *m/z* of 357.26 corresponding to HAA (C_{10}-C_{10}), showing a retention time of 11.36 min (B) MS spectra of the sample at 11.36 min (C) MS/MS Mass spectrum from the 11.36 min chromatographic peak (collision energy -35 ± 15 V). (D) Fragment mass assignments of observed fragments in MS/MS spectrum of HAA (C_{10}-C_{10}). Calculated monoisotopic mass: 358.2728 Da.

3.4. 3-hydroxydecanoic acid methyl ester derived from HAA hydrolysis and esterification is detected in RhlA lines by GC-FID

The UHPLC-QTOF analysis presented above indicates that the four detected HAA congeners that are apparently more abundant (C_{10}-C_{10}, C_{10}-C_8, C_{10}-C_{12}, and C_{10}-C_6) are composed by at least one C_{10} 3-hydroxydecanoic acid. Therefore, 3-HA(C_{10}) must be the most abundant 3-hydroxylated fatty acid composing the HAA produced in RhlA-expressing lines. The 3-HA methyl ester (3-HA-ME) resulting from the hydrolysis and esterification reaction of HAA (Fig. 5A) was monitored by GC-FID to quantify intra and extracellular HAA production in RhlA transplastomic lines (Fig. 5B). Analysis of the extracellular medium of saturated cultures, equivalent to the samples analyzed above by UHPLC-QTOF (Suppl. Fig. S2), detected a peak at 4.0 min, equivalent to the elution time of the 3-HA(C_{10}) standard submitted to the same methyl esterification reaction, in the RhlA-expressing line but not in the WT (Fig. 5B). The identity of this peak as 3-HA(C_{10})-ME was further supported by GC-MS analysis (Suppl. Fig. S6).

This analysis was also extended to the intracellular fraction to test whether HAA can also accumulate as a novel intracellular pool. In this case, cells from saturated WT 137c and RhlA#1 strain cultures were collected by centrifugation, and the intracellular lipidic fraction was extracted with organic solvent as for the extracellular medium. Then the 3-HA(C_{10})-ME resulting from the hydrolysis and esterification reaction of HAA was analyzed by GC-FID. As shown in Fig. 5B, the complexity of the chromatogram is much higher in the intracellular fraction compared to the extracellular medium as expected. Importantly, this analysis also confirmed the presence of an intracellular pool of HAA in RhlA lines but not in WT. The absence of 3-HA(C_{10}) in the WT indicates that an endogenous pool of free 3-HA(C_{10}) does not exist as intermediary of the Fatty Acid Synthase cycle, or that it accumulates at a concentration that is below the detection limit by GC-FID.

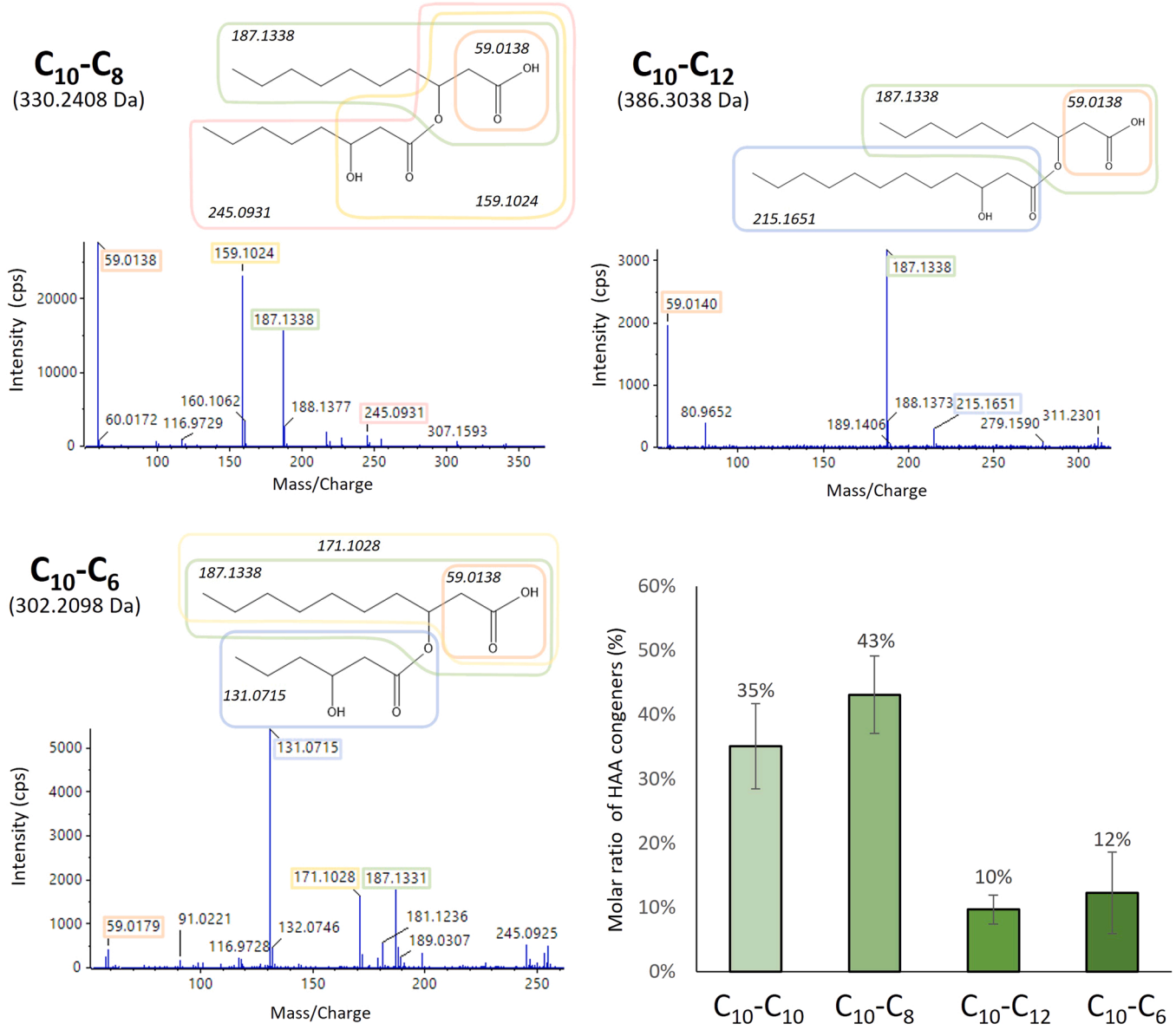

Fig. 4. Identification of HAA congeners other than C_{10}-C_{10} in RhlA-HA expressing lines by UHPLC-QTOF. The extracellular fraction of RhlA#1 strain cultures, grown to saturation, was extracted with chloroform/methanol and analyzed by UHPLC-QTOF. The MS/MS mass spectrum from the indicated HAA congeners (collision energy −35 ± 15 V), and the proposed mass fragment assignments are shown. The corresponding extracted ion chromatogram and the MS spectra are found in Fig. S5. The monoisotopic mass for each compound is indicated in parenthesis. Molar ratio of each congener (lower right panel) corresponds to the ratio of peak areas assuming the same response factor for the $[M\text{-}H]^-$ of each congener (standard deviation; n = 3).

