Algal Research 38 (2019) 101395

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Nitrogen scavenging from amino acids and peptides in the model alga *Chlamydomonas reinhardtii*. The role of extracellular L-amino oxidase

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ARTICLE INFO

Keywords:
Algae
Chlamydomonas
Extracellular L-amino acid oxidase
LAAO
LAO1
Amino acids
Peptides
Nitrogen mineralization

ABSTRACT

Phytoplankton live under constantly changing environments in which concentrations of inorganic nitrogen can be limiting but organic nitrogen sources (urea, amino acids or peptides) are available. Understanding how algae, as primary producers, assimilate organic nitrogen is of eco-physiological importance although such studies are rare. Given genetic tractability, the green microalga *Chlamydomonas reinhardtii* is an excellent model system for elucidating amino acid assimilation by algal species. This alga can extracellularly deaminate most amino acids using a L-Amino acid Oxidase (LAO1), which generates ammonium that can then be taken up as a source of nitrogen. In this work, we have used *lao1* mutant strains to investigate the impact of the absence of this enzyme on *C. reinhardtii* growth using amino acids and di-/tri-peptides as sole nitrogen sources. Our results show that LAO1 enzyme is crucial for growth on most proteinogenic amino acids and peptides by this alga. We present findings from an analysis of algal genomes that reveal a new evolutionary branch for algal L-amino acid oxidase genes (*ALAAO*) that includes Rhodophyta, Alveolata, Heterokonta, Haptophyta, and Dinophyta species. Interestingly, *C. reinhardtii* appears to be the only green algal that contains an ALAAO homolog, and we present a hypothesis about the possible origins of *ALAAO* genes in algae based on a comparative analysis of currently available and assembled algal genomes.

1. Introduction

Inorganic nitrogen is often the most abundant form of nitrogen in marine and freshwater systems, with concentrations that can regularly change spatially or temporarily reach low levels, while organic nitrogen sources like urea and free amino acids are often at higher concentrations (reviewed by [1]). In many aquatic systems, terrestrial leaking and runoff are important inputs of organic nitrogen. Sewage and other anthropogenic activities lead to significant infusions of organic nitrogen into rivers that are eventually transported to estuarine and coastal waters. These organic inputs into water systems have dramatically increased over the last century due to industrial growth and global warming, and have great impact on the biodiversity of associated ecosystems [1–4]. Although bacteria have long been considered to be the dominant consumers of organic nitrogen, many phytoplankton species have been shown to also use organic nitrogen as evidenced during algal blooms [1,3,5,6]. Thus, primary producers may compete with other heterotrophic organisms like bacteria for organic nitrogen, which should be factored into models of ecosystem balance.

The green microalga *Chlamydomonas reinhardtii* has served as a model organism for physiological studies for more than fifty years [7] and nitrogen metabolism in this alga has been one of the most extensively studied of any alga [8]. This alga preferentially assimilates inorganic nitrogen over other sources [9]. Under inorganic nitrogen deficiency, *C. reinhardtii* can grow on organic nitrogen sources such as urea, purines and amino acids. L-arginine is the only reported amino acid that is imported by a high affinity transporter in *C. reinhardtii* [10]. Once intracellular, L-arginine is deaminated by an arginine deiminase (ADI), producing ammonium is incorporated into carbon skeletons by the GS-GOGAT cycle [11]. *C. reinhardtii* also bears a periplasmic L-amino acid oxidase encoded by the gene *LAO1* that deaminates a wide range of L-amino acids according to the reaction:

$$\text{L-amino acid} + H_2O + O_2 \rightarrow \text{keto acid} + NH_4^+ + H_2O_2$$

The resulting ammonium is then imported into the cell by ammonium transporters (AMTs); the α-keto acids are not further metabolized and remain extracellular [12]. The LAO1 protein is highly expressed during nitrogen starvation but is repressed in the presence of

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https://doi.org/10.1016/j.algal.2018.101395
Received 9 October 2018; Received in revised form 19 December 2018; Accepted 20 December 2018
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ammonium [13]. An intracellular LAO1 homolog exists and is encoded by the gene *LAO3* [14].

