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Isoprenoid biosynthesis in the diatom *Haslea ostrearia*

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19 **Summary**

- 20 • Diatoms are eukaryotic, unicellular algae that are responsible for about 20% of the Earth's
21 primary production. Their dominance and success in contemporary oceans have prompted
22 investigations on their distinctive metabolism and physiology. One metabolic pathway that
23 remains largely unexplored in diatoms is isoprenoid biosynthesis, which is responsible for the
24 production of numerous molecules with unique features.
- 25 • We selected the diatom species *Haslea ostrearia* because of its characteristic isoprenoid content
26 and carried out a comprehensive transcriptomic analysis and functional characterization of the
27 genes identified.
- 28 • We functionally characterized one farnesyl diphosphate synthase, two geranylgeranyl
29 diphosphate synthases, one short-chain polyprenyl synthase, one bifunctional isopentenyl
30 diphosphate isomerase - squalene synthase and one phytoene synthase. We inferred the
31 phylogenetic origin of these genes and used a combination of functional analysis and
32 subcellular localization predictions to propose their physiological roles.
- 33 • Our results provide insight into isoprenoid biosynthesis in *H. ostrearia* and propose a model of
34 the central steps of the pathway. This model will facilitate the study of metabolic pathways of
35 important isoprenoids in diatoms, including carotenoids, sterols and highly branched
36 isoprenoids.

37 **Keywords:** Heterokonts, isoprenoids, phytoene synthase, prenyltransferase, squalene synthase

38

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49

50

51 **Introduction**

52 Diatoms (phylum Heterokontophyta, class Bacillariophyceae) are one of the most diverse and
53 ecologically important groups of phytoplankton. With more than 100,000 species that are widely
54 distributed in aquatic environments, it is estimated that they contribute to about 20% of the global
55 primary production. Consequently, they play central roles in aquatic food webs and in the
56 biogeochemical cycling of nutrients (Nelson *et al.*, 1995; Field *et al.*, 1998; Falkowski, 2002). This
57 profound ecological success has created great interest in distinctive physiological features of diatoms.
58 Even though, several studies have already investigated unique facets of their metabolism (Kroth *et al.*,
59 2008; Ast *et al.*, 2009; Gruber *et al.*, 2009; Allen *et al.*, 2011; Fabris *et al.*, 2012; Smith *et al.*, 2012;
60 Obata *et al.*, 2013), there are still important gaps in our understanding of key biochemical pathways.
61 One such unexplored area is the biosynthesis of isoprenoids, a large class of metabolites that have vital
62 biological functions in all domains of life (Holstein & Hohl, 2004).

63 Despite their structural diversity, all isoprenoids are assembled from the same five-carbon atom
64 precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Two
65 distinct biosynthetic routes are responsible for the synthesis of these five-carbon precursors: the
66 mevalonate (MVA) pathway and the methyl-erythritol-phosphate (MEP) pathway. Beyond these early
67 steps, IPP and DMAPP are condensed to form prenyl diphosphate molecules of various lengths that
68 serve as substrates for the synthesis of the different isoprenoid classes (geranyl diphosphate (GPP) for
69 C₁₀ monoterpenoids; farnesyl diphosphate (FPP) for C₁₅ sesquiterpenoids, C₃₀ triterpenoids and sterols;
70 geranylgeranyl diphosphate (GGPP) for C₂₀ diterpenoids and C₄₀ carotenoids; etc.). This set of
71 condensation reactions comprise the central steps of the pathway and are catalyzed by
72 prenyltransferase-type enzymes. After this central part, synthesis of carotenoids and sterols proceeds
73 through the commitment of FPP and GGPP to squalene and phytoene, respectively, by
74 squalene/phytoene synthase-type enzymes (Fig. S1) (Vranová *et al.*, 2012).

Initial work using feeding experiments with labelled precursors and specific pathway inhibitors has shown that both the MVA and MEP pathways are present in diatoms (Cvejić & Rohmer, 2000; Massé *et al.*, 2004). Following the genomic sequencing of model diatom species *Thalassiosira pseudonana* (Armbrust *et al.*, 2004) and *Phaeodactylum tricornutum* (Bowler *et al.*, 2008), the focus of investigation has moved towards the final steps of the pathway, i.e. the carotenoid biosynthetic branch that provides important light harvesting and photoprotective molecules (Coesel *et al.*, 2008; Dambek *et al.*, 2012; Eilers *et al.*, 2016) and the synthesis of sterols, which serve as membrane structural components (Fabris *et al.*, 2014). An additional branch of isoprenoid biosynthesis that has attracted special interest is that of the highly branched isoprenoids (HBI). HBIs are only synthesized by a limited number of diatom species. They are extensively used as geochemical and paleoenvironmental markers (Massé *et al.*, 2011; Belt & Müller, 2013) and show potential for use as pharmaceuticals and as an alternative form of fuels (Rowland *et al.*, 2001; Ferriols *et al.*, 2015, 2017).

Nevertheless, the central steps of the isoprenoid pathway are less well explored and there is limited understanding on the function, subcellular localization and regulation of prenyltransferases from diatoms. Considering that the synthesis of prenyl diphosphates is a key regulatory step in the pathway that determines the flux towards different branches, a thorough investigation of the central steps will significantly improve our overall understanding of isoprenoid biosynthesis in diatoms. To this end, we selected the diatom species *Haslea ostrearia* for further studies, as this species is also able to synthesize HBIs (Wraige *et al.*, 1999) (Fig. S2), thus providing a more comprehensive system to study isoprenoid biosynthesis. Through transcriptomic analysis, we *in silico* reconstructed the MVA and MEP pathways and confirmed the expression of five putative prenyltransferases and two putative phytoene/squalene synthases. We cloned six of these genes and characterized their function in a heterologous host or *in vitro*. By the combination of phylogenetic analysis, subcellular localization prediction and functional characterization, we proposed a model of the isoprenoid pathway in *H.*

99 *ostrearia*. This model will serve as a basis for the elucidation of the biosynthesis of HBIs or other
100 useful isoprenoids from diatoms.

101

102 **Materials and Methods**

103 **Diatom cultures**

104 The *H. ostrearia* clone NCC 153.8 used in this study was a product of heterothallic
105 reproduction between clones NCC 141 and NCC 171, both of which were isolated in 2003
106 from natural populations of the oyster pond Lainard (La Barre de Monts 46° 53' 33" N - 02° 07'
107 59" W). Clone NCC 153.8 was kindly provided by Dr. Vona Medeler (University of Nantes)
108 and cultured at the University of Crete Greece, in f/2 medium (Guillard, 1975) at 20 °C under
109 an irradiance of 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (12h:12h light:dark cycle) prior to analysis. The
110 ability of the strain NCC 153.8 to synthesize HBIs was confirmed by extraction and gas
111 chromatography-mass spectrometry GC-MS analysis (Methods S1, Fig. S3).

