Metabolite profiling of *Phycomyces blakesleeanus* carotene mutants reveals global changes across intermediary metabolism

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The filamentous fungus *Phycomyces blakesleeanus* provides a renewable biosource of industrial high-value compounds such as carotenes, other isoprenoids (ubiquinone and sterols), organic acids and fatty acids. Several *Phycomyces* mutants involved in the formation of β-carotene are available. For example, the *carA* mutants have a leaky mutation in the phytoene synthase and produce significantly lower amounts of carotenes, while the *carB* and *carR* mutants produce phytoene and lycopene, respectively, due to a null mutation in the genes encoding the phytoene dehydrogenase and lycopene cyclase, respectively. The *carS* mutants are mutated in the gene encoding the oxygenase responsible for the conversion of β-carotene into apo-carotenoids and, as a result, β-carotene accumulates. In order to ascertain further the biochemical changes arising in these potential industrial strains, a metabolite profiling workflow was implemented for *Phycomyces*. GC-MS and ultra-performance liquid chromatography–photodiode array platforms enabled the identification of over 100 metabolites in 11 *carA*, *carB*, *carR* and *carS* mutant strains and their wild-type comparator. All mutant strains possessed decreased TCA cycle intermediates, galactose, alanine and ribitol, while dodecanol and valine showed a general increase. As predicted, other terpenoid levels were affected in the *carB*, *carR* and *carS* mutants but not in the *carA* mutants. The global changes across intermediary metabolism of the mutants suggest that complex metabolic networks exist between intermediary and secondary metabolism or that other mutations beyond the carotene pathway may exist in these mutants. These data show the utility of the methodology in metabolically phenotyping *Phycomyces* strains with potential industrial exploitation.

**INTRODUCTION**

The filamentous fungus *Phycomyces blakesleeanus* is a species of the Mucorales (Zycha *et al.*, 1969). It has been utilized for decades as a model system in studies relating to classical genetics, metabolism (including carotene biosynthesis) and phototropism (Cerdá-Olmedo, 1987, 2001). More recently, renewed commercial interest in *Phycomyces* has arisen as a renewable biosource of industrial high-value compounds such as carotenes, other isoprenoids (ubiquinone and sterols), organic acids and fatty acids. One of the drivers behind this potential exploitation as a new or underutilized source of small molecules has been our dwindling sources of fossil fuels (Cherubini, 2010; Clark *et al.*, 2012; Connor & Atsumi, 2010; Fesenko & Edwards, 2014). Presently, chemical synthesis is the predominant method for carotenoid and steroid production. The precursors used in this process are derived directly from the petrochemical industry as by-products.

An important feature of *Phycomyces* that has facilitated its use as a system for the advancement of science and its potential exploitation has been its amenability to mutagenesis. For example, a plethora of mutants involved in the β-carotene biosynthetic pathway in *Phycomyces* have been isolated. These include mutant alleles in two structural genes that encode for three proteins, *carB* for phytoene dehydrogenase and *carRA* for phytoene synthase and lycopene cyclase (Arrach *et al.*, 2001; Eslava & Cerdá-Olmedo, 1974; Torres-Martínez *et al.*, 1980). The *carA* mutants are white and deficient in all carotenes, the *carB* mutants are white and rich in phytolene and the *carR* mutants are red and rich in lycopene. Two oxygenases, encoded by the genes *carS* and *acaA*, cleave β-carotene to produce the
apocarotenoids (Medina et al., 2011), precursors of the pheromones involved in the sexual interaction of opposite sex strains (Sutter, 1975). The carS mutants (Murillo & Cerdá-Olmedo, 1976; Tagua et al., 2012) are unable to catabolize β-carotene into an array of apocarotenoids (Tagua et al., 2012) (Fig. 1). This blockage in the pathway results in the accumulation of β-carotene at high levels. Phenotypically, this creates mycelia that have a deep yellow/orange coloration rather than the light yellow colour observed in the wild-type comparator.

Carotenes are isoprenoids and thus are related to the ubiquinones and sterols found in Phycomyces at high concentrations via the universal C5 biosynthetic isoprenoid precursor, isopentenyl diphosphate. Within Phycomyces, the isopentenyl diphosphate generated is formed by the mevalonate pathway, which uses acetyl-CoA as its three-carbon precursor (Ruiz-Albert et al., 2002). This common precursor and its position at the interface of intermediary and secondary metabolism link isoprenoid formation to fatty acid/lipid formation. Despite these direct biochemical interactions between precursors and products, radiolabelled tracer experiments have inferred that the carotene, ubiquinone and triacylglycerol biosynthetic pathways work separately from acetyl-CoA in Phycomyces, while sterols and ubiquinone pathways exchange common precursors (Kuzina et al., 2006) but are not co-regulated (Kuzina & Cerdá-Olmedo, 2007).