L-Amino Acid Oxidases (LAAOs, EC 1.4.3.2.) are widely distributed in nature and are present in diverse organisms including bacteria [15], fungi [16,17], algae [13,18–20], fishes [21], the sea hare [22], mammals [23,24], and in snake venom [25,26]. LAAOs catalyze a similar reaction in different organisms with similar structures that may derive from a common evolutionary origin but evolved to cope with different physiological functions in distinct groups [27,28]. While the major role of some LAAO enzymes has been suggested to be defence against pathogens by H_2O_2 generation [29], it has also been shown that fungal and algal LAAOs help to scavenge nitrogen from amino acids [30,31]. Consistent with this function in algae and fungi, LAAO enzymes show broad substrate specificity and are induced under nitrogen-limiting conditions [13,32,33]. LAAOs may provide an ecological advantage in instances where inorganic nitrogen is limiting, and organic nitrogen is otherwise available. However, information about how algae use amino acids as a nitrogen source is in general poorly understood.

In this work we used *C. reinhardtii lao1* mutants to study the impact of extracellular LAAO on nitrogen scavenging from amino acids and peptides. We also analyzed LAAO genes from available sequenced algal genomes to gain insights into the evolutionary origins of L-amino acid oxidase-based mineralization of amino acids/peptides. We discuss three independent pathways for nitrogen acquisition from amino acids/peptides in algae: (1) amino acid transport, (2) extracellular amine oxidation, and (3) bacterial metabolic complementation.

2. Materials and methods

2.1. Strains

The *C. reinhardtii lao1* mutant LMJ.RY0402.044073 and its parental strain CC-5325 were obtained from the *Chlamydomonas Resource Center* (https://www.chlamycollection.org/). This *lao1* mutant contains a putative CIB gene cassette insertion at the *Cre12.g551352* locus, corresponding to the *LAO1* gene [34]. This cassette contained a paromomycin resistance marker gene. We confirmed this mutant genotype via colony PCR using primers that flank the regions of insertion (LAO1F: *5′-TCG AAG CAA GTA CAC GCA AC-3′* and LAO1R *5′-AAC TGT GGG AGT GTG GGA AG-3′*), and in combination with primers matching the inserted cassette [34] (CIB 5′ (*5′-GCA CCA ATC ATG TCA AGC CT-3′*)-LAO1F and CIB 3′ (*5′-GAC GTT ACA GCA CAC CCT TG-3′*)-LAO1R). Amplicons with expected sizes were sequenced (*SCA*I Sequencing Facility, University of Córdoba) to confirm the insertion of the cassette in the *LAO1 gene* (Phytozome ID (v4 genome): *Cre12.g551300.t1.1*). The corroborated flanking region sequences were: 5′ flanking region: 5′-*CAC GAA GCC TGT GTG GGT GGT GCG TGC GTG* -3′; and 3′ flanking region: 5′-*GTG ACG TTC GTG GAG GAG CTG ACA AAG CTG*-3′). In referencing the experimentally verified cDNA sequence for *LAO1* (NCBI accession *U78797.1*) [35], we verified that the current *C. reinhardtii* genome annotation (Phytozome v5.5; https://phytozome.jgi.doe.gov/) incorrectly splits *LAO1* into two gene models, *Cre12.g551352.t1.1* and *Cre12.g551253t.1.1*.

The genetic cross between wild-type CC-1690 and *lao1* mutant was performed by the random spore plating method [36]. One hundred randomly selected colonies were plated on TAP agar (1.6%) plates containing either ammonium (8 mM) or ammonium and paromomycin (25 $\mu g \cdot mL^{-1}$) as sole nitrogen source for segregant analysis.

2.2. Growth experiments on L-amino acids and peptides

C. reinhardtii pre-cultures were grown on Tris-Acetate-Phosphate (TAP) medium as previously described [37], supplemented with 8 mM of ammonium chloride (TA medium) for 2–3 days (mid-log growth phase) at 23 °C under continuous light with agitation. Cells were harvested by centrifugation (3 min at 3000*g*) and washed three times with TAP medium without nitrogen. Cells were transferred to TAP medium containing the indicated L-amino acid (8 mM) or peptide (4 mM) as the sole nitrogen source, with the exception of L-tyrosine, which was used at 2 mM due to poor solubility. The initial cell concentration was A_{750} of 0.025 for liquid culture tests. For growth tests on solid media, 5 µL of each cultures with A_{750} of 0.125 were plated. Final cell concentrations of liquid cultures were determined using a cell counting (Sysmex Microcellcounter F-500).