3.5. Time-course quantification of 3-HA(C_{10})-ME derived from secreted and intracellular HAA

An accurate quantification of total HAA levels is not easy because it is produced as a complex mixture of congeners (Figs. 3 and 4) and because, standards are apparently not available. In order to develop a fast and easy method that provide an estimate of the relative production of HAA under different culture conditions and cell lines, it was opted to quantify by GC-FID the 3-HA(C_{10})-ME resulting from HAA hydrolysis and esterification (Fig. 5), since the four identified major HAA congeners all contain this 3-hydroxylated-acyl C_{10} chain. First a standard curve was set up by adding known amounts of 3-HA(C_{10}) standard to the extracellular medium and intracellular fraction of WT 137c cultures that do not produce HAA. The lipid fraction was then extracted with chloroform and methyl-esterified before GC-FID analysis (standard curve in Suppl. Fig. S7).

Quantification of 3-HA(C_{10})-ME derived from extracellular HAA produced by RhlA-expressing lines #1 to #5 was performed on saturated cultures grown at the same OD. 3-HA(C_{10})-ME was detected in all five RhlA lines, indicating that stable expression of RhlA-HA in the chloroplast consistently results in HAA secretion to the extracellular medium (Fig. 6A and Suppl. Fig. S8). Variation in the production of HAA was observed among lines from different transformation batches (Fig. 6A), possibly due to biological variation or different culture production dynamics, with RhlA lines #1 and #2 accumulating more prominent levels.

In order to explore how the production of HAA evolves during cell culture growth, a time-course analysis of 3-HA(C_{10}) from produced HAA was performed in biological triplicates of the best producing RhlA line #2. Cell growth of the RhlA-expressing line was equivalent to the WT (as monitored by OD 750 nm), thus indicating that RhlA expression did not compromise cell viability or fitness under these culture conditions (Fig. 6B). Samples of 50 mL were harvested at the indicated time points (Fig. 6 C and Suppl. Fig. S9), and secreted and intracellular levels of 3-

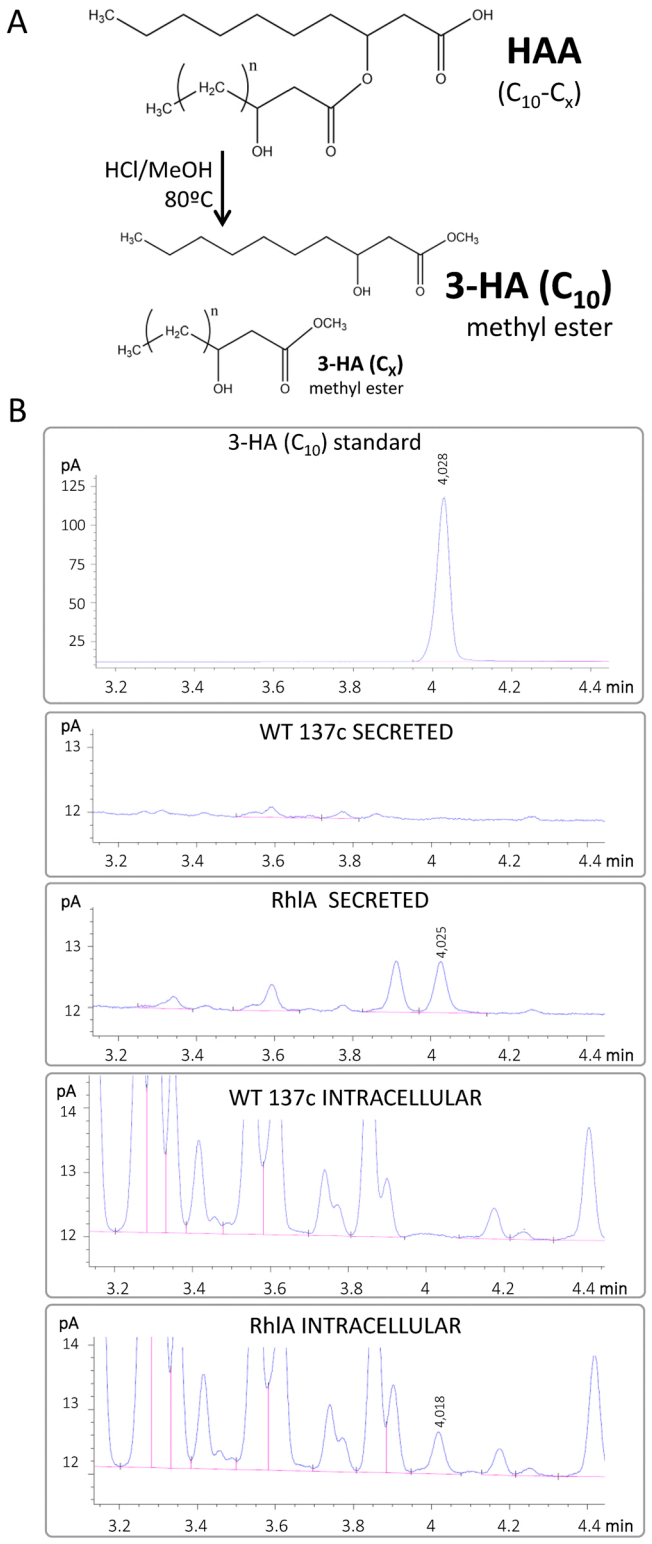

Fig. 5. Analysis of HAA production by Gas Chromatography. (A) Methyl esterification reaction of C_{10}-C_X HAA identified congeners, in which one molecule of HAA results in one 3-hydroxy-methylester-decanoic acid (3-HA (C_{10}) methyl ester) and one 3-hydroxyacyl (C_x) methyl ester (that could be C_{10} or another). (B) Detection by Gas Chromatography of the 3-HA (C_{10}) methyl ester resulting from the hydrolysis of HAA. RhlA-HA expressing line #1 and WT 137c as negative control were grown to saturation, and upon centrifugation samples were prepared from the supernatant (secreted fraction) and from the cell pellet (intracellular fraction). The chromatogram of the methyl ester of a 3-HA (C_{10}) standard is also included (top image), which elutes at 4.0 min as indicated.