112

113 **RNA extraction and transcriptome sequencing**

114 Total RNA was extracted with Spectrum™ Plant Total RNA Kit (Sigma Aldrich, USA) and
115 quantified using the Qubit™ RNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). Messenger
116 RNA (mRNA) was isolated from total RNA using the NEBNext® Poly(A) mRNA Magnetic
117 Isolation Module (New England Biolabs). Complementary DNA (cDNA) library was
118 constructed using the NEBNext Ultra Directional RNA Library Kit for Illumina (New England
119 Biolabs), according to the manufacturer's instructions. Library quantification was conducted
120 with the KAPA Library Quantification kit for Illumina sequencing platforms (KAPA
121 BIOSYSTEMS, U.S.A.) on a Rotor-Gene Q thermocycler (Qiagen). Sequencing was

122 performed at the Institute of Applied Biosciences of the Centre for Research and Technology
123 Hellas, on an Illumina NextSeq500 platform (Illumina, USA) using the NextSeq™ 500/550
124 Mid Output Kit (2 x 150 cycles) (Illumina, USA).

125

126 **Transcriptome Analysis**

127 The overall bioinformatics strategy included the following steps: (i) Trim and clean-up of the
128 sequencing reads using the trim galore wrapper (<https://github.com/FelixKrueger/TrimGalore>)
129 with default parameters, except for --length 40 and the --fastqc option, so as to remove
130 adaptors and low-quality sequences; (ii) de novo assembly of the read using the Trinity
131 software suite (Grabherr *et al.*, 2011) with default parameters. All the analysis was
132 implemented on a Linux/based HPC cluster assigning one node with 32 cores and 256 GB
133 RAM.

134

135 **Gene identification, sequence and phylogenetic analysis**

136 Identification of candidate biosynthetic genes from *H. ostrearia* was based on homology with
137 characterized and annotated homologues from red, green algae and heterokonts retrieved from the
138 Genbank protein database (NCBI & Resource, 2017) and DiatomCyc database (Fabris *et al.*, 2012). For
139 the phylogenetic analysis all diatom homologues were retrieved from the the Marine Microbial
140 Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling *et al.*, 2014) database using Blast
141 search. The species names and corresponding MMETSP ID numbers are listed on Table S1. Sequences
142 from other heterokonts, red algae, green algae, land plants, cyanobacteria, bacteria, archaea, fungi and
143 metazoa were retrieved from GenBank protein database (NCBI & Resource, 2017). Sequences were
144 aligned using ClustalW (Larkin *et al.*, 2007) and alignments were manually edited by exclusion of
145 ambiguously aligned regions. Phylogenies were inferred using the maximum likelihood method

146 (Whelan & Goldman, 2001) and JTT matrix-based model in MEGA version 7 (Kumar *et al.*, 2016). All
147 positions with less than 85% site coverage were eliminated. Branch support was generated using non-
148 parametric bootstrap analysis with 100 replicates. Conserved motifs in the selected sequences were
149 identified using NCBI Conserved Domain Search (Marchler-Bauer *et al.*, 2015). Prediction of
150 subcellular localization was carried out through ASAFind (Gruber *et al.*, 2015) and Hectar (Gschloessl
151 *et al.*, 2008) in combination with SignalP 3.0 (Bendtsen *et al.*, 2004) and TMHMM v. 2.0 (Krogh *et al.*,
152 2001) for signal peptide and transmembrane domain prediction, respectively.

153

154 **Gene amplification and cloning**

155 Basic sequence analysis and design of primers (Table S2) was performed using CLC workbench
156 (QIAGEN). Total RNA was extracted using Trizol and cDNA was synthesized using SuperScript III
157 RT (Thermo) and DNaseI (Roche) treatment. Both full length and truncated variants of the selected
158 genes were PCR amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs) and
159 cDNA as template. PCR products were gel purified, A-overhangs were added using MyTaq
160 Polymerase (BIOLINE) and the products were subsequently cloned into vector pCRII-TOPO using
161 TOPO TA cloning kit (Thermo). After digestion with the appropriate restriction enzymes, the digests
162 were ligated to bacterial and yeast expression vectors. For bacterial expression, pRSET (N-terminal
163 6xHis tag) or pET102-His (C-terminal 6xHis tag) or pET102-Trx-His (N-terminal TRX and C-terminal
164 6xHis tag) plasmid backbones, digested with the same restriction enzymes were used. For yeast
165 expression, vectors pUTDH3myc, pWTDHmyc and pHTDH3myc (Ignea *et al.*, 2012) were used. Final
166 constructs were verified by sequencing. Gene expression in bacteria and yeast and protein purification
167 protocols are described in Methods S1.

168

169 **In vitro enzymatic assays**

170 For the characterization of the prenyltransferases 200 µl enzymatic assays were carried out in glass
171 vials. The reaction mixture contained 10 mM MOPS buffer (pH 7.0), 5 mM MgCl₂, 1 mM DTT, 1
172 mg/ml BSA, 100 µM prenyl diphosphate substrate. The substrates used were: dimethylallyl
173 pyrophosphate (D4287, Sigma-Aldrich), isopentenyl diphosphate (I0503, Sigma-Aldrich), geranyl
174 diphosphate (G6772, Sigma-Aldrich), farnesyl diphosphate (F6892, Sigma-Aldrich), geranylgeranyl
175 diphosphate (G6025, Sigma-Aldrich). The reactions were initiated by addition of 50 ng of purified
176 enzyme. After 16 h incubation at 25 °C, the reactions were terminated by addition of equal volume of
177 2N HCl in 83% EtOH and after 20 min incubation they were neutralized with 0.14 mL of 10% NaOH.
178 The hydrolyzed diphosphates were extracted three times using 300 µl of hexane. The hexane extracts
179 were concentrated to a final volume of 50 µl and 1 µl of each reaction was used for GC-MS analysis
180 (Methods S1). Individual compounds were identified by comparing their GC retention indices and mass
181 spectra with those of authentic standards.

182 **Results**

183 **Transcriptomic analysis and identification of candidate biosynthetic genes**

184 *H. ostrearia* NCC 153.8 strain was cultured and its ability to produce HBIs was confirmed (Fig. S3)
185 prior to RNA extraction, library construction and sequencing on Illumina platform. After quality
186 filtering and trimming a total of 38631556 pair-end reads were *de novo* assembled into contigs using
187 the Trinity suite (Haas *et al.*, 2013). A total of 45508 contigs were obtained.