2.3. Gene expression quantification

C. reinhardtii CC-1690 cells were grown in liquid TA medium for 3 days with continuous light and agitation. Cultures were harvested by centrifugation (2 min at 3000 rpm) and washed twice with MM without nitrogen (M-N). Cells were transferred to fresh M-N media supplemented with 4 mM of ammonium chloride, potassium nitrite or potassium nitrate, to study the effect of different nitrogen sources. After 10 h, cells were harvested by centrifugation (2 min at 2000 *g*), resuspended in lysis buffer (50 mM Tris·HCl pH 8, 300 mM NaCl, 5 mM EDTA pH 8 and 2% SDS) and frozen at −80 °C. Samples were thawed and RNA extracted using a standard phenol-chloroform extraction protocol [38]. Total RNA was precipitated overnight with 4 mM LiCl, quantified spectrophotometrically, and run on a gel to check RNA integrity. One ng of total RNA was used to synthesize cDNA using iScript Reverse Transcriptase (Bio-Rad, Madrid, Spain). The resulting cDNA was used to quantify the relative *CreLAO1* expression by qPCR using SsoFast EvaGreen Supermix (Bio-Rad, Madrid, Spain) and specific primers for *CreLAO1* gene expression (LAO1QC1: *5′-GAG ACT GTG ATG CCC AAA AAG TG-3′*; LAO1QR1: *5′-GCT TGC CCA GGC CGC GAA TGG AA-3′)* [85]. Simultaneously, we measured the gene expression of ubiquitin ligase, a housekeeping gene control for which expression was reported to be constitutive under the conditions used in this work [39]. The mixed reactions were run and detected using the MyiQ2 detection system (Bio-Rad, Spain) with the following conditions: initial denaturation for 2 min at 98 °C, 5 s of denaturation at 98 °C (40 cycles) and 10 s of annealing and extension at 60 °C. All qPCR runs contained two technical replicates of reactions with the same cDNA sample for each of the three biological replicates, along with no-DNA negative controls. The Ct values obtained were normalized to the housekeeping gene control and expressed as fold-changes ($2^{-\Delta Ct}$, where $\Delta Ct = Ct_{sample} - Ct_{control}$).

2.4. Phylogenetics analysis

Different genome resources were used to identify LAO1 orthologs: NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the JGI Genome Portal (https://genome.jgi.doe.gov/portal/) and Phytozome (https://phytozome.jgi.doe.gov/). Putative subcellular location was also analyzed for each ortholog using the PredAlgo program, tailored to algae [40].

Evolutionary trees of LAAOs and other enzymes with amine oxidase activity were constructed using the Maximum Likelihood method and default settings in MEGA7 [41]. Alignments were performed using the Clustal method, and evolutionary distances were computed using the Poisson correction model [42]. The bootstrap consensus tree was inferred from 500 replicates [43]. This analysis involved 60 amino acid sequences (Supplemental Table S1).

3. Results and discussion

3.1. The impact of LAO1 on the use of amino acids and peptides in Chlamydomonas reinhardtii

LAAOs have a role on L-amino acid catabolism. In fact, in natural waters where algae are present, LAAOs are proposed to alter amino acid distributions [44]. The *C. reinhardtii* genome contains two *LAAO* genes:

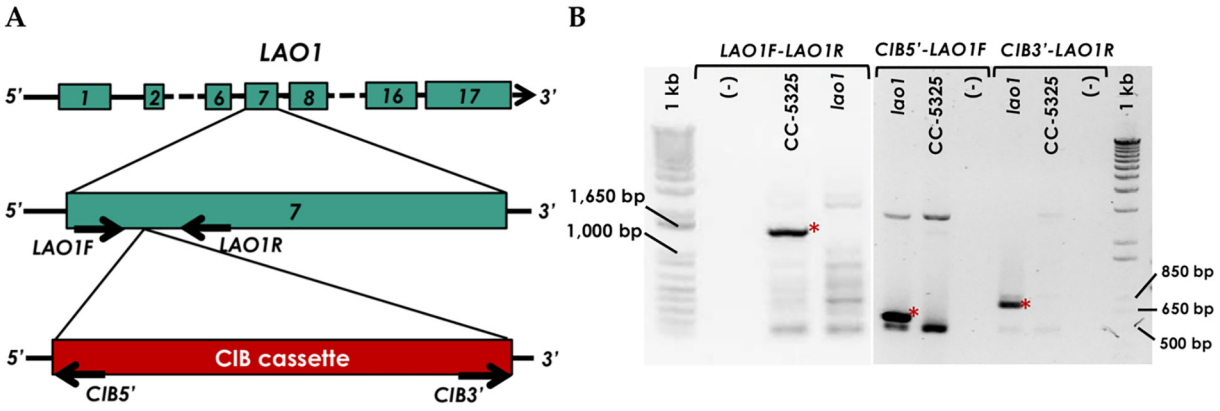

Fig. 1. A, Schematic view of *LAO1* gene with the insertion site of the CIB cassette in *lao1* mutant. Primers used for characterization by amplification are indicated by black arrows. **B,** *lao1* mutant characterization by PCR. Primer sequences and PCR specifications for amplification are indicated in *Materials and Methods* section. Expected fragments for parental strain (CC-5325) and *lao1* mutant (LMJ.RY0402.044073) are indicated by asterisks. (−) indicates negative controls without DNA template.