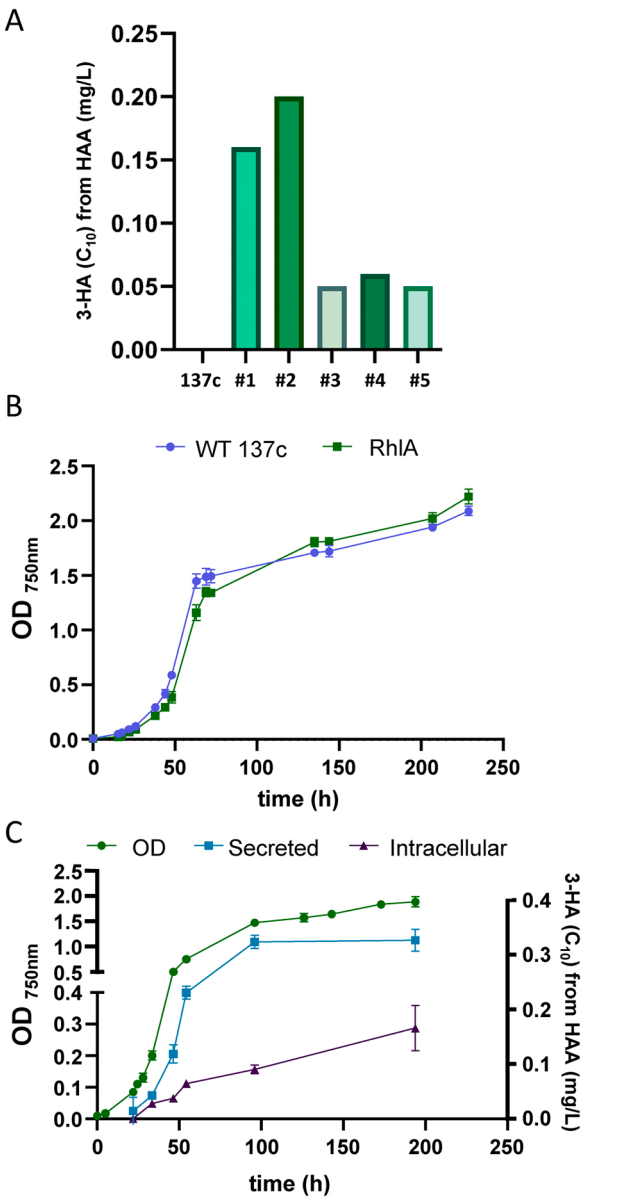

Fig. 6. Quantitative assessment of HAA production during cell culture growth. (A) Quantification of 3-HA(C_{10}) from produced HAA in the secreted fraction of saturated cultures of different RhlA-HA-expressing lines #1 to #5 and WT 137c as a negative control. The methyl ester 3-HA(C_{10}) resulting from the hydrolysis of HAA was detected and quantified by GC-FID as indicated (Fig. S7). (B) Analysis of growth of HAA-producing line RhlA#2 line and WT 137c. The $OD_{750\ nm}$ was measured at different times for 7 days. (C) Time-course quantification of 3-HA(C_{10}) from produced HAA in RhlA line #2. Zero-time point corresponds to a culture that was diluted and split in 50 mL aliquots. Secreted and intracellular fractions from the 50 mL cultures were grown and harvested at different times, the optical density was measured at 750 nm, and samples were processed for the analysis by GC-FID as in (A). Values in B and C represent the mean and standard deviation of 3 biological replicates.

HA(C_{10})-ME derived from HAA were quantified as before (Suppl. Fig. S7). It was observed that secreted C_{10}-containing HAA followed the same progression than culture growth, with an exponential accumulation between 22 h (OD 750 nm ~ 0.1) and 96 h (OD 750 nm ~ 1.5) that reaches a plateau at about 0.33 mg/L of 3-HA(C_{10}) derived from HAA (Fig. 6 C). In contrast to the secreted fraction, the intracellular production of HAA is much lower and follows a linear progression, reaching 0.166 mg/L at 194 h (Fig. 6 C).

Interestingly, in the analysed chromatographic window, peaks other

than 3-HA differentially accumulate in the extracellular medium of RhlA lines compared to WT, such as the one eluting at ~3.9 min (Fig. 5 and Suppl. Fig. S9). Although no attempt was made to identify this compound, it was noted that the peak corresponding to 3-HA(C_{10})-ME (eluting at ~ 4.0 min) appeared first and was relatively more abundant than the ~ 3.9 min peak in young cultures up to 54 h (Suppl. Fig. S9), whereas the relative abundance of the latter increased in aged cultures (196 h).

Altogether, combining the results of abundances obtained in the UHPLC-QTOF and the quantification of 3-HA(C_{10}) into the GC-FID the amount of each HAA congener per liter of culture could be estimated (Table 1).

Together, these results indicate that (i) HAA composed by the ten carbon 3-HA congener is produced in RhlA lines; (ii) it accumulates in the intracellular fraction; and (iii) it is secreted exponentially during cell growth until reaching a plateau in saturated cultures.