Although Phycomyces cannot be stably transformed, the diverse array of mutants available has warranted the development of ‘omic’ resources in this organism, the genome of which has recently been sequenced (Corrochano et al., 2016). The metabolome of an organism represents the end products and intermediates of biological processes and is complementary to the genome. Its analysis can reveal the underlying biochemical mechanisms associated with certain traits and provide holistic characterization of chemical composition at different growth stages in response to environmental perturbation, as well as validating gene function.

The present study represents, to our knowledge, the first metabolite-profiling approach in a mucoral fungus. Comparisons between the metabolite profiles of wild-type with carA, carB, carR and carS mutant strains using GC-MS and ultra-performance liquid chromatography–photodiode

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**Fig. 1.** Biosynthetic pathway of carotene in Phycomyces. Phytoene is produced from two molecules of geranylgeranyl pyrophosphate (GGPP). Four dehydrogenations produce the lycopene and two cyclizations produce the β-carotene. In boldface, protein names are as follows: CarA, phytoene synthase; CarB, phytoene dehydrogenase; CarR, lycopene cyclase; CarS, β-carotene cleavage enzyme.
array (UPLC-PDA) have been performed in order to ascertain whether perturbations to the carotene pathway have widespread effects on the steady-state metabolite levels across cellular metabolism in these fungi.

METHODS

Phycomyces strains and culture conditions. The wild-type P. blakesleeanus strain NRR11555, (-) sex, was used in this study and mutants derived from this parent strain. The mutant strains included the carA mutants S40, carA79 (-) (Bergman et al., 1973), S45, carA84 (-) (Bergman et al., 1973) and C2, carA5 (-) (Meissner & Delbruck, 1968); the carB mutants C5, carB10 (-) (Eslava & Cerdá-Olmedo, 1974), S21, carB60 (-) (Bergman et al., 1973) and S22, carB61 (-) (Bergman et al., 1973); the carR mutants C9, carR21 (-) (Meissner & Delbruck, 1968) and C11, carR23 (-) (Ootaki et al., 1973) and the carS mutants S324, carS180 (-) (Tagua et al., 2012), S178, carS153 madB104 (-) (Bejarano et al., 1988) and C115, carS42 mad-107 (-) (Meissner & Delbruck, 1968). Cultures were grown for 3 days at 22 °C in light (16–20 µmol m⁻² s⁻¹) on minimal agar with glucose, L-asparagine, thiamine and mineral salts, enriched with yeast extract (2 g l⁻¹) as described in Murillo & Cerdá- Olmedo (1976).

Quenching the Phycomyces metabolome. Quenching of metabolism in Phycomyces was performed by rapidly (60 s) adding four volumes of pre-chilled (-20 °C) methanol [60 % (v/v)] containing NaCl [0.9 % (w/v)] to one volume of the Phycomyces culture. After centrifugation at 3500 g for 5 min at 0 °C (Eppendorf 5810 R), the supernatant was discarded and pelleted mycelia were frozen at −80 °C. Then, the supernatant was freeze-dried and stored at −80 °C prior to extraction.

Extraction of polar metabolites and carotenoids. Methanol (500 µl) [50 % (v/v)] was added to dried biomass (10 mg) and the mixture was placed in a sonication bath (Sonicor SC-120TH; Sonicor Instrument) at room temperature. Sonication was performed for 15 min at 50/60 Hz. To the resulting suspension, chloroform (1 ml) was added and the mixture was centrifuged at 10 000 r.p.m. for 5 min to facilitate phase separation. The epiphase (500 µl), containing polar metabolites, and the hypophase, containing carotenoids, were separated and stored dry at −20 °C until further analysis.

Extraction of non-polar metabolites. Prior to extraction, alkaline saponification was carried out by adding aqueous 10 % NaOH (w/v) to the dried biomass, at room temperature, then sonication was performed for 10 min. The NaOH solution was removed by centrifugation and the pellet was extracted as described in the previous section. The hypophase, containing non-polar metabolites, was separated from the epiphase and stored at −20 °C until further analysis. Phases for polar and non-polar metabolites obtained were dried under vacuum using Genevac EZ-2 Plus (Genevac).