LAO1, located on chromosome 12 that encodes the periplasmic LAAO (NCBI protein ID: AAB97101.1); and *LAO3*, located on chromosome 5 that encodes a putative intracellular LAAO (NCBI protein ID: XP_001700756.1). The LAO1 enzyme has been more extensively characterized and shown to generate ammonium from L-amino acids, which *C. reinhardtii* uses for growth, while the corresponding α-keto acid by-product is not assimilated [12]. The purified LAO1 protein was shown to have activity over a broad range of proteinogenic amino acids [13]. The cDNA corresponding to the catalytic subunit was verified by cloning [35], and LAO1 gene expression is a hallmark cell response to nitrogen deprivation [13,45].

Here we analyzed nitrogen scavenging from amino acid and peptides by *C. reinhardtii* strains containing or lacking a functional *LAO1* gene. The *C. reinhardtii lao1* insertional mutant (strain *LMJ.RY0402.044073*) was first verified for the integration of the paromomycin resistant cassette in *LAO1* gene (Fig. 1). According to the cDNA sequence (*U78797.1*) [35] and the *LAO1* transcript model (Phytozome ID (v4 genome), *Cre12.g551300.t1.1*) the cassette is integrated in the exon seven of LAO1.

To further confirm that the paromomycin resistant phenotype was due to a unique integration event in *LAO1*, a genetic cross between the *lao1* mutant (mt-) and a wild-type strain CC-1690 (Sager 21 g, mt+) was performed. Of the segregants, 51% were resistant to paromomycin, suggesting a single integration event. Six paromomycin-resistant and six paromomycin-sensitive representative segregants were chosen to test growth on L-alanine or L-serine. As shown in Fig. 2, all paromomycin-resistant segregants and parental lao1 mutants showed impaired growth on these amino acids. In contrast, all the paromomycin-sensitive segregants and wild-type parental strains were able to use the L-amino acids for growth.

The ability of *C. reinhardtii* to use the 20 proteinogenic amino acids and several di- and tri-peptides as a sole nitrogen source was assessed (Fig. 3). We selected peptides that result in positive respiratory activity (LA, FA and LG_3) and one (G_3) that does not [46], with the idea that respiration is likely closely linked to the ability to use the peptide for growth. *C. reinhardtii* growth was analyzed at 4 and 12 days. At 12 days, cultures were evaluated for positive or negative growth (Fig. 3B, C). At 4 days (Fig. 3A), cell number was also quantified in order to relate the efficiency of growth with the LAO1 activity reported for each amino acid [13]. We observed three different phenotypes for growth: LAO1-independent, LAO1-dependent, and no growth.

LAO1-independent growth was evident for L-arginine (Fig. 1B). A specific transporter for L-arginine likely enables the intracellular processing of this amino acid [10]. Although the molecular identification of this arginine transporter has not yet been determined, the *C. reinhardtii* genome encodes six APC (Amino acid Polyamine organoCation) transporters and among them, AOC5 has been suggested as a candidate [14].

Strict LAO1-dependent growth was observed for 16 amino acids, 15 of which are known substrates for LAO1 (L-glutamine, L-alanine, L-phenylalanine, L-serine, L-asparagine, L-leucine, L-methionine, L-valine, L-lysine, L-tyrosine, L-histidine, L-isoleucine, L-threonine, L-tryptophan, and glycine). Consistent with the lower specific activity of LAO1 for some L-amino acids (L-histidine, L-aspartic acid, L-glutamic acid, L-threonine and glycine) [13], slower growth was also observed (12 days, Fig. 3A and B). L-cysteine showed a utilization defect in the *lao1* mutant, suggesting that LAO1 is involved in L-cysteine-dependent growth (Fig. 3A). L-cysteine is not deaminated by LAO1 but can be spontaneously oxidized in aqueous solution to form the dimer cystine [47], which is then efficiently deaminated by LAO1 [13].

