Letters

Retention, erosion, and loss of the carotenoid biosynthetic pathway in the nonphotosynthetic green algal genus *Polytomella*

Introduction

The evolutionary loss of carotenoid biosynthesis is often tied to the loss of photosynthesis, which is not surprising. In plants and algae, carotenoids are primarily associated with photosynthetic processes, from light absorption to photosystem assembly to protection from photodamage (Lohr, 2009; Cazzonelli, 2011; Santabarbara *et al.*, 2013), and they are also key constituents of algal eyespots – specialized, typically plastid-located optical devices that detect light and direct phototaxis (Kreimer, 2009; Ladygin & Semenova, 2014). In fact, a nonphotosynthetic mutant of the model green alga *Chlamydomonas reinhardtii* lacking phytoene synthase – one of the first enzymes in carotenoid biosynthesis (Fig. 1a) – bears a remarkable resemblance to naturally occurring colourless algae, exhibiting starch accumulation, a disorganized eyespot, and no pyrenoid (Inwood *et al.*, 2008). This observation has led some to suggest that mutations to the carotenoid pathway could be responsible for the evolution of nonphotosynthetic algae (Inwood *et al.*, 2008), many of which are missing the genes for carotenoid production (Borza *et al.*, 2005; Pombert *et al.*, 2014; Figueroa-Martinez *et al.*, 2015), with some notable exceptions (Tonhosolo *et al.*, 2009).

One algal system that could prove particularly useful for investigating the relationship between carotenoid biosynthesis and a heterotrophic existence is *Polytomella*: a monophyletic genus of nonphotosynthetic, free-living unicells closely related to *C. reinhardtii* (Pringsheim, 1955; Smith *et al.*, 2013), and not to be confused with the polyphyletic genus *Polytoma*, which lost photosynthesis independently of *Polytomella* (Figueroa-Martinez *et al.*, 2015). There are currently four well-described *Polytomella* lineages (Fig. 1b), represented by *P. magna*, *P. capuana*, *P. piriformis*, and *P. parva* (Göttingen Culture Collection of Algae, SAG, strains 63-9, 63-5, 63-10 and 63-3, respectively) (Smith *et al.*, 2013). The three latter species have no discernable eyespot and are white in colour, whereas *P. magna*, the deepest-branching of the four species, has an eyespot and is pinkish (Pringsheim, 1955; Smith *et al.*, 2013; MacDonald & Lee, 2015).

Recently, our laboratory group in collaboration with Robert W. Lee of Dalhousie University (Halifax, NS) generated large amounts of Illumina RNA and DNA sequencing data from all four *Polytomella* lineages, which can be found in GenBank's Sequence Read Archive (accession numbers SRX363995, SRX377397, SRX377560, SRX551283, and SRX710730–SRX710732). These data have already been used to explore certain plastid-located pathways, revealing that *Polytomella* spp. likely do not have a plastid genome (Smith & Lee, 2014), and to validate the species name *P. piriformis* (MacDonald & Lee, 2015). In our ongoing work towards developing a complete and polished *Polytomella* genome and transcriptome, we discovered an interesting feature regarding the carotenoid biosynthetic pathway, which to the best of our knowledge has heretofore not been observed in any other group of nonphotosynthetic or photosynthetic algae.

Presence and progressive loss of the carotenoid pathway in *Polytomella*

The genetic architecture of the carotenoid pathway in *C. reinhardtii* is well characterized (Lohr *et al.*, 2005; Lohr, 2009) and involves a series of enzymatic steps, all of which are encoded by the nuclear genome but occur within the plastid, downstream of the nonmevalonate methylerythritol phosphate (MEP) pathway of isoprenoid biosynthesis (Fig. 1a). In an effort to identify these same pathways in *Polytomella*, we searched (using BLAST) the nucleotide and deduced amino acid sequences of the *C. reinhardtii* MEP and carotenoid genes against the draft transcriptome and genome assemblies of *P. magna*, *P. capuana*, *P. piriformis*, and *P. parva* (Fig. 1c; Supporting Information Methods S1; Table S1). Full-length transcripts and genes for all of the MEP enzymes were easily identified in each of the four *Polytomella* spp. (Fig. 1c; Table S1), indicating that the MEP pathway is intact and functional. The presence and absence of enzymatic genes for carotenoid biosynthesis, however, varied across the genus.

For *P. magna*, we recovered complete transcript and gene sequences for all of the enzymes in the carotenoid pathway up to and including lycopene β-cyclase (LCYB), which synthesizes β-carotene through the cyclization of lycopene (Fig. 1a,c). This finding is consistent with *P. magna* having an eyespot – an organelle that is known to be rich in carotenoids (Ladygin & Semenova, 2014). Entire *P. magna* transcripts and genes were also collected for carotene β-ketolase (BKT) and carotene β-hydroxylase (CHYB), which are involved in canthaxanthin and zeaxanthin synthesis, respectively. The nucleotide sequences for the remaining carotenoid enzymes, however, were not found in either the transcriptome or genome of *P. magna*, implying that its carotenoid pathway ends shortly downstream of LCYB (Fig. 1c).