GC-MS analysis of polar and non-polar metabolites. Polar and non-polar extracts, generated using the methods described in previous sections, were analysed by GC-MS using procedures described in Perez-Fons et al. (2014) with modifications. In brief, aliquots from each polar extract (200 µl) and non-polar extract (500 µl) were removed, dried under vacuum and solubilized in the derivatization reagents. Samples were derivatized to their methoxylated and silylated forms according to Halket et al. (2005). Methoxyamine hydrochloride (20 mg ml⁻¹ in anhydrous pyridine; 30 µl) was added to samples and incubated at 40 °C for 1 h. Following this reaction, the suspensions were treated with MSTFA [N-methyl-N-(trimethylsilyl)trifluoroacetamide] (70 µl) and heated at 40 °C for 2 h. The final solution (1 µl) was injected in split mode (1 : 10) into a 7890A GC in-line with a 5975C mass spectrometer (Agilent Technologies). Metabolites were separated on a DB-5MS 30 m × 0.25 mm × 0.25 µm column (J&W Scientific), equipped with a 10 m guard column and using a temperature gradient ranging from 70 to 320 °C at 5 °C min⁻¹. Helium was employed as the carrier gas and the flow rate was 0.5 ml min⁻¹. The inlet was heated to 280 °C and the mass spectrometer transfer line was at 250 °C. A mixture of n-alkanes, ranging from 8 to 32 carbons, was used for retention index external calibration. Authentic standards, D₈-succinic acid and D₇'-myristic acid, for polar and non-polar extracts, respectively, were added to the samples at a concentration of 1 mg ml⁻¹ as aliquots (10 µl) before drying and being subjected to the derivatization procedure identical to that used with the extracts.

Data analysis. Levels of metabolites analysed by GC-MS were quantified relative to the internal standard and corrected for the dried weight of the biomass. Compounds generating multiple peaks in the chromatogram as a consequence of the methoxylation and silylation reactions were quantified by summing the areas of the different derivatives. AMDIS version 2.7 (NIST) software was used for peak deconvolution and establishing the authors’ libraries for polar and non-polar metabolites. Identification of metabolites for library construction was done by comparing mass spectra and retention indexes to NIST [version 2.0 (2008)] and Golm metabolome (http://gmd.mpimp-golm.mpg.de/) mass spectral databases and confirmed with authentic standards whenever possible. Those compounds not identified were named as Unknown (UNK), followed by the sub-index p or np for polar or non-polar extracts, respectively, and by the corresponding retention time. The identification criteria used and annotation of unknowns were performed as described in Bino et al. (2004) and Sumner et al. (2007). Data matrices were transformed using the pareto-scaled method (van den Berg et al., 2006) and multivariate analysis was performed using SIMCA-P+13.03 (Umetrics). Pathway diagrams were created using the in-house-developed software BioSynLab (Royal Holloway, University of London) or Inkscape 0.48.5 (www.inkscape.org). Means, standard deviation and P values [for false discovery rates, see Benjamini & Hochberg (1995)] were calculated in Excel (Microsoft).

Separation and detection by UPLC-PDA. Carotenoids, sterols and ubiquinone (coenzyme Q₉) extracted by the method described previously were separated and identified by LC with photodiode array detection (Waters). An Acquity UPLC system (Waters) was used with an Ethylene Bridged Hybrid (BEH C₁₈) column (2.1 × 100 mm, 1.7 mm) with a BEH C₁₈ VanGuard precolumn (2.1 × 50 mm, 1.7 mm). The mobile phase used was A, methanol/water (50/50), and B, acetonitrile (ACN)/ethyl acetate (75 : 25), and the flow rate was 0.5 ml min⁻¹ and t. All solvents used were HPLC grade and filtered prior to use through a 0.2 mm filter. The gradient was 30 % A : 70 % B for 0.5 min and then stepped to 0.1 % A : 99.9 % B for 5.5 min and then to 30 % A : 70 % B for the final 2 min. Column temperature was maintained at 30 °C and the temperature of samples at 8 °C. Online scanning across the UV-visible range was performed in a continuous manner from 250 to 600 nm, using an extended-wavelength photodiode array detector.