The LAO1-dependent growth was also observed for some di- and tri-peptides. The use of peptides as nitrogen source by *C. reinhardtii* has not been extensively studied. Vallon et al. [13] reported that purified LAO1 enzyme is not active on di-peptides, although the di-peptides tested were not specified. A high-throughput screen based on cellular respiration of *C. reinhardtii* in response to a large array of metabolites has shown that *C. reinhardtii* is metabolically active on a number of di- and tri-peptides (108 out of 267 di-peptides, and 3 out of 14 tri-peptides) [46]. Whether these peptides are nitrogen sources for *C. reinhardtii* growth is unknown, however. We tested three respiration-positive (L-Leu-Ala, L-Phe-Ala and L-Leu-Gly-Gly) and one respiration-negative (Gly-Gly-Gly) peptides [46]. Peptides that showed a respiration-positive phenotype supported growth of the lao + *C. reinhardtii* parental strain CC-5325, while the respiration-negative Gly-Gly-Gly tri-peptide did not (Fig. 3C). The *lao1* mutant was unable to grow using these peptides (Fig. 3C). These data suggest that LAO1 has a role in peptide assimilation, either by deaminating the peptide directly or via deamination of free amino acids generated through the activity of some extracellular peptidase.

C. reinhardtii does not grow on L-proline, consistent with the absence of activity of the LAO1 enzyme on this amino acid. Recently, it has been shown that a specific mutualistic interaction *Chlamydomonas-Methylobacterium aquaticum* results in metabolic complementation and algal growth on proline [48]. This mutualism on L-proline is the consequence of the amino acid mineralization by *M. aquaticum* producing ammonium that is assimilated by *C. reinhardtii*. In return, *C. reinhardtii* produces glycerol as a result of photosynthetic carbon fixation, which is used by the bacteria. The interaction of *Chlamydomonas* with different *Methylobacterium* spp. also results in algal growth on peptides that *Chlamydomonas* cannot use by itself [48].

LAO1 seems to endow *C. reinhardtii* with the advantage of scavenging nitrogen from a broad range of L-amino acids and peptides. Given