188 We screened the assembled transcriptome to identify genes that putatively encode enzymes of the
189 MVA and MEP pathways. Exploiting the high degree of sequence conservation among genes of
190 isoprenoid biosynthesis, we based our screening on other characterized or annotated algal sequences
191 (See Materials and methods section). A pair-wise alignment between the queries and the contigs

192 obtained from the transcriptome assembly was performed with the Massblast tool (Veríssimo *et al.*,
193 2017). Through this analysis, we identified seven contigs corresponding to full-length protein
194 sequences with high similarities to enzymes that catalyze the seven steps of the MEP pathway. We
195 additionally identified six contigs with homology to genes involved in the conversion of acetyl-CoA to
196 IPP, through the MVA route. The last step of the MVA pathway involves the isomerization of IPP to
197 DMAPP by isopentenyl diphosphate isomerase (IDI) (Berthelot *et al.*, 2012). Searching for an IDI, we
198 were only able to identify a contig that corresponds to a fusion of an IDI with a squalene synthase
199 (*HoIDI-SQS*). Squalene synthases catalyze dimerization of two farnesyl diphosphate molecules to form
200 squalene, the precursor of all sterols (Spanova & Daum, 2011). It was not possible to identify
201 independent transcripts of IDI or SQS in our sequencing data and we concluded that only the fusion of
202 the two genes was expressed under the specific conditions.

203 We continued our analysis focusing on the central steps, which are catalyzed by prenyltransferase-type
204 enzymes. Mining our assembled transcriptome, we were able to retrieve five contigs with similarity to
205 annotated trans-prenyltransferases (PTS). These included one putative farnesyl pyrophosphate (FPP)
206 synthase, sharing 57% sequence similarity at the amino acid level, with a functionally characterized
207 homologue from *Rhizosolenia setigera* (Ferriols *et al.*, 2015) (from now on referred to as *HoPTS1*) and
208 four other putative polyprenyl diphosphate synthases (named *HoPTS2-5*) that exhibited similarities to
209 other diatom and algal prenyltransferases (Fig. 1a, Table 1). We investigated whether this set of the
210 five trans-prenyltransferases is conserved among different diatom species by examining the publicly
211 available diatom transcriptomes in the Marine Microbial Eukaryote Transcriptome Sequencing Project
212 (MMETSP) database (Keeling *et al.*, 2014). Based on homology searches, *HoPTS1*, *HoPTS2* and
213 *HoPTS4* orthologs are present in all diatom species investigated (a total of twenty-six species,
214 representing both centric and pennate diatoms). An *HoPTS5* ortholog seems to be missing only in one
215 species (*Skeletonema menzeli*), while a *HoPTS3* ortholog seems to be absent from seven diatom

216 species (Table S3, Fig. S4). Among the measured transcripts, *HoPTS5* showed significantly high
217 expression levels (Fig. S5).

218 Sequence analysis of the identified prenyltransferases, led to identification of conserved polyprenyl
219 synthase domains (Fig. 1b) and conserved motifs. Among them, the two aspartic acid-rich motifs
220 DDxx(xx)D (First Aspartic acid-Rich Motif -FARM; and the Second Aspartic acid-Rich Motif -
221 SARM), found in characterized prenyltransferases from all domains of life. These motifs are involved
222 in the binding of magnesium ions and are essential for prenyltransferase activity. The identification of
223 intact motifs in all five PTSs suggested that these genes likely encoded for active enzymes. Conserved
224 amino acids were also observed at positions 4 and 5 upstream of FARM. It has previously been shown
225 that these residues are involved in the regulation of the product chain length (Tarshis *et al.*, 1996;
226 Wang & Ohnuma, 1999; Liang *et al.*, 2002). In *HoPTS1*, like in other characterized FPP synthases, this
227 region contains aromatic residues that are bulkier and block further chain elongation (Fig. S6a). By
228 contrast, smaller residues were observed at this region in the remaining four PTSs, suggesting that
229 these likely synthesize longer chain products (Fig. S6b).

230 The prenyl diphosphates synthesized in the central steps are allocated to different branches of the
231 pathway for the synthesis of final isoprenoid products, with sterol and carotenoid pathways being the
232 main ones. Specific enzymes commit FPP and GGPP to these pathways. As described above, we
233 identified a squalene synthase, N-terminally fused to an IDI (*HoIDI-SQS*). In the absence of additional
234 SQS transcripts, this bi-functional protein could be involved in committing FPP to the synthesis of
235 sterols. We also identified a single transcript, termed *HoPSY*, which shares 72% similarity with a
236 characterized phytoene synthase from *P. tricornutum* (Dambek *et al.*, 2012) and could catalyze the first
237 committed step of carotenoid biosynthesis in *H. ostrearia*. Both *HoIDI-SQS* and *HoPSY* were found to
238 contain the conserved phytoene/squalene synthase domain (Fig. 1b).

239

240 **Phylogenetic analysis**

241 Diatoms have a distinctive evolutionary history. Red and green algae, as well as glaucophytes evolved
242 after a primary endosymbiotic event, when a heterotrophic eukaryote engulfed a cyanobacterial cell
243 (McFadden, 2001; Rodríguez-Ezpeleta *et al.*, 2005). Diatoms arose from secondary endosymbiosis,
244 during which a different heterotrophic eukaryote captured a cell of red algal origin (Yoon *et al.*, 2004;
245 Prihoda *et al.*, 2012). After incorporation, the engulfed cell was transformed into the plastid organelle
246 (Bhattacharya *et al.*, 2007), lost its mitochondria and nucleus and genetic information was transferred
247 into the nucleus of the heterotrophic host, in a process termed endosymbiotic gene transfer (EGT)
248 (Timmis *et al.*, 2004). The evolutionary linkage between diatoms and red algae is usually observed in
249 phylogenetic surveys (Armbrust *et al.*, 2004; Bowler *et al.*, 2008; Frommolt *et al.*, 2008). However,
250 such surveys have also revealed a green phylogenetic signal. It has been proposed that this is due to a
251 cryptic endosymbiotic event that involved a green algal cell, which was later replaced by the red algal
252 endosymbiont (Moustafa *et al.*, 2009; Chan *et al.*, 2011). Alternatively, the green related genes could
253 have been acquired through repeated horizontal gene transfer (HGT) events from green algae, early
254 after the evolution of the first diatoms (Oborník & Green, 2005; Dorrell & Smith, 2011). This
255 multisourced genetic background has created unique, chimeric metabolic pathways that combine
256 features from multiple lineages.