RESULTS

Culture conditions and quenching methods All strains were cultivated under identical condition and harvested at the same time point to generate adequate biomass for experimentation and to ensure that biological variation was minimized. The average dry weights of the cultures after 3 days of incubation were similar in all strains analysed (7.01 g l⁻¹±1 g, standard deviation). The quenching method using a pre-chilled (−20 °C)
quenching solution [methanol 60% (v/v) containing NaCl 0.9%] and the extraction method based on methanol and chloroform showed good quenching recovery and extraction efficiency, respectively, based on repeated extractions.

**Metabolite profiling of Phycomyces**

The GC-MS library for Phycomyces was created in an *AMDIS* format, using retention time locking and concurrent chromatography with a hydrocarbon standard mixture, in order to generate retention indices (Table S1, available in the online supplementary material). Identification of chromatographic components was carried out by firstly performing automated matching of mass spectrum to those present in the NIST and/or Golm metabolome databases (http://gmd.mpimp-golm.mpg.de/). These putative matches were then confirmed with authentic standards. Alternatively, authentic standards representing metabolites known to be present in *Phycomyces* and possessing chemistry amenable to GC-MS analysis were chromatographed and their characteristic online mass spectrum added to the library. The library contains more than 400 metabolites and its curation is ongoing. This analytical platform allowed the identification and quantification of 69 metabolites (Table S2) among the polar and non-polar extracts of *Phycomyces* strains. In accordance with the recommendations proposed by the metabolomics standards initiative (Bino et al., 2004; Sumner et al., 2007), chromatographic components were annotated, providing valuable metadata and validation of peak identities (Table S1). To provide an indication of the biological and technical variation within the system, a series of analyses were carried out on material cultivated under standardized conditions. To facilitate the visualization of these datasets, pathway networks were constructed to link the metabolites detected by GC-MS and UPLC-PDA.

The pathways were reconstructed from information present in the KEGG (Kanehisa et al., 2006) databases, and bespoke templates were created using in-house software (Perez-Fons et al., 2014) and Inkscape. This approach enabled the representation of metabolite perturbations across the network in a semi-automated manner.

Over one hundred GC-MS peaks were detected in the extracts of the quenching samples in *Phycomyces* strains, 69 were identified as known components and 7 were identified as unknown. Amino acids, fatty acids, organic acids and sugars are mainly represented. Furthermore, seven terpenoids, β-carotene, phytoene, lycopene, ubiquinone (Q0), ergosterol and two unidentified sterols (annotated by their retention time) were detected by UPLC-PDA. The metabolites identified included a range of intermediates and end products of central and secondary metabolism (Table 1). These data enabled the construction of a quantitative biochemical network for Phycomyces.

**A comparison of the metabolite profiles determined for the carotene mutants and their wild-type strain**

Three strains with different mutant alleles for each gene (*carA*, *carB* and *carS*) and two for *carR* were used. Visual inspection of the GC-MS chromatograms indicated similar profiles for all mutants compared to the wild-type (Fig. S1). A data matrix was created combining the variables from all the analytical platforms used and then the data were subjected to principal component analysis (PCA), in order to identify variables (metabolites) contributing to the overall changes in chemical composition between the wild-type and mutants strains, *carA* (Fig. 2a, e), *carB* (Fig. 2b, f), *carR* (Fig. 2c, g) and *carS* (Fig. 2d, h). Carotenoids were not included in the PCA to avoid influencing the clustering. The score plot from the PCA separates the mutant strains, *carB* (Fig. 2b), *carR* (Fig. 2c) and *carS* (Fig. 2d), from the wild-type strain. These data indicated that, despite similar visual chromatographic profiles, the comparative chemical composition among all the strains is significantly different. The loading plot of the PCA indicated that the separation of the strains was not solely due to the influence of carotenoid pigments, but could be attributed to other metabolites or sectors of metabolism. For example, amino acids, sugars and organic acids contributed to the clustering of the *carB* mutants away from the wild-types. While those families of metabolites, along with free fatty acids, glycerolipids and some unknown compounds, contributed to the clustering of the *carR* and *carS* mutants, this separation was not shown between *carA* mutant and the wild-type strains in the PCA.