3.6. HAA congeners are produced under photoautotrophic conditions in minimal HSM medium

Production of HAA in Figs. 3–6 above was assessed under mixotrophic conditions in standard TAP medium, with acetate as organic carbon source, and in the presence of photosynthetic light. To assess whether HAA is also produced under photoautotrophic conditions where growth is exclusively supported by atmospheric CO_2, wild-type and RhlA#1 and RhlA#2 transplastomic lines were grown in minimal HSM medium under constant light in shake flasks. Although it was observed that growth in HSM medium was significantly reduced compared to growth in TAP as expected, RhlA lines showed similar growth curves in HSM medium compared to wild type (Suppl. Fig. S10). Moreover, HAA congeners were detected in the secreted fractions of both RhlA lines in HSM medium by UHPLC-QTOF (Suppl. Table S3 and Fig. S11). Similar to the growth in TAP, C_{10}-C_8 and C_{10}-C_{10} were identified as the main HAA congeners in HSM medium, and C_{10}-C_6 as a minor congener (Fig. 7 and Suppl. Table S3). In contrast, the minor congener in TAP C_{10}-C_{12} was not detected in HSM medium, indicating that this congener is not produced under photoautotrophic conditions, or that it accumulates below detection levels in the low-density (not saturated) cultures used here. Although no attempt was made to quantify the production of HAA under these non-optimized HSM conditions, the chromatographic peak areas for the main HAA congeners were similar to those observed in TAP medium (compare Suppl. Tables S2 and S3). These findings are thus consistent with recent reports describing efficient photoautotrophic production of recombinant products in *Chlamydomonas reinhardtii* [52,53].

Table 1
Relative abundance of each HAA congener secreted by recombinant *C. reinhardtii*[a].

HAA congener	MW (Da)	molar ratio (%)	mg HAA cong. per L culture	HAA congener (% w/v)
HAA (C_{10}-C_8)	330.2408	43.1	0.18	41.6
HAA (C_{10}-C_{10})	358.2728	35.1	0.16	36.7
HAA (C_{10}-C_6)	302.2098	12.2	0.05	10.8
HAA (C_{10}-C_{12})	386.3038	9.7	0.05	10.9
			0.44	100

[a] The amount (mg) of each HAA congener per L of culture is estimated from the total amount of 3-HA(C_{10}) quantified by GC-FID (0.326 mg/L culture) and the molar ratio of each HAA congener (from HPLC-MS, assuming equal response factor for $[M-H]^-$ ions).

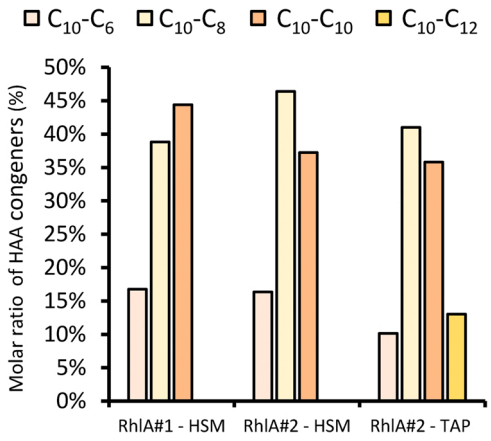

Fig. 7. Relative abundance of HAA congeners identified in RhlA-HA expressing lines under photoautotrophic conditions. RhlA#1 and RhlA#2 transplastomic lines were grown under photoautotrophic conditions in HSM media up to an $OD_{750\ nm}$ of 0.65 according to Fig. S10. The extracellular fraction was harvested and extracted with chloroform/methanol and analyzed by UHPLC-QTOF. The molar ratio of each congener corresponds to the ratio of peak areas (Table S3) assuming the same response factor for the $[M-H]^-$ of each congener. The MS and MS/MS mass spectrums are shown in Fig. S11. The molar ratio of each congener in mixotrophic conditions in TAP medium (Fig. 4) is also included for comparison.

4. Discussion

Several bacterial hosts have been explored for the sustainable, safe, and cost-effective production of RL biosurfactants [12,15] but, as far as we are aware, there is only one attempt in a eukaryotic cell (the yeast *Saccharomyces cerevisiae*), in which RL production was indirectly detected by non-specific lipid staining [54]. Microalgae are emergent cell factories for sustainable industrial biotechnology [25], constituting a suitable platform for RL production. Here, transplastomic lines of the eukaryotic green microalgae *C. reinhardtii* were generated stably expressing the RhlA acyltransferase from *P. aeruginosa* in the chloroplast. Importantly, UHPLC-QTOF and GC-FID analysis showed that these lines produce and accumulate HAA, the hydrophobic precursor of RLs, thus providing initial demonstration of microalgal potential as a platform for RL production. In natural RL producers, HAA is not an end-product and is detected at trace amounts as an intermediary in RL biosynthesis [12,17]. Although HAA is a biosurfactant with valuable bioactive properties in itself [10,12], the long-term purpose of the project is not only to produce HAA as end product, but also to use this HAA as an in vivo building block for RL production in subsequent engineered microalgal strains.

RhlA acyltransferase condenses two 3-HA chains to render HAA by catalyzing the formation of an ester bond between the hydroxyl group of one of the chains, and the carboxylic acid group of the other (Fig. 1). In *P. aeruginosa*, RhlA seems to capture either the hydroxyacyl chains activated with Acyl Carrier Protein (ACP) as intermediaries of the FAS cycle or with CoA resulting from the fatty acid degradation cycle [11,12,22]. In any case, *P. aeruginosa* RhlA has higher specificity for the ten-carbon intermediate 3-hydroxydecanoic acid (3-HA-(C_{10})), mainly yielding the C_{10}-C_{10} HAA congener [12]. Like most photosynthetic organisms, *C. reinhardtii* synthesizes fatty acids through the FAS cycle linked to the ACP [41,42], a biosynthetic pathway located in the chloroplast. The resulting fatty acids are incorporated into chloroplast glycerolipids (such as the abundant galactoglycerolipids, structural components of the thylakoid membranes) through the prokaryotic pathway, or travel outside the chloroplast to the endoplasmic reticulum, where they are incorporated into phospholipids through the eukaryotic pathway of glycerolipid biosynthesis [41]. Importantly, although the end products of the *C. reinhardtii* FAS cycle are C_{16}, C_{18}, and $C_{18:1}$ fatty

acids [41,42], it was hypothesized that RhlA expressed in the chloroplast would access and pull out the 3-HA-ACP intermediaries. RhlA-HA fusion protein was stably detected in all homoplasmic transplastomic lines analyzed (Fig. 2) and four HAA congeners were detected and identified by UHPLC-QTOF analysis (Figs. 3 and 4). These results confirm that an active *P. aeruginosa* RhlA-HA expressed in *C. reinhardtii* chloroplasts accesses 3-HA-ACP substrates to redirect lipid metabolism towards a novel product.