257 To investigate how this is reflected to isoprenoid biosynthesis we inferred the phylogenetic origin for
258 each of the candidate biosynthetic genes. Three different possibilities were distinguished: (1) origin
259 from the secondary heterotrophic host (2) origin from the red algal endosymbiont and (3) origin from a
260 green algal cell, either through EGT or HGT.

261 It is evident that the MEP pathway genes can only have algal origin, as only the autotrophic cells
262 involved in the evolution of diatoms had the plastidial route of isoprenoid biosynthesis. Taking this into
263 account, we set out to investigate whether it was a red or a green algal cell that mediated each of the

MEP pathway gene transfers to diatoms (Fig. S7). According to our analysis, 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 2-C-methyl-D-erythritol-4-phosphate-cytidyltransferase (MCT) were transferred from the red algal endosymbiont. This is evident from the highly supported clustering of diatom and red algal proteins. By contrast, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (CMK) and (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthases (HDS) genes were transferred from a green algal related cell. For the remaining two, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS) and hydroxymethylbutenyl diphosphate reductase (HDR), diatom proteins form well separated clades, in distance from their algal homologues. This topology suggests that the corresponding genes have diverged so that their origin cannot be traced.

Prenyltransferases and phytoene/squalene synthases could have been acquired from any of the involved cells. The poorly supported branching patterns obtain for *HoPTS1* and its homologues did not allow us to trace its exact evolutionary origin. *HoPTS3* clusters closely to prenyltransferases from opisthokonts (fungi/metazoan), while other algal and cyanobacterial homologues are branching separately. This topology suggests heterotrophic host origin. By contrast, the topologies obtained for rest of the prenyltransferases, as well as the phytoene/squalene synthases are suggestive of algal origin. For *HoPTS5* and *HoPSY* this is the red algal endosymbiont, as diatom and red algal proteins form well-supported branches together. The *HoIDI-SQS* gene is only conserved among heterokonts, haptophytes and dinoflagellates, so it is likely that the fusion occurred in the common ancestor of these groups (Davies *et al.*, 2015). We investigated the evolutionary origin of each domain separately and both for IDI and SQS inferred phylogenies indicate green algal origin. While for SQS the corresponding cluster is poorly supported, for IDI, branching is more robust (Fig. S8). *HoPTS2* and *HoPTS4* have also been acquired from an algal cell, however, due to divergence it was not possible to pinpoint their exact origin.

288 This divergence could alternatively be explained by gene duplication events. To resolve this, we
289 examined three other diatom species (*P. tricornutum*, *T. pseudonana* and *Fragilariopsis cylindrus*) that
290 have their genomes sequenced. We obtained the homologues of the *H. ostrearia* genes with non-
291 resolved origin and we identified their localization in the respective genome. In all cases, those genes
292 are localized in different genomic regions (Table S4), indicating that they have been likely
293 independently acquired.

294 For genes of the MVA pathway see the extended discussion on the phylogenetic analysis that is
295 included in the Supporting Information (Notes S1).

296

297 **Prediction of subcellular localization**

298 To facilitate analysis of the physiological roles of the identified genes, we investigated the presence of
299 signal/target peptides and obtained insight into the subcellular localization of the encoded proteins. The
300 different endosymbiotic events provided diatoms with secondary plastids surrounded by four
301 membranes (Kroth & Strotmann, 1999). As a result, nuclear encoded diatom proteins targeted to the
302 chloroplast contain a bipartite N-terminal pre-sequence, consisting of a signal peptide with a conserved
303 motif at its cleavage site (ASAFAP motif), followed by a chloroplast transit peptide. These pre-
304 sequences are essential for efficient transportation through all of the four membranes (Gruber *et al.*,
305 2007). Two independent computational tools, ASAFind (Gruber *et al.*, 2015) and HECTAR
306 (Gschloessl *et al.*, 2008), developed for identification of such pre-sequences were used. A different tool
307 that is used to predict transmembrane domains in protein sequences (TMHMM Server v. 2.0) (Krogh *et*
308 *al.*, 2001) was included to further support this analysis. The results are summarized on Table S5. As
309 expected, enzymes of the MEP pathway are predicted to be targeted to the chloroplast and those of the
310 MVA to the cytosol. *HoPTS1* was found to contain a Type II signal anchor and analysis with TMHMM

311 suggested a strong possibility for the presence of transmembrane domain at the N-terminal region.
312 Taken together, these results suggest that *HoPTS1* is likely localized on the ER membrane. The ER
313 membrane is continuous with the outermost chloroplastic membrane, so the possibility that *HoPTS1* is
314 related to the periplastic space (the space between the second and third outermost membranes) cannot
315 be ruled out. For *HoPTS3* and *HoIDI-SQS*, no target or signal peptides were predicted, indicating
316 cytosolic localization. *HoPTS2*, *HoPTS5* and *HoPSY* were predicted to be targeted to the chloroplast,
317 and *HoPTS4* to the mitochondria.

318

319 **Functional characterization of prenyltransferases**

320 Since the subcellular compartmentalization analysis revealed the presence of signal/target peptides for
321 the majority of the identified prenyltransferases, both the full length and different truncated variants of
322 the enzymes (Table 2) were cloned into bacterial vectors, in-frame with a C- or N- terminal His-tag.
323 This allowed the purification of the expressed proteins and the assessment of their activity in *in vitro*
324 enzymatic reactions with prenyl diphosphate substrates. IPP was always used as the homoallylic
325 substrate, while DMAPP, GPP, FPP and GGPP were used as the allylic substrates. Control reactions
326 with cells transformed with empty vectors and subjected to the same purification steps were ran in
327 parallel. Following acid hydrolysis that facilitated the removal of diphosphates from the substrates and
328 products, all reactions were extracted and analyzed by GC-MS. Compound identification was based on
329 combination of accurate mass and retention time comparisons between reaction products and acid
330 hydrolysis products of authentic standards.

331 To characterize *HoPTS1*, the full-length enzyme was initially incubated with DMAPP or GPP and IPP.
332 As no activity was detected, two truncated variants, *HoPTS1*(Ser73-end) and *HoPTS1*(Val89-end),
333 were tested with the same substrates. Analysis of the reaction extracts resulted in the identification of

334 linalool (LOH) and nerolidol (NOH), the acid hydrolysis products of GPP and FPP, respectively. (Fig.
335 2a). In the presence of DMAPP and IPP both alcohols were detected, while in the presence of GPP and
336 IPP, NOH was the only product identified. No other substrate combination resulted in new product
337 formation, demonstrating that *HoPTS1* is an FPP synthase.