More detailed analysis of the individual changes in metabolites was achieved through pairwise statistical analysis, using the significance derived from Student’s *t*-tests (Table S3). These changes were then visualized by illustrating the significant changes over a biochemical network (Fig. 3). Those metabolites considered significantly different were identified on the basis of consistent alteration across all mutant strains with the defined carotene phenotypes. The differences were recorded relative to the wild-type strain showing statistical robustness. Interestingly, several consistent changes were evident in all the carotene mutants analysed. For example, all mutant strains possessed decreased TCA cycle intermediates, such as aconitid acid or citric acid, galactose, alanine and ribitol, while dodecanol and valine showed a general increase in most of the mutant strains. Furthermore, decreased contents of isoleucine, gluconic acid, inositol and ubiquinone were detected in the *carR* mutants, and of aspartic acid, dihydouracil, inositol-P and inositol in the *carS* mutants. The glucose content increased significantly in the *carA* mutants only. There were trends in the content of different metabolites in all the phenotypes, but these changes were not similar in all the mutant strains affected in the same gene, or the differences with the wild-type strain were not significant. As predicted, other terpeneoid levels were affected by the *car* gene mutations. The ergosterol level, the main sterol in *Phycomyces* (Barrero et al., 1998, 2002), did not show a clear trend but the
unidentified sterol 2.85 showed a significant decrease in the carS mutant strains and a clear trend in carB and carR mutant strains. In the carR and carS mutant strains, the ubiquinone levels were lower than those determined in the wild-type mycelia.

**DISCUSSION**

The metabolism of *Phycomyces* has been well studied but not in a holistic manner; instead, specific pathways have been progressively studied (Hilgenberg et al., 1987). A collection comprising hundreds of mutants, selected on the basis of phenotype, and numerous natural strains exist that represent a vast unexplored metabolic resource. This genetic resource has the potential to become a new bio-source of numerous important industrial products. This study reports on the methodologies used to analyse the metabolome of *Phycomyces* strains and their potential utility. Although the front-end of a metabolite profiling workflow is an area of continuous development where compromises have to be made in order to maximize metabolite coverage, quenching of the samples with the pre-chilled (∼20 °C) quenching solution was effective in obtaining good recovery and metabolite representation for robust metabolome analysis in this case. In the present study, we have illustrated that the procedures are robust and can be used to address biological questions. The mutations in the *carA*, *carB*, *carR* and *carS* genes are well characterized in the phytoene synthase, phytoene dehydrogenase, lycopene cyclase and β-carotene cleavage enzymes, respectively. However, our metabolite profiling data presented in the present study show that the effects of these mutations go beyond the target carotene pathway and changes arise across metabolism. All the mutants used were produced by a chemical mutagen that can yield additional mutations. The use of different strains mutated in the same gene allowed us to distinguish between differences due to the gene mutation, to different background or to additional mutations. A key question to decipher is whether these changes arise directly as a consequence of biochemical partitioning resulting in perturbations to the carotene pathway or small-molecule regulation. β-carotene is essential for the photomorphogenesis in *Phycomyces* and cannot be replaced by other carotenes (Corrochano & Cerdá-Olmedo, 1992), and the substitution or alteration of β-carotene content could affect the general metabolism of the cell in various ways.

Separation of *Phycomyces* organelles by centrifugation from sporangiophores showed that most of the β-carotene appears in free-floating oil and in a layer composed of

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### Table 1. Metabolites found in wild-type and carotene mutant cultures of *Phycomyces* using GC-MS-based metabolite profiling

The criteria used to assign the identity of the metabolites are detailed in Methods.