One important parameter regarding the performance of HAA congeners and RLs as biosurfactants is their carbon chain length, as it determines their physical properties [5,12,15]. In a pivotal study, it was shown that RhlA specificity towards 3-HA substrates was the main feature defining the length of the carbon chains incorporated in the HAA congeners, whereas the cell environment played a relatively minor role [12]. This was supported by the observation that expression of *P. aeruginosa* RhlA (with higher specificity for C_{10} chains) in *E. coli* resulted in a HAA congener profile containing mainly C_{10}-C_{10} (49.5%) and C_{10}-C_{12} (32.5%), similar to the *P. aeruginosa* endogenous profile [12, 51,55]. In contrast, RhlA from the bacteria *Burkholderia plantarii* preferentially uses longer C_{14} chain and produces C_{14}-C_{14} and C_{14}-$C_{14:1}$ congeners with longer chains, both in the natural host and upon heterologous RhlA expression in *E. coli* [12,56]. In the present case, four HAA congeners were detected and identified in RhlA-expressing lines, C_{10}-C_{10} and C_{10}-C_8 apparently being the more abundant (35% and 43%, respectively) compared to the C_{10}-C_6 and C_{10}-C_{12} congeners (12% and 10%, respectively) (Table 1). The results support the suggestion that *P. aeruginosa* RhlA prefers the 3-HA(C_{10}) chains as a substrate when expressed in *C. reinhardtii*, as C_{10} hydroxyacyl chains represent about 67% of the hydroxyacyl chains observed in the profile. Intriguingly, whereas the C_{10}-C_8 is one of the main HAA congener in *C. reinhardtii*, it is relatively minor in *P. aeruginosa* RLs, or in *E. coli* expressing P. *aeruginosa* RhlA [12,51,55]. Although the reason for this divergence is unknown, it supports the idea that the environment where RhlA is acting also plays a certain role in determining the 3-HA chains incorporated into HAA [12]. It will be interesting to investigate in the future whether expression of RhlA enzymes from species other than *P. aeruginosa* in *C. reinhardtii* chloroplast also results in HAA congeners with different chain lengths, as this would pave the road towards the production of tailor-made HAA and RLs in microalgae [5,12,15]. In addition to the four HAA congeners, the non-targeted UHPLC-QTOF approach detected five other compounds that differentially accumulated in RhlA-expressing lines (Suppl. Table S2). Although their identification was beyond the scope of the present study, it will be interesting to understand the nature and origin of these other unknown compounds.

In bacterial hosts, RLs and HAA are secreted to the extracellular medium [8,12,15]. The behavior of HAA accumulation was unpredictable in this *C. reinhardtii* platform because the transport mechanisms are different in prokaryotic and eukaryotic cells, and because RhlA expression and HAA production were targeted to the chloroplast compartment (Fig. 1). However, HAA congeners were detected in the extracellular fraction, indicating that HAA must be able to cross the inner and the outer chloroplast membranes and, for its extracellular export, the plasma membrane and cell wall of *C. reinhardtii*. It remains unclear whether HAA uses an endogenous microalgal transport system for this export, or whether the ability to cross lipid bilayers is an intrinsic capability of HAA. To rapidly and easily monitor HAA production and cellular dynamics in RhlA-expressing lines, and considering that the produced HAA congeners are mainly conformed by C_{10} hydroxyacyl chains (Fig. 4), a method was established based on GC-FID similar to that reported in [22]. This method enabled detection and quantification of the hydroxydecanoic acid methyl ester (3-HA(C_{10})-ME), resulting from the hydrolysis and methyl esterification reaction of HAA, at different times over a growth curve and in different fractions (intracellular and secreted) (Figs. 5B and 6 C). In agreement with the UHPLC-QTOF analysis, 3-HA(C_{10}) derived from HAA was detected in the secreted fraction of transplastomic lines but not in the WT strain. In addition, 3-HA(C_{10}) derived from HAA was also detected in the intracellular fraction of RhlA expressing lines, consistent with the plastid biosynthesis of HAA. The 3-HA(C_{10}) derived from HAA accumulated exponentially in the secreted fraction that parallels the culture growth curve, whereas intracellular accumulation seemed to follow a more linear progression. The production of 3-HA(C_{10}) derived from secreted HAA was 0.326 mg/L, corresponding to 0.44 mg/L HAA (Table 1). Likewise, 3-HA (C_{10}) measured in the intracellular fraction was 0.166 mg/L. Assuming the same congener composition in both intracellular and secreted fractions, the intracellular HAA production was estimated to be 0.22 mg/L, accounting for an overall production of 0.66 mg/L. This estimated volumetric production was achieved under standard *C. reinhardtii* TAP medium and culture conditions in flasks at laboratory scale, and is still far from the current productivity of bacterial hosts after years of optimizing producing strains, media formulation and growth conditions [12, 15,22]. However, the observed HAA production in *C. reinhardtii* is not far from initial non-optimized production of other recombinant bioproducts that ranged from μg/L such as Patchoulol [57] to a few mg/L such as astaxanthin, cadaverine, sclareol or putrescine [35,36,52,53]. Importantly, in these examples, the initial, apparently low, volumetric productivity was significantly improved by complex metabolic engineering addressed to optimize biosynthetic or precursor pathways, together with further bioprocess development to reach high-density cultures. Prominent optimization examples with about 100-fold increase include sclareol production (from 5.4 mg/L to 656 mg/L) [52], and cadaverine production (from 4.2 mg/L to 248 mg/L) [36]. Future studies must be focused on optimizing HAA production in microalgae, either by metabolic engineering to increase lipidic 3-HA-ACP precursor availability, or by bioprocess engineering to optimize media formulation and growth conditions allowing high-density cultures, or by boosting lipid metabolic flux under nitrogen deprivation conditions [39,41,42].