338 Initial expression of full length and truncated versions of *HoPTS2* resulted in formation of inclusion
339 bodies. To obtain soluble proteins the variants were subcloned in frame with thioredoxin at the N-
340 terminus and a C-terminal 6xHis-tag. Soluble *HoPTS2* fusions were obtained and purified. The
341 activities of the full-length enzyme and *HoPTS2*(Gly140-end) variant were tested with all substrate
342 combinations but no new product was formed. However, variant *HoPTS2*(Arg45-end) was active and
343 gave a range of C₂₀-C₃₀ prenyl diphosphates in the presence of FPP and IPP and C₂₅-C₃₀ prenyl
344 diphosphates when GGPP and IPP were used as reaction substrates. We were able to identify these
345 products only after hydrolysis treatment and formation of the corresponding prenyl alcohols (Fig. 2b).
346 The fact that the variant *HoPTS2*(Gly140-end) was inactive indicates the presence of a functionally
347 important region between amino acids 45 and 140. Taken together, these results suggest that *HoPTS2*
348 is likely a short chain polyprenyl synthase.

349 As there was no prediction for the presence of any signal/target peptide in *HoPTS3*, the full-length
350 enzyme was expressed, purified and tested with different substrates. In presence of DMAPP and IPP,
351 there was no formation of new products. However, when GPP and IPP were used as reaction substrates,
352 GGPP was produced, detected as its acid hydrolysis product geranyl linalool (GGOH). The same
353 product was formed when the enzyme was incubated with FPP and IPP (Fig. 2c). These results, in
354 combination with the lack of a signal peptide predicted for *HoPTS3*, suggest that *HoPTS3* is possibly a
355 cytosolic GGPP synthase in *H. ostrearia*.

356

357 Efforts to characterize *HoPTS4* were unsuccessful, as there was no activity detected in any reaction
358 extract analyzed, either when using the full-length protein or the truncated variant *HoPTS4*(Leu121-
359 end). The prediction of a mitochondrial target peptide at the N-terminal region suggests that this,
360 possibly, affects the catalytic activity of *HoPTS4* and more truncated variants should be tested to
361 identify the optimal cleavage position for obtaining an active enzyme.

362 Finally, full length *HoPTS5* and one truncated variant, *HoPTS5*(Ser36-end), were tested with different
363 substrate combinations. Even though the full-length enzyme was inactive, the truncated variant
364 *HoPTS5*(Ser36-end) produced GGPP as the main product. This was detectable by the formation of
365 GLOH after acid hydrolysis of the reaction products. Minor peaks of LOH and NOH were detected in
366 when DMAPP and IPP were used as substrates while NOH could be detected when GPP and IPP were
367 used instead, indicating that the formation of GGPP proceeds via GPP and FPP (Fig. 2d). Taken
368 together with the prediction of a chloroplastic target peptide in its N-terminus, these results suggest that
369 *HoPTS5* possibly encodes for a GGPP synthase, likely functioning in the chloroplast providing the
370 substrates for carotenoid and phytol biosynthesis.

371

372 **Functional characterization of the squalene/phytoene synthase family members**

373 IDI and SQS are enzymes that catalyze two non-consecutive reactions. IDI isomerizes IPP to DMAPP,
374 while SQS catalyzes the formation of squalene using two FPP molecules. In order to characterize the
375 *HoIDISQS* fusion, we studied the two domains separately. Initially, we evaluated the functionality of
376 the SQS domain by introducing the full-length gene into a *Saccharomyces cerevisiae* strain engineered
377 to produce high amounts of FPP (Ignea *et al.*, 2012). Analysis of the non-saponifiable lipid extract of
378 yeast cells expressing the fusion showed production of higher amounts of squalene when *HoIDISQS*
379 was expressed, comparing to the levels of squalene produced by the endogenous yeast squalene

380 synthase (Fig. 3a). This suggests that the SQS domain of *HoIDISQS* likely encodes for a functional
381 synthase.

382 The functionality of the IDI domain was assessed both in the fusion (*HoIDISQS*) and as a separately
383 cloned IDI domain (*HoIDI*). These two enzymes were coexpressed with a monoterpene synthase from
384 *Salvia fruticosa*, 1,8-cineole synthase (*SfCinS1*) (Kampranis *et al.*, 2007), in a yeast strain engineered
385 to produce GPP (Ignea *et al.*, 2012). By catalyzing the isomerization of IPP to DMAPP, IDI provides
386 substrates to the endogenous yeast enzyme, Erg20p, which produces GPP. It has been shown that
387 functional IDI expression in this strain increased synthesis of 1,8-cineole by *SfCinS1* (Ignea *et al.*,
388 2011). We used the endogenous yeast IDI (*ScIDI*) as positive control. We sampled the head space of all
389 yeast cell cultures expressing the different combinations with solid-phase microextraction (SPME).
390 Their analysis showed that when *HoIDISQS*, *HoIDI* or *ScIDI* were expressed, significantly higher
391 amounts of 1,8-cineole were produced (Fig. 3a), indicating that the IDI domain is functional both
392 individually and in the fusion.

393 We also employed the yeast expression system for the characterization of the candidate phytoene
394 synthase, *HoPSY*. During our signal/target peptide analysis, *HoPSY* was predicted to have a
395 chloroplastic signal peptide. Taking into account the cleavage position predictions, we introduced the
396 truncated variant *HoPSY*(Ser78-end) into a yeast expression vector. In order to evaluate the
397 functionality of the variant, we employed an *in vivo* chromogenic assay. In this assay, the candidate
398 phytoene synthase is coexpressed with a GGPP synthase and a phytoene desaturase. The GGPP
399 synthase provides the substrate for phytoene biosynthesis. If the examined enzyme is functional and
400 produces phytoene, the desaturase will take it up to produce lycopene (Fig. 3c). As yeast colonies that
401 accumulate lycopene become orange colored, this assay provides a quick and reliable means for the
402 evaluation of phytoene synthase activity. We used the yeast strain AM94, which is engineered for the
403 efficient production of isoprenoids (Ignea *et al.*, 2012), and introduced *HoPSY*(Ser78-end) together

404 with Erg20p(Y95A), an engineered GGPP synthase (Ignea *et al.*, 2015), and crtI, a phytoene desaturase
405 from *Xanthophyllomyces dendrorhous* (Verwaal *et al.*, 2007). While control cells that were
406 transformed with an empty vector, instead of *HoPSY*(Ser78-end), did not develop any color,
407 coexpression of the three genes resulted in orange colored yeast colonies, confirming the ability of
408 *HoPSY* to synthesize phytoene (Fig. 3b).

409

410 Discussion

411 Aiming to shed light to isoprenoid biosynthesis in diatoms, we investigated different aspects of the
412 pathway in the species *H. ostrearia*. By combining transcriptomic analysis with functional
413 characterization of enzyme activities and predictions for the subcellular localization of the
414 corresponding proteins, we can propose a model of isoprenoid biosynthesis in *H. ostrearia* that is
415 summarized in Fig. 4 and discussed below.