In contrast to *P. magna*, the entire carotenoid pathway appears to be missing from both *P. parva* and *P. piriformis*, which are the two most derived and closely related of the four

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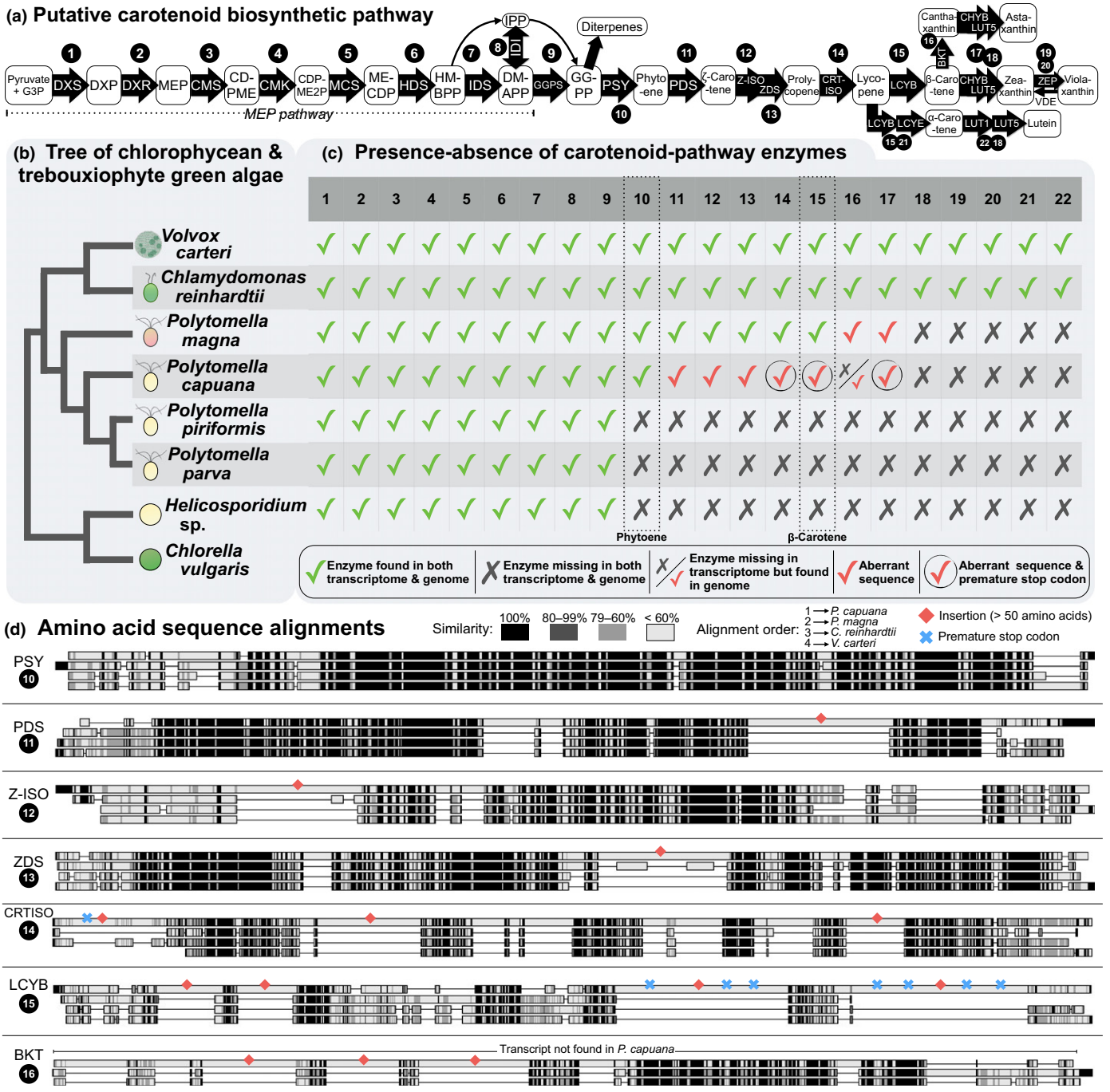

Polytomella spp. explored here (Fig. 1b). Neither transcripts nor genes for any of the carotenoid enzymes could be identified from *P. parva* or *P. piriformis* (Fig. 1c), which is in line with their lack of both pigmentation and eyespot structures (Pringsheim, 1955). Pseudogenes showing resemblance to carotenoid enzymes were also undetectable in these two taxa.