<table>
<thead>
<tr>
<th>Classification of metabolites</th>
<th>No.</th>
<th>Metabolites</th>
</tr>
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<tbody>
<tr>
<td>Amino acid</td>
<td>17</td>
<td>Alanine, asparagine, aspartic acid, cysteine, glycine, isoleucine, leucine,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lysine, methionine, phenylalanine, proline, pyroglutamic acid, serine,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>threonine, valine, β-alanine and γ-aminobutyric acid</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>14</td>
<td>C14:0, C15:0, C16:0, C17:0, C18:0, C18:1 cis9, C18:1 trans9, C18:2 cis9,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12, C18:2 trans9, 12, C18:3 cis9, 9, 12, C20:0, C22:0, C24:0 and C26:0</td>
</tr>
<tr>
<td>Glycerolipid</td>
<td>5</td>
<td>Glycero-1-C14:0, glycerol-1-C16:0, glycerol-1-C18:0, glycerol-2-C16:0 and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glycerol-2-C18:0</td>
</tr>
<tr>
<td>Non-amino-acid N-containing</td>
<td>2</td>
<td>Dihydouracil and pyrimidine</td>
</tr>
<tr>
<td>compound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic acid</td>
<td>8</td>
<td>Aconitic acid, butanoic acid, citric acid, docosanoic acid, fumaric acid,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>isocitric acid, lactic acid and malonic acid</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>Diethylphenanthrene</td>
</tr>
<tr>
<td>Phosphorylated</td>
<td>3</td>
<td>Glucose-6-phosphate, glycerol-3-phosphate and inositol-6-phosphate</td>
</tr>
<tr>
<td>Phosphate</td>
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<td>Phosphate</td>
</tr>
<tr>
<td>Polyamine</td>
<td>1</td>
<td>Putrescine</td>
</tr>
<tr>
<td>Polyol</td>
<td>2</td>
<td>Dodecanol and ribitol</td>
</tr>
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<td>Sugar</td>
<td>10</td>
<td>Arabinose/xylene/pentose, arabinose/ribose/xylene, erythrose, fructose,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>galactose, glucose, maltose, sedoheptulose, sucrose and threhalose</td>
</tr>
<tr>
<td>Sugar, acid</td>
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<td>Erythronic acid, gluonic acid and glyceric acid</td>
</tr>
<tr>
<td>Sugar, alcohol</td>
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<td>Glycerol and inositol</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>7</td>
<td>Ergosterol*, esterol 2.85 min*, esterol 3.65 min*, lycopene*, phytoene*,</td>
</tr>
<tr>
<td></td>
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<td>ubiquinone* (coenzyme Q9) and β-carotene*</td>
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<td>Total no. of identified</td>
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</tr>
<tr>
<td>Unknown metabolites</td>
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*Identified by UPLC-PDA.
Fig. 2. PCA of *Phycomyces* strains. (a–d) Score plot showing the clustering pattern of wild-type strain (green circle) with (a) carA mutant strains (purple), with (b) carB mutant strains (blue), with (c) carR mutant strains (red) and with (d) carS mutant strains (yellow). (e–h) Loading plot showing those metabolites responsible for the clustering of *Phycomyces* strains.
lipoproteins and large, irregular vesicles (Zalokar, 1969). In agreement with this, the density gradient centrifugation of the Phycomyces carS mutant mycelia showed that most of the \( \beta \)-carotene is found in protein-coated oil drops (Riley & Bramley, 1976, 1982). Non-polar carotenoids are located in the hydrophobic phase of the lipid bilayer of the membrane. The conjugated double bonds of the polyene chain play a key role in the stabilization of the lipid membrane and the proteins (Gruszecki & Strząłka, 2005). The glycerolipids represent an abundant source of lipid material in Phycomyces, 80 and 60% in the sporangiophore and the mycelia, respectively (DeBell & Jack, 1975).

Fatty acids and membrane precursors were altered among the different carotene mutants, but there was no consistent trend linking the qualitative and quantitative levels of carotene to lipid/fatty acid content.

Total carotene content was increased in the carB, carR and carS mutants while the sterol intermediate (RT2.85min) and ubiquinone were both decreased, which suggests that a dynamic precursor/product mechanism of regulation exists within the terpenoid pathway of Phycomyces. This conclusion is supported by the concurrent increased levels of the intermediary metabolites valine and leucine, which in Phycomyces act as intermediates in carotene formation (Chichester et al., 1959; Goodwin & Lijinsky, 1951). In the carA mutant, an increase in the precursors valine and leucine was detected but there was no variation in components of the sterol or ubiquinone pathways. In contrast, the carB mutants contained reduced or unchanged levels of intermediary precursors responsible for carotene formation, reflecting increased total carotene levels.

Regulation of carotene production is a complex process that has been studied extensively. The carS gene was first postulated to be a regulatory gene involved in the \( \beta \)-carotene biosynthetic pathway (Murillo & Cerdá-Olmedo, 1976). This hypothesis was dismissed when the carS gene was found to be located in the Phycomyces genome (Tagua et al., 2012) and to encode an oxygenase that cleaves \( \beta \)-carotene to produce apocarotenoids (Medina et al., 2011), which are

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**Fig. 3.** Pathway map visualization of metabolic changes in carA, carB, carR and carS mutant strains (p<0.05) using wild-type strain as reference. In pale orange background are those metabolites that showed changes. The pathway diagram was created using the in-house software BioSynlab and Inkscape.
ACKNOWLEDGEMENTS

The authors are grateful to Dr Laura Perez (Royal Holloway University of London) for expert advice and encouragement and to Dr Luis M Corrochano and Eva M Luque (Dept. of Genetics, University of Seville) for their help in providing the strains. This work was funded in part through the ERA-IB PROCAR project, BBSRC/BB/M025829/1.

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