416 *H. ostrearia* retains a functional cytosolic MVA pathway and a functional plastidial MEP pathway. The
417 majority of the sequenced diatom species investigated to date, have both routes for IPP and DMAPP
418 synthesis. However, these precursors seem to be differentially allocated towards the final isoprenoid
419 products across different species. A plant-like dichotomy has been observed in *P. tricornutum*,
420 *Nitzschia ovalis* and *R. setigera*. Accordingly, sterols are synthesized using precursors from the MVA
421 route, while the biosynthesis of carotenoids and the diterpenoid phytol proceeds via MEP generated
422 precursors in the chloroplast (Cvejić & Rohmer, 2000; Massé *et al.*, 2004). On the contrary, *H.*
423 *ostrearia* uses chloroplast-derived precursors to synthesize its main sterol (24-ethylcholest-5-en-3-ol)
424 (Massé *et al.*, 2004). Contribution of the MEP pathway to sterol (24-methyl-cholesta-5,24(28)-dien-3b-
425 ol) biosynthesis is also observed in the centric species *T. pseudonana* under fast growing, nitrogen-
426 replete culture conditions (Zhang *et al.*, 2009). Similar differentiations were shown for HBI

427 biosynthesis, with *R. setigera* incorporating C₅ precursors derived from the MVA route and *H.*
428 *ostrearia* synthesizing HBIs with precursors generated via the MEP pathway (Massé *et al.*, 2004).
429 These different patterns on precursor partitioning indicate different regulation mechanisms that in
430 addition to being responsive to external conditions (for example nutrient availability), also possibly
431 involve precursor transportation between cytosol and chloroplast. MVA and MEP pathway cross-talk
432 has already been reported in some plants (Bick & Lange, 2003; Hemmerlin *et al.*, 2003a). Even though
433 there are substantial differences between the organization of the primary plastids of plants and the
434 secondary plastids of diatoms, the four membranes of diatoms' plastids were previously shown to be
435 permeable through specific transporters (Ast *et al.*, 2009).

436 The first key step towards prenyl diphosphate synthesis is the isomerization of IPP to DMAPP by IDI.
437 Photosynthetic heterokonts including diatoms and brown algae, haptophytes and dinoflagellates are
438 characterized by the expression of a bifunctional protein fusion between IDI and SQS that catalyzes the
439 dimerization of FPP towards sterol synthesis. In *H. ostrearia* this enzyme fusion does not appear to
440 have any target peptide, while it is predicted to contain a pair of transmembrane helices at the C-
441 terminal part, suggesting ER localization. No other contig encoding for an IDI could be identified in
442 our transcriptomic data. Analysis of thirty three diatom species by Ferriols and coworkers (2017)
443 (Ferriols *et al.*, 2017) showed that all but one specific strain of *R. setigera*, express an IDI-SQS fusion
444 and 19 of them additionally express an independent IDI. Many of these fall within the same
445 phylogenetic clade with the independent IDI from *R. setigera* that contains a putative chloroplast
446 targeting peptide. A growing body of evidence recently supports the occurrence of alternative splicing
447 in diatoms (Rastogi *et al.*, 2018). Thus, the possibility that diatoms that only contain the IDI-SQS
448 fusion gene may also produce an alternatively spliced form producing only the IDI protein cannot be
449 ruled out.

450 The intermediate step between the reactions catalyzed by IDI and SQS is FPP synthesis and according
451 to our functional characterization is likely catalyzed by *HoPTS1*. Sequence analysis of *HoPTS1*
452 suggested the presence of a type II signal anchor. This is responsible to anchor the enzyme to a
453 membrane. It is reasonable to assume that this is the ER membrane, which is continuous with the
454 outermost chloroplastic membrane in diatoms. Since *HoPTS1* and *HoIDISQS* catalyze consecutive
455 reactions, we can speculate that they might also physically interact, forming an enzymatic complex that
456 is localized at the ER. Previous studies showed that *H. ostrearia*'s main sterol, 24-ethylcholest-5-en-3-
457 ol, incorporates precursors generated from the plastid localized MEP pathway (Massé *et al.*, 2004).
458 Taken together, these results indicate again precursor transportation from the chloroplastic stroma to
459 the cytosol. The mechanism and the exact regulation of such events are unknown. Recently, a novel
460 isoprenoid regulatory mechanism that possibly involves precursor transportation between subcellular
461 compartments has been described in plants. According to this, a cytosolic isopentenyl phosphate kinase
462 (IPK) and specific Nudix hydrolases regulate IPP supply to the pathway by active phosphorylation-
463 dephosphorylation of IP/IPP. Perturbation of IP supply in *Nicotiana tabacum* was shown to affect both
464 MVA and MEP derived isopenoids, highlighting a correlation with precursor transportation (Henry *et*
465 *al.*, 2015, 2018). Mining the transcriptome of *H. ostrearia* for similar genes, we were able to identify
466 homologues of IPK and Nudix hydrolase (candidate sequences can be found in Supporting Information
467 Notes S2) that are also present in transcriptomes of other sequenced diatom species. Whether a similar
468 regulatory mechanism is present in diatoms and how this controls IP/IPP supply and/or transportation
469 are open questions that remain to be answered.

470 Our model suggests that *HoPTS5* acts in the plastids to generate the GGPP that is essential for
471 carotenoid synthesis. The high relative expression levels of *HoPTS5* (Fig. S5) probably reflect the high
472 demand for plastidial isoprenoid biosynthesis (primarily GGPP-derived carotenoids and phytol) under
473 the specific growth conditions. The first committed step towards carotenoids is catalyzed by the

474 phytoene synthase *HoPSY*. Both of these enzymes were acquired from the red algal endosymbiont, a
475 fact that corroborates their predicted targeting to the chloroplast. *HoPTS2*, which was also predicted to
476 have chloroplastic localization, showed activity as a short-chain poly prenyl synthase. The lack of
477 detailed information on the isoprenoid content of diatoms prevents us from assigning a role to *HoPTS2*.
478 It is possible that this enzyme synthesizes polyprenyl diphosphates or the corresponding alcohols *in*
479 *vivo*. In plants these compounds have been shown to be incorporated in thylakoid membranes and
480 modulate their fluidity, influencing the overall photosynthetic performance (Bajda *et al.*, 2009;
481 Surmacz & Swiezewska, 2011; Akhtar *et al.*, 2017).