The most unexpected observations came from *P. capuana*: the genetic architecture of its carotenoid pathway is intermediate to those of *P. magna* and *P. parval P. piriformis*. Like with *P. magna*, we uncovered *P. capuana* transcript and gene sequences for each of the carotenoid enzymes leading to and including LCYB and CHYB (Fig. 1c; Table S1). Unlike *P. magna*, however, the nucleotide and putative amino acid sequences of the *P. capuana* transcripts and genes were abnormal, containing large insertions and showing very poor sequence identity relative to the carotenoid transcripts/genes of *P. magna*, *C. reinhardtii* and *Volvox carteri*, and in three cases they had premature stop codons (Fig. 1c,d). Moreover, the enzyme BKT, which was present in the transcriptome of *P. magna*, was missing from that of *P. capuana*, but a likely BKT pseudogene was discovered in the *P. capuana* genome (Table S1). Together, these data suggest that the *P. capuana* carotenoid pathway is in a state of decay.

Fig. 1 Retention and loss of the carotenoid biosynthetic pathway in photosynthetic and nonphotosynthetic green algae. (a) The putative carotenoid biosynthetic pathway for *Chlamydomonas reinhardtii* and other green algae based on Lohr *et al.* (2005) and Lohr (2009). All of the enzymes are encoded in the nuclear genome and are located in disparate chromosomal regions. Enzymes are numbered and placed within black arrows; products are boxed in white. Enzymes: DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; CMK, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase; MCS, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; IDS, isopentenyl-/dimethylallyl diphosphate synthase; IDI, isopentenyl-diphosphate delta-isomerase; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, zeta-carotene isomerase; ZDS, zeta-carotene desaturase; CRTISO, carotenoid isomerase; LCYB, lycopene β-cyclase; BKT, carotene β-ketolase; CHYB, carotene β-hydroxylase; LUT5, carotenoid β-hydroxylase cytochrome P450 type; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; LCYE, lycopene ε-cyclase; LUT1, carotene ε-hydroxylase cytochrome P450 type. Products: DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-*C*-methyl-D-erythritol-4-phosphate; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate; MECDP, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate; HMBPP, 1-hydroxyl-2-methyl-2-(E)-butenyl-4-diphosphate; DMAPP, dimethylallyl-diphosphate; IPP, isopentenyl-diphosphate; GGPP, geranylgeranyl-diphosphate. (b) Tree of green algae based on phylogenic analyses of Smith *et al.* (2013) and Figueroa-Martinez *et al.* (2015). Nonphotosynthetic lineages include all four *Polytomella* spp. and *Helicosporidium* sp. (c) The presence or absence of genes encoding carotenoid biosynthetic enzymes in the transcriptomes and genomes of various photosynthetic and nonphotosynthetic green algae. Enzymes are numbered based on pathway shown in (a). A single green checkmark signifies that a complete transcript and gene was identified in the transcriptome and genome, respectively. Red arrows denote the identification of aberrant sequences within the transcriptome and/or genome, including those with large insertions (> 150 nt), poor sequence identity, and premature stop codons (the latter is shown with a circle). Sources for transcriptomic and genomic data as well as search methods are listed in Supporting Information Table S1. (d) Amino acid sequence alignments of carotenoid biosynthetic enzymes (numbering and abbreviations as shown earlier). Alignments were generated with MUSCLE (Edgar, 2004) implemented through GENEIOUS v8.1.4 (Biomatters, Auckland, New Zealand) using default settings and eight iterations. Similarity shading generated with GENEIOUS using the BLOSUM62 score matrix and a threshold value of one.

Polytomella capuana genes for carotenoid biosynthesis: another one bites the dust

Close inspection of the *P. capuana* genomic and transcriptomic sequence data indicate that the genes encoding carotenoid enzymes are under relaxed selective constraints and are accumulating deleterious mutations (Fig. 1d; Table S1). Indeed, three of the seven recovered transcripts contained premature stop codons (Fig. 1c) and six contained one or more large (> 50 amino acid) insertions; these same features were also observed in the corresponding *P. capuana* genomic sequences. One of the most extreme examples of genetic degeneration within the *P. capuana* carotenoid pathway is the transcript representing LCYB: not only does it have an internal stop codon, but its putative coding sequence – again, because of a series of large insertions – is potentially > 1000 nt longer than the LCYB transcripts from *P. magna*, *C. reinhardtii* and *V. carteri* (Fig. 1d; Table S1). Likewise, the *P. capuana* CHYB transcript contained so many insertions that it was not possible to accurately align it to those from other chlamydomonadalean algae (Table S1). Signs of relaxed selection were also observed in the CHYB and BKT genes of *P. magna*, both of which catalyse reactions at the very end of its carotenoid pathway (Fig. 1c,d). Similar to the carotenoid genes from *P. capuana*, the *P. magna* BKT coding sequence had three large (> 150 nt) insertions and was > 600 nt longer than the BKT transcripts of *C. reinhardtii* and *V. carteri* (Fig. 1d; Table S1).