The ability of *C. reinhardtii* cells to accumulate HAA upon RhlA acyltransferase expression supports a goal to continue developing this microalga as a platform to produce RLs. In subsequent studies, RhlB rhamnosyltransferase must be co-expressed with RhlA, enabling transformation of HAA into mono-RLs (Fig. 1). Moreover, the dTDP-Rha sugar-nucleotide precursor pathway may also need to be introduced or optimized in the microalgal cell, as was performed in yeast [54]. Since chloroplast offers advantages as a compartment for synthetic biology and biotechnology [58], it is deemed reasonable to propose chloroplast genome engineering to co-express RhlA, RhlB, and/or dTDP-Rha biosynthetic enzymes to produce mono-RLs. Moreover, because HAA is also produced in photoautotrophic conditions (Fig. 7, Table S3), it is envisaged that this platform will be able to produce mono-RL in the sole presence of CO_2 as a carbon source upon required optimization. This idea of exploiting the chloroplast compartment as photosynthetic biofactories to produce biocompounds of interest has extensively been pursued in plants, including the production of antioxidant ketocarotenoids [59], antimalarial artemisinin [60], or bioplastics [61]. Together with other recent examples of production of diterpenoids [34, 52] or astaxanthin [35] in the chloroplast of *C. reinhardtii*, this work should contribute to the continuous development of microalgae as emerging sustainable hosts for molecular pharming.

In conclusion, these results indicate that (i) HAA composed by the ten-carbon 3-HA congener is produced in RhlA lines; (ii) it accumulates in the intracellular fraction; (iii) it is secreted exponentially during cell growth until it reaches a plateau in saturated cultures; (iv) four major HAA congeners are produced, with HAA(C_{10}-C_8) and HAA(C_{10}-C_{10}) being the major products; and (iv) HAA congeners are also produced under photoautotrophic conditions, with atmospheric CO_2 as the only carbon source.

Author contributions

Bernat Miró-Vinyals: Experimental investigation; Data analysis; Methodology; Writing – original draft. **Margalida Artigues:** Data

analysis; Methodology; Writing – original draft. **Katia Wostrikoff:** Methodology; Resources; Writing – review & editing. **Elena Monte:** Resources, Writing – review & editing. **Francesc Broto:** Data analysis; Methodology; **Pablo Leivar:** Conceptualization; Methodology; Resources; Supervision; Writing – original draft; Writing – review & editing, Funding acquisition. **Antoni Planas:** Conceptualization; Methodology; Resources; Supervision; Writing – review & editing, Funding acquisition; Project administration.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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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.2023.03.005.