482 We propose that cytosolic isoprenoid biosynthesis is supported by precursors generated by the MVA
483 pathway and prenyl diphosphates synthesized by *HoPTS3*. Our analysis showed that this is probably
484 the only enzyme that is not conserved among different diatom species. In *H. ostrearia*, the *HoPTS3*
485 gene likely originates from the heterotrophic host of secondary endosymbiosis.

486 Although it was not possible to characterize the activity of *HoPTS4*, we presume that this enzyme
487 likely acts as a prenyl transferase in the mitochondria. As there was no other prenyltransferase
488 predicted to be targeted to mitochondria, *HoPTS4* probably uses substrates transported from the cytosol
489 or chloroplast. Crosstalk between mitochondria and chloroplast in diatoms has been previously
490 proposed for other metabolic processes (Prihoda *et al.*, 2012).

491 Even though the majority of prenyltransferases catalyze the linear condensation of prenyl diphosphates,
492 there are examples where these enzymes are involved in the synthesis of irregular, neither head-to-tail
493 nor head-to-head, isoprenoids (Rivera *et al.*, 2001; Demissie *et al.*, 2013). A characteristic example is
494 an FPPS in *Artemisia tridentata* that among other reactions catalyzes the head-to-middle linkage of two
495 DMAPP molecules to produce the branched isoprenoid, lavandulyl diphosphate (Hemmerlin *et al.*,
496 2003b). It is thus likely that HBI biosynthesis also involves prenyltransferase-type enzymes.
497 Furthermore, different HBI producing diatom species were shown to use different precursors for the

498 synthesis of these molecules (Massé *et al.*, 2004), suggesting that the pathway is regulated differently
499 among species. By providing here a model of the isoprenoid pathway in *H. ostrearia* that illustrates the
500 subcellular distribution of different prenyltransferases we believe that we provide a basis for future HBI
501 biosynthetic studies that will test the currently identified or different enzymes, substrates and/or
502 conditions.

503

504 **Conclusions**

505 In conclusion, our investigation has significantly contributed to our understanding of isoprenoid
506 biosynthesis in diatoms. At least five prenyltransferases mediate the prenyl diphosphate synthesis and
507 provide substrates to downstream branches of the pathway. It is likely that precursors are transported
508 from the plastids to the cytosol. Further studies that will confirm the localization of the enzymes *in vivo*
509 and explore the regulatory mechanisms and crosstalk between the MVA and MEP pathways are
510 essential for a thorough elucidation of the mechanisms involved. Our phylogenetic analysis provided an
511 insight into the multisourced genetic background of diatoms that is reflected clearly on isoprenoid
512 biosynthesis. While there is still a general debate on the extent of the contribution of each lineage (red
513 and green) to the genomes of diatoms (Dagan & Martin, 2009; Burki *et al.*, 2012; Deschamps &
514 Moreira, 2012), it is commonly accepted that these events have armed diatoms with the genetic
515 potential and the metabolic plasticity to succeed in contemporary oceans. Finally, our results provide
516 the blueprint for the elucidation of the biosynthetic pathways leading to unique diatom isoprenoids,
517 such as HBIs.

518 **Accession Numbers**

519 All sequences from *H. ostrearia* mentioned in this study have been submitted to the GenBank database
520 (www.ncbi.nlm.nih.gov) with accession numbers provided on Table 1. Transcriptomic data have been

521 submitted to the European Nucleotide Archive (ENA) Database under experiment accession
522 ERX2834706 and Run accession ERR2827962.

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530

531 **Author contributions**

532 AA, EG, SM, FV, CI carried out experiments. SCK, FV, KK, AMM, AAr, GM designed experiments.
533 AA, EG, SM, CI, FV, SCK, AMM, GM, KK, AAr analyzed results. AA and SCK wrote the
534 manuscript. All authors have read and commented on the manuscript.

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760 **Supporting Information**

761 **Additional supporting information may be found in the online version of this article.**

762 **Methods S1** Supplementary methods

763 **Table S1** IDs of the transcriptomes from the MMETSP used in the phylogenetic analysis

764 **Table S2** List of primers used in this study

765 **Table S3** Identification of *HoPTS1-HoPTS5* homologues in other diatom species

766 **Table S4** Genomic location of isoprenoid biosynthetic genes in the *P. tricornutum*, *T. pseudonana*
767 and *F. cylindrus*

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770 central, final steps)

771 **Fig. S2** Chemical structures of some of the isoprenoids synthesized by *H. ostrearia*

772 **Fig. S3** HBI profile of *Haslea ostrearia* NCC 153.8 strain

773 **Fig. S4** Phylogenetic relationship of prenyltransferases from diatoms

774 **Fig. S5** Expression patterns of genes involved in isoprenoid biosynthesis as detected by RNA-seq

775 **Fig. S6** (a) Multiple sequence alignment of *HoPTS1* with known farnesyl diphosphate synthases
776 from other species (b) Multiple sequence alignment of *HoPTS2-5* with known polyprenyl
777 diphosphate synthases from other species

778 **Fig. S7** Phylogenetic trees of MEP pathway proteins

779 **Fig. S8** Phylogenetic trees of prenyltransferases and squalene/phytoene synthase family members

780 **Notes S1** Extended discussion on the phylogenetic analysis

781 **Notes S2** Diatom sequences

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800 **Table 1** Candidate isoprenoid biosynthetic genes, their closest homologues their evolutionary origin and
801 subcellular localization as indicated by analysis in this study