The aberrant carotenoid gene sequences in *Polytomella capuana* and *P. magna*, and the complete loss of these genes from *P. parva* and *P. piriformis* signify that the carotenoid pathway in *Polytomella* is at various stages of degradation and loss, which is presumably linked to the presence or absence of an eyespot among its members. Furthermore, this degradation follows a phylogenetic pattern whereby the pathway is present in the deepest branching lineage (*P. magna*), lost in the most derived one (*P. parva*/*P. piriformis*), and is in an intermediate stage of loss in *P. capuana*, which branches between the former two lineages (Fig. 1b). This pattern could make *Polytomella* an attractive group for studying the evolution, function, and loss of carotenoid biosynthesis in algae.

Polytomella: a model system for studying the retention and loss of carotenoids

Research into carotenoids has major implication for medicine, industry, and evolution (Cazzonelli, 2011; Shumskaya & Wurtzel, 2013). Carotenoids are crucial for human health, providing precursors for vitamin A biosynthesis, but they need to be acquired through diet, which has led to the genetic engineering of β-carotene-rich crops, such as 'golden rice' (Ye *et al.*, 2000). Carotenoids are also manufactured on an industrial scale for use in nutritional supplements, medicines, and cosmetics (Cazzonelli, 2011). Among the best-studied organisms for synthesizing, harvesting, and genetically modifying carotenoids are chlamydomonadalean algae (e.g. Cordero *et al.*, 2011), including *Haematococcus pluvialis* and *Dunaliella salina* (Fassett & Coombes, 2011), which are close relatives of *Polytomella* (Figueroa-Martinez *et al.*, 2015). Consequently, *Polytomella* is well situated within the tree of green algae for comparative studies on carotenoid biosynthesis with model photosynthetic species. The next obvious step is a detailed biochemical characterization of the pigments within *Polytomella* taxa. Whether or not *Polytomella* spp. could be suitable for harvesting carotenoids largely depends on the amount and types of carotenoids they can produce, and if they are confined to an eyespot or exist in additional storage compartments.

Although there has yet to be a transformation system developed for *Polytomella* spp., it is conceivable that they could serve as a biological factory for carotenoid production. *Polytomella* spp. are fast growing (*c.* 6 h doubling time) and can be cultivated at room temperature in a simple, well-defined medium. They also constitute a potentially cost-effective industrial system since, in contrast to photosynthetic algae, their growing conditions do not require a photobioreactor, and the absence of a cell wall makes cell disruption easy.

More broadly, *Polytomella* spp. could yield insights into why the carotenoid pathway (as well as an eyespot) is maintained in some heterotrophic algae and lost in others. Although the presence of a carotenoid biosynthetic pathway is considered quite rare among well-studied nonphotosynthetic species, new data from diverse lineages are showing that it is not as rare as once thought. For instance, the nonphotosynthetic apicomplexan parasite *Plasmodium falciparum* has a red-algal-derived plastid (but no eyespot) and can synthesize carotenoids, which appear to have an important metabolic role (Tonhosolo *et al.*, 2009); and the same might be true for other apicomplexan parasites, such as *Toxoplasma gondii* (Nagamune *et al.*, 2008). *Polytomella*, however, appears to be the first example of a nonphotosynthetic genus in which some members have retained the carotenoid pathway whereas others have lost it. If anything, these data support the idea that in nature the evolutionary loss of photosynthesis (at least with respect to *Polytomella* spp.) can lead to the loss of carotenoid biosynthesis, rather than the other way around, which some have hypothesized (Inwood *et al.*, 2008); that said, the data do not exclude the alternative scenario from happening in other groups. It will be interesting to see if other aspects of *Polytomella* nuclear genomic architecture follow a similar pattern to those observed here for the carotenoid pathway. An earlier study on *Polytomella* mitochondrial genomes showed that palindromic genes were lost or retained to various degrees in the four known lineages highlighted here (Smith *et al.*, 2013). If we have learned anything about *Polytomella* genomic architecture over the past decade, it is that anything can go.

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Author contributions

S.R.A. and D.R.S. designed the research, performed the experiments, analysed the data, and wrote the manuscript.

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Supporting Information

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

Table S1 Carotenoid pathway transcript sequences and their sources for *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Polytomella* spp.

Methods S1 Mining carotenoid genes from *Polytomella* transcriptomes and genomes.

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