References

[1] Abdel-Mawgoud AM, Stephanopoulos G. Simple glycolipids of microbes: chemistry, biological activity and metabolic engineering. Synth Syst Biotechnol 2018;3:3–19. https://doi.org/10.1016/j.synbio.2017.12.001.
[2] Marchant R, Banat IM. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol 2012;30:558–65. https://doi.org/10.1016/j.tibtech.2012.07.003.
[3] Jarvis FG, Johnson MJ. A glyco-lipide produced by pseudomonas aeruginosa. J Am Chem Soc 1949. https://doi.org/10.1021/ja01180a073.
[4] Abdel-Mawgoud AM, Lépine F, Déziel E. Rhamnolipids: diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 2010;86:1323–36. https://doi.org/10.1007/s00253-010-2498-2.
[5] Wittgens A, Rosenau F. On the road towards tailor-made rhamnolipids: current state and perspectives. Appl Microbiol Biotechnol 2018;102:8175–85. https://doi.org/10.1007/s00253-018-9240-x.
[6] Vatsa P, Sanchez L, Clement C, Baillieul F, Dorey S. Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. Int J Mol Sci 2010;11:5095–108. https://doi.org/10.3390/ijms11125095.
[7] Sajid M, Ahmad Khan MS, Singh Cameotra S, Safar Al-Thubiani A. Biosurfactants: Potential applications as immunomodulator drugs. Immunol Lett 2020;223:71–7. https://doi.org/10.1016/j.imlet.2020.04.003.
[8] Chong H, Li Q. Microbial production of rhamnolipids: opportunities, challenges and strategies. Micro Cell Fact 2017;16:137. https://doi.org/10.1186/s12934-017-0753-2.
[9] Randhawa KKS, Rahman PKSM. Rhamnolipid biosurfactants-past, present, and future scenario of global market. Front Microbiol 2014;5:1–7. https://doi.org/10.3389/fmicb.2014.00454.
[10] Schellenberger R, Crouzet J, Nickzad A, Shu LJ, Kutschera A, Gerster T, et al. Bacterial rhamnolipids and their 3-hydroxyalkanoate precursors activate Arabidopsis innate immunity through two independent mechanisms. Proc Natl Acad Sci USA 2021;118:1–10. https://doi.org/10.1073/PNAS.2101366118.
[11] Soberón-Chávez G, González-Valdez A, Soto-Aceves MP, Cocotl-Yañez M. Rhamnolipids produced by Pseudomonas: from molecular genetics to the market. Micro Biotechnol 2020;14:136–46. https://doi.org/10.1111/1751-7915.13700.
[12] Germer A, Tiso T, Müller C, Behrens B, Vosse C, Scholz K, et al. Exploiting the natural diversity of RhlA acyltransferases for the synthesis of the rhamnolipid precursor 3-(3-Hydroxyalkanoyloxy)alkanoic acid. Appl Environ Microbiol 2020:1–16. https://doi.org/10.1128/AEM.02317-19.
[13] Bator I, Karmainski T, Tiso T, Blank LM. Killing two birds with one stone – strain engineering facilitates the development of a unique rhamnolipid production process. Front Bioeng Biotechnol 2020;8:1–16. https://doi.org/10.3389/fbioe.2020.00899.
[14] Xu A, Zhang X, Cao S, Zhou X, Yu Z, Qian X, et al. Transcription-associated fluorescence-activated droplet sorting for Di-rhamnolipid hyperproducers. ACS Synth Biol 2022;11:1992–2000. https://doi.org/10.1021/acssynbio.1c00622.
[15] Wittgens A, Rosenau F. Heterologous rhamnolipid biosynthesis: advantages, challenges, and the opportunity to produce tailor-made rhamnolipids. Front Bioeng Biotechnol 2020;8:1–11. https://doi.org/10.3389/fbioe.2020.594010.
[16] Sivapuratharasan V, Lenzen C, Michel C, Muthukrishnan AB, Jayaraman G, Blank LM. Metabolic engineering of Pseudomonas taiwanensis VLB120 for rhamnolipid biosynthesis from biomass-derived aromatics. Metab Eng Commun 2022;15:e00202. https://doi.org/10.1016/j.mec.2022.e00202.
[17] Tiso T, Zauter R, Tulke H, Leuchtle B, Li WJ, Behrens B, et al. Designer rhamnolipids by reduction of congener diversity: production and characterization. Micro Cell Fact 2017;16:1–14. https://doi.org/10.1186/s12934-017-0838-y.
[18] Tiso T, Ihling N, Kubicki S, Biselli A, Schonhoff A, Bator I, et al. Integration of genetic and process engineering for optimized rhamnolipid production using pseudomonas putida. Front Bioeng Biotechnol 2020;8:1–24. https://doi.org/10.3389/fbioe.2020.00976.
[19] Ochsner UA, Fiechter A, Reiser J. Isolation, characterization, and expression in Escherichia coli of the Pseudomonas aeruginosa rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J Biol Chem 1994;269:19787–95. https://doi.org/10.1016/S0021-9258(17)32089-6.
[20] Tavares LFD, Silva PM, Junqueira M, Mariano DCO, Nogueira FCS, Domont GB, et al. Characterization of rhamnolipids produced by wild-type and engineered Burkholderia kururiensis. Appl Microbiol Biotechnol 2013;97:1909–21. https://doi.org/10.1007/s00253-012-4454-9.
[21] Han L, Liu P, Peng Y, Lin J, Wang Q, Ma Y. Engineering the biosynthesis of novel rhamnolipids in Escherichia coli for enhanced oil recovery. J Appl Microbiol 2014;117:139–50. https://doi.org/10.1111/jam.12515.
[22] Zhu K, Rock CO. RhlA converts beta-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the beta-hydroxydecanoyl-beta-hydroxydecanoate component of rhamnolipids in Pseudomonas aeruginosa. J Bacteriol 2008;190:3147–54. https://doi.org/10.1128/JB.00080-08.
[23] Rahim R, Ochsner UA, Olvera C, Graninger M, Messner P, Lam JS, et al. Cloning and functional characterization of the Pseudomonas aeruginosa rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol Microbiol 2001;40:708–18. https://doi.org/10.1046/j.1365-2958.2001.02420.x.
[24] Gangl D, Zedler JAZ, Rajakumar PD, Martinez EMR, Riseley A, Włodarczyk A, et al. Biotechnological exploitation of microalgae. J Exp Bot 2015;66:6975–90. https://doi.org/10.1093/jxb/erv426.
[25] Fabris M, Abbriano RM, Pernice M, Sutherland DL, Commault AS, Hall CC, et al. Emerging technologies in algal biotechnology: toward the establishment of a sustainable, algae-based bioeconomy. Front Plant Sci 2020;11:1–22. https://doi.org/10.3389/fpls.2020.00279.
[26] Salomé PA, Merchant SS. A series of fortunate events: introducing chlamydomonas as a reference organism. Plant Cell 2019;31:1682–707. https://doi.org/10.1105/tpc.18.00952.
[27] Scaife MA, Nguyen GTDT, Rico J, Lambert D, Helliwell KE, Smith AG. Establishing Chlamydomonas reinhardtii as an industrial biotechnology host. Plant J 2015;82:532–46. https://doi.org/10.1111/tpj.12781.
[28] Schroda M, Remacle C. Molecular advancements establishing chlamydomonas as a host for biotechnological exploitation. Front Plant Sci 2022:13. https://doi.org/10.3389/fpls.2022.911483.
[29] Greiner A, Kelterborn S, Evers H, Kreimer G, Sizova I, Hegemann P. Targeting of photoreceptor genes in Chlamydomonas reinhardtii via zinc-finger nucleases and CRISPR/Cas9. Plant Cell 2017;29:2498–518. https://doi.org/10.1105/tpc.17.00659.
[30] Fauser F, Vilarrasa-Blasi J, Onishi M, Ramundo S, Patena W, Millican M, et al. Systematic characterization of gene function in the photosynthetic alga Chlamydomonas reinhardtii. Nat Genet 2022;54:705–14. https://doi.org/10.1038/s41588-022-01052-9.
[31] Crozet P, Navarro FJ, Willmund F, Mehrshahi P, Bakowski K, Lauersen KJ, et al. Birth of a photosynthetic chassis: a moclo toolkit enabling synthetic biology in the microalga chlamydomonas reinhardtii. ACS Synth Biol 2018;7:2074–86. https://doi.org/10.1021/acssynbio.8b00251.
[32] Rasala BA, Mayfield SP. The microalga Chlamydomonas reinhardtii as a platform for the production of human protein therapeutics. Bioeng Bugs 2010;2:50–4. https://doi.org/10.4161/bbug.2.1.13423.
[33] Kiefer AM, Niemeyer J, Probst A, Erkel G, Schroda M. Production and secretion of functional SARS-CoV-2 spike protein in Chlamydomonas reinhardtii. Front Plant Sci 2022;13:1–12. https://doi.org/10.3389/fpls.2022.988870.