	Gene Name	Genebank ID	Closest homologue (percentage similarity)	Evolutionary origin	Subcellular localization prediction
MEP PATHWAY	1-deoxy-D-xylulose 5-phosphate synthase (DXS)	MH731010	<i>Phaeodactylum tricornutum</i> XP_002176386.1 (83%)	Red algae	Chloroplast
	1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)	MH731011	<i>Thalassiosira pseudonana</i> XP_002295597.1 (84%)	Red algae	Chloroplast
	2-C-methyl-D-erythritol-4-phosphate- cytidyltransferase (MCT)	MH731012	<i>Fistulifera solaris</i> GAX13480.1 (78%)	Red algae	Chloroplast
	4-diphosphocytidyl-2c-methyl-d-erythritol kinase (CMK)	MH731013	<i>Phaeodactylum tricornutum</i> XP_002178363.1 (77%)	Green algae	Chloroplast
	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS)	MH731014	<i>Phaeodactylum tricornutum</i> XP_002180038.1 (76%)	Algae	Chloroplast
	(E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS)	MH731015	<i>Fragilariopsis cylindrus</i> OEU20628.1 (77%)	Green algae	Chloroplast
	Hydroxymethylbutenyl diphosphate reductase (HDR)	MH731016	<i>Phaeodactylum tricornutum</i> XP_002178617 (73%)	Algae	Chloroplast
MVA PATHWAY	Acetyl-coa c-acetyltransferase 1 (AACT)	MH731017	<i>Phaeodactylum tricornutum</i> XP_002185228.1 (69%)		Cytosol
	Hydroxy-methylglutaryl-CoA synthase (HMGS)	MH731018	<i>Fragilariopsis cylindrus</i> OEU16767.1 (73%)		Cytosol
	Hydroxyl-methylglutaryl-CoA reductase (HMGR)	MH731019	<i>Fragilariopsis cylindrus</i> OEU16221.1 (82%)		Cytosol
	Mevalonate kinase (MVK)	MH731020	<i>Thalassiosira pseudonana</i> XP_002287787.1 (73%)		Cytosol
	Phospho-mevalonate kinase (PMK)	MH731021	<i>Fragilariopsis cylindrus</i> OEU13939.1 (49%)		Cytosol
	Mevalonate diphosphate decarboxylase (MVD)	MH731022	<i>Fragilariopsis cylindrus</i> OEU17781.1 (69%)		Cytosol
	Isopentenyl-diphosphate delta-isomerase fused to squalene synthase (<i>Ho</i> IDISQS)	MH720297	<i>Fistulifera solaris</i> GAX27897.1 (63%)	Green algae	Cytosol
CENTRAL STEPS	Farnesyl diphosphate synthase (<i>Ho</i> PTS1)	MH720291	<i>Rhizosolenia setigera</i> AKH49589.1 (56%)	Not traced	ER or PPC
	Polyprenyl diphosphate synthase (<i>Ho</i> PTS2)	MH720292	<i>Thalassiosira oceanica</i> EJK71722.1 (75%)	Algae	Chloroplast
	Geranylgeranyl diphosphate synthase (<i>Ho</i> PTS3)	MH720293	<i>Phaeodactylum tricornutum</i> XP_002181666.1 (74%)	Heterotrophic host	Cytosol
	Putative polyprenyl synthase (<i>Ho</i> PTS4)	MH720294	<i>Phaeodactylum tricornutum</i> XP_002185039.1 (70%)	Algae	Mitochondria
	Geranylgeranyl diphosphate synthase (<i>Ho</i> PTS5)	MH720295	<i>Phaeodactylum tricornutum</i> XP_002178555.1 (75%)	Red algae	Chloroplast
	Phytoene synthase (<i>Ho</i> PSY)	MH720296	<i>Phaeodactylum tricornutum</i> XP_002178776.1 (69%)	Red algae	Chloroplast

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Table 2 Functionally characterized enzymes in this study

	Full length ORF	Variants studied	Accepted substrates	Products
<i>HoPTS1</i>	1299 bp	Full length	no activity	-
		Ser73-end	DMAPP+IPP	GPP, FPP
			GPP+IPP	FPP
		Val89-end	DMAPP+IPP	GPP, FPP
			GPP+IPP	FPP
<i>HoPTS2</i>	1584 bp	Full length	no activity	-
		Arg45-end	FPP+IPP	C20-C30 PPP
			GGPP+IPP	C25-C30 PPP
		Gly140-end	no activity	no activity
<i>HoPTS3</i>	1014 bp	Full length	GPP+IPP	FPP, GGPP
			FPP+IPP	GGPP
<i>HoPTS4</i>	1416 bp	Full length	no activity	-
		Leu121-end	no activity	
<i>HoTPS5</i>	1011 bp	Full length	no activity	-
		Ser36-end	DMAPP+IPP	GPP,FPP,GGPP
			GPP+IPP	FPP, GGPP
			FPP+IPP	GGPP
<i>HoPSY</i>	1485 bp	Full length	Not tested	-
		Ser78-end	GGPP+GGPP	phytoene
<i>HoIDISQS</i>	2277 bp	Full length	FPP+FPP	squalene

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824 Figure legends

825 **Fig. 1. (a) Neighbor joining phylogenetic tree of the selected sequences and their closest**
826 **homologues and protein domain structures.** Numbers on the branches indicate bootstrap support
827 values from 1,000 trees; values under 70 were removed **(b) Protein domain structure of selected**
828 **sequences.** BSP; Bipartite Signal Peptide, MSP; Mitochondrial Signal Peptide, TD; Transmembrane
829 domain.

830 **Fig. 2. GC-MS profile of (a) *Ho*PTS1(Ser73-end), (b) *Ho*PTS2(Arg45-end), (c) *Ho*PTS3, (d)**
831 ***Ho*PTS5(Ser36-end) in *vitro* reaction products using different prenyl diphosphates as substrates.**
832 Identification of compounds was based on comparison of accurate mass and retention time between
833 reaction substrates/products and acid hydrolysis products of authentic standards (bottom panel). Ions
834 m/z 137, 205, 273, 341 and 409 are derived from precursor ions (m/z 154, 222, 290, 358 and 426
835 respectively) by loss of H₂O in positive ion mode

836 **Fig. 3. Functional characterization of squalene/phytoene synthase family members. (a)** Expression
837 of *Ho*IDISQS in the yeast strain AM94 resulted in high accumulation of squalene (up). Coexpression of
838 *Ho*IDISQS with SfCinS1 resulted in high accumulation of 1,8-cineole. *Ho*=*Haslea ostrearia*,
839 *Sf*=*Salvia fruticosa*, *Sc*=*Saccharomyces cerevisiae*. **(b)** Yeast colonies coexpressing ERG20(Y95A),
840 crtI and *Ho*PSY(Ser78-end) (up) in comparison with the control colonies that carry an empty vector
841 instead of a phytoene synthase (down) and **(c)** Lycopene biosynthetic pathway.

842 **Fig. 4 A model for isoprenoid biosynthesis in *H. ostrearia*.** Isopentenyl diphosphate (IPP) and
843 dimethyl allyl diphosphate (DMAPP) are synthesized via the mevalonate (MVA) and methylerythritol
844 phosphate (MEP) pathways in the cytosol and plastid. Each of these precursor pools are used for the
845 synthesis of prenyl diphosphates by prenyltransferases at different subcellular compartments. *Ho*PTS1

846 is responsible for farnesyl diphosphate (FPP) synthesis at the endoplasmic reticulum (ER), in close
847 proximity to *HoDISQS*. *HoPTS3* mediates synthesis of geranylgeranyl diphosphate (GGPP) in the
848 cytosol. In the chloroplast *HoPTS2* and *HoPTS5* synthesize precursors for polyprenol and carotenoid
849 synthesis. The first committed step of carotenoid biosynthesis is catalyzed by *HoPSY*. *HoPTS4* likely
850 mediates prenyl diphosphate synthesis in mitochondria. Dashed arrows indicate possible precursor
851 transportation between cytosol and plastids.

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