[34] Lauersen KJ. Eukaryotic microalgae as hosts for light-driven heterologous isoprenoid production. Planta 2019;249:155–80. https://doi.org/10.1007/s00425-018-3048-x.
[35] Perozeni F, Cazzaniga S, Baier T, Zanoni F, Zoccatelli G, Lauersen KJ, et al. Turning a green alga red: engineering astaxanthin biosynthesis by intragenic pseudogene revival in Chlamydomonas reinhardtii. Plant Biotechnol J 2020;18:2053–67. https://doi.org/10.1111/pbi.13364.
[36] Freudenberg RA, Baier T, Einhaus A, Wobbe L, Kruse O. High cell density cultivation enables efficient and sustainable recombinant polyamine production in the microalga Chlamydomonas reinhardtii. Bioresour Technol 2021;323:124542. https://doi.org/10.1016/j.biortech.2020.124542.
[37] Chaogang W, Zhangli H, Anping L. Biosynthesis of poly-3-hydroxybutyrate (PHB) in the transgenic green alga Chlamydomonas reinhardtii. J Phycol 2010;402: 396–402. https://doi.org/10.1111/j.1529-8817.2009.00789.x.
[38] Chia WY, Ying Tang DY, Khoo KS, Kay Lup AN, Chew KW. Nature's fight against plastic pollution: Algae for plastic biodegradation and bioplastics production. Environ Sci Ecotechnology 2020;4:100065. https://doi.org/10.1016/j.ese.2020.100065.
[39] Kong F, Yamaoka Y, Ohama T, Lee Y, Li-Beisson Y. Molecular genetic tools and emerging synthetic biology strategies to increase cellular oil content in Chlamydomonas reinhardtii. Plant Cell Physiol 2019;60:1184–96. https://doi.org/10.1093/pcp/pcz022.
[40] Kim JW, Park SBin, Tran QG, Cho DH, Choi DY, Lee YJ, et al. Functional expression of polyethylene terephthalate-degrading enzyme (PETase) in green microalgae. Micro Cell Fact 2020;19:1–9. https://doi.org/10.1186/s12934-020-01355-8.
[41] Li-Beisson Y, Beisson F, Riekhof W. Metabolism of acyl-lipids in *Chlamydomonas reinhardtii*. Plant J 2015;82:504–22. https://doi.org/10.1111/tpj.12787.
[42] Li-Beisson Y, Thelen JJ, Fedosejevs E, Harwood JL. The lipid biochemistry of eukaryotic algae. Prog Lipid Res 2019;74:31–68. https://doi.org/10.1016/j.plipres.2019.01.003.
[43] Harris EH, Stern DB, Witman GB. The Chlamydomonas Sourcebook. 2nd ed. Oxford: Academic Press; 2009. https://www.sciencedirect.com/book/9780123708731/the-chlamydomonas-sourcebook#book-description.
[44] Sueoka N. Mitotic replication of deoxyribonucleic acid in chlamydomonas reinhardi. Proc Natl Acad Sci 1960;46:83–91. https://doi.org/10.1073/pnas.46.1.83.
[45] Young REB, Purton S. Codon reassignment to facilitate genetic engineering and biocontainment in the chloroplast of Chlamydomonas reinhardtii. Plant Biotechnol J 2016;14:1251–60. https://doi.org/10.1111/pbi.12490.
[46] Michelet L, Lefebvre-Legendre L, Burr SE, Rochaix JD, Goldschmidt-Clermont M. Enhanced chloroplast transgene expression in a nuclear mutant of Chlamydomonas. Plant Biotechnol J 2011;9:565–74. https://doi.org/10.1111/j.1467-7652.2010.00564.x.
[47] Guzmán-Zapata D, Macedo-osorio KS, Almaraz-delgado AL, Durán-figueroa N, Badillo-corona JA. Production of Recombinant Proteins in the Chloroplast of the Green Alga Chlamydomonas reinhardtii. Methods Mol Biol 2016:1385. https://doi.org/10.1007/978-1-4939-3289-4.
[48] Kuras R, Wollman FA. The assembly of cytochrome b6/f complexes: An approach using genetic transformation of the green alga Chlamydomonas reinhardtii. EMBO J 1994;13:1019–27. https://doi.org/10.1002/j.1460-2075.1994.tb06350.x.
[49] Cabrera-Valladares N, Richardson AP, Olvera C, Treviño LG, Déziel E, Lépine F, et al. Monorhamnolipids and 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) production using Escherichia coli as a heterologous host. Appl Microbiol Biotechnol 2006;73:187–94. https://doi.org/10.1007/s00253-006-0468-5.
[50] Déziel E, Lépine F, Milot S, Villemur R. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 2003;149:2005–13. https://doi.org/10.1099/mic.0.26154-0.
[51] Lépine F, Déziel E, Milot S, Villemur R. Liquid chromatographic/mass spectrometric detection of the 3-(3-hydroxyalkanoyloxy)alkanoic acid precursors of rhamnolipids in Pseudomonas aeruginosa cultures. J Mass Spectrom 2002;37: 41–6. https://doi.org/10.1002/jms.244.
[52] Einhaus A, Steube J, Freudenberg RA, Barczyk J, Baier T, Kruse O. Engineering a powerful green cell factory for robust photoautotrophic diterpenoid production. Metab Eng 2022;73:82–90. https://doi.org/10.1016/j.ymben.2022.06.002.
[53] Freudenberg RA, Wittemeier L, Einhaus A, Baier T, Kruse O. Advanced pathway engineering for phototrophic putrescine production. Plant Biotechnol J 2022;20: 1968–82. https://doi.org/10.1111/pbi.13879.
[54] Bahia FM, De Almeida GC, De Andrade LP, Campos CG, Queiroz LR, Da Silva RLV, et al. Rhamnolipids production from sucrose by engineered Saccharomyces cerevisiae. Sci Rep 2018;8:1–10. https://doi.org/10.1038/s41598-018-21230-2.
[55] Déziel E, Lépine F, Dennie D, Boismenu D, Mamer OA, Villemur R. Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by Pseudomonas aeruginosa strain 57RP grown on mannitol or naphthalene. Biochim Biophys Acta - Mol Cell Biol Lipids 1999;1440:244–52. https://doi.org/10.1016/S1388-1981(99)00129-8.
[56] Costa SGVAO, Lépine F, Déziel E. Characterization of rhamnolipid production by Burkholderia glumae. Lett Appl Microbiol 2011;53:620–7. https://doi.org/10.1111/j.1472-765X.2011.03154.x.
[57] Lauersen KJ, Baier T, Wichmann J, Wördenweber R, Mussgnug JH, Hübner W, et al. Efficient phototrophic production of a high-value sesquiterpenoid from the eukaryotic microalga Chlamydomonas reinhardtii. Metab Eng 2016;38:331–43. https://doi.org/10.1016/j.ymben.2016.07.013.
[58] Jackson HO, Taunt HN, Mordaka PM, Smith AG, Purton S. The Algal Chloroplast as a Testbed for Synthetic Biology Designs Aimed at Radically Rewiring Plant Metabolism. Front Plant Sci 2021;12:1–15. https://doi.org/10.3389/fpls.2021.708370.
[59] Lu Y, Stegemann S, Agrawal S, Karcher D, Ruf S, Bock R. Horizontal Transfer of a Synthetic Metabolic Pathway between Plant Species. Curr Biol 2017;27: 3034–3041.e3. https://doi.org/10.1016/j.cub.2017.08.044.
[60] Fuentes P, Zhou F, Erban A, Karcher D, Kopka J, Bock R. A new synthetic biology approach allows transfer of an entire metabolic pathway from a medicinal plant to a biomass crop. Elife 2016;5:1–26. https://doi.org/10.7554/eLife.13664.
[61] Bohmert-Tatarev K, McAvoy S, Daughtry S, Peoples OP, Snell KD. High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate. Plant Physiol 2011; 155:1690–708. https://doi.org/10.1104/pp.110.169581.