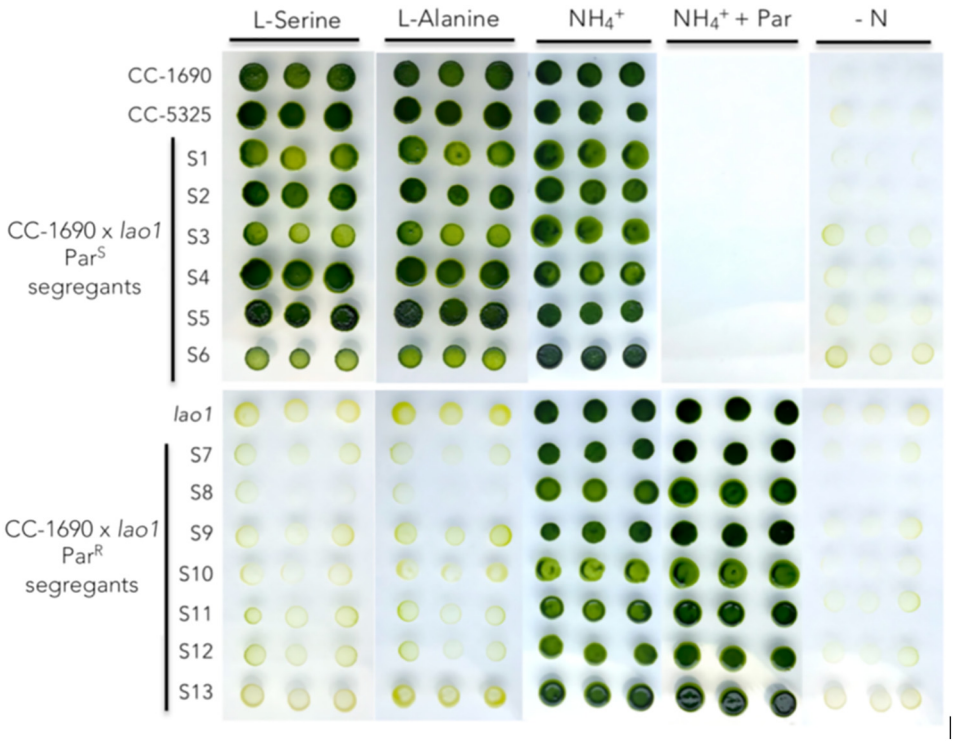

Fig. 2. Growth phenotypes of segregants from a genetic cross between the *C. reinhardtii lao1 LMJ.RY0402.044073* mutant and wild-type CC-1690 (Sager 21 gr) strains. Growth phenotypes on the amino acids L-serine and L-alanine (8 mM) were imaged after seven days. A control without any supplemented nitrogen source was included (- N). Growth on ammonium (8 mM NH_4Cl) was tested in the presence and absence of paromomycin (25 $\mu g \cdot mL^{-1}$) after four days. CC-5325 is the wild-type parental strain of the *lao1 LMJ.RY0402.044073* mutant. S1–13 are representative random segregants of the genetic cross between the lao1 mutant and wild-type CC-1690 strains. These results represent one of at least three different experiments.

the impact on algal growth and potential ecological relevance of these results, we investigated the occurrence of LAAO genes in other algal genomes.

3.2. LAO1 gene expression is down-regulated by the presence of inorganic nitrogen

In *C. reinhardtii,* the uptake activity of L-arginine is inhibited by ammonium and nitrate but induced by the absence of nitrogen [10,49].

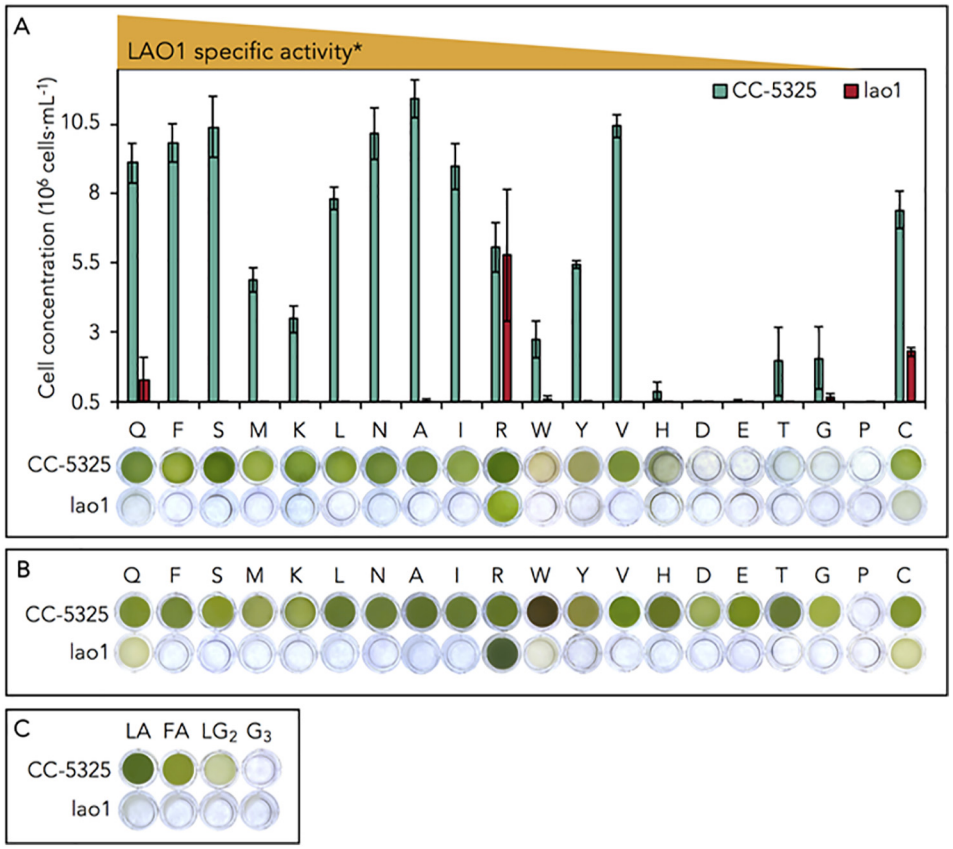

Fig. 3. *C. reinhardtii* growth on amino acids and peptides as sole nitrogen source. (**A**) Short-term growth on L-amino acids (4 days). (*) From left to right, amino acids are arranged from higher to lower LAO1-specific activity, previously reported [13]. Long-term growth (12 days) on L-amino acids (**B**) and di−/tri-peptides (**C**). Parental lao1 + strain CC-5325 and the derived *lao1* mutant (*LMJ.RY0402.044073*) were grown on each amino acids or peptides as the sole nitrogen source (8 mM, except for L-Tyr: 2 mM) in TAP liquid media. Q: L-glutamine; F: L-phenylalanine; S: L-serine; M: L-methionine; K: L-lysine; L: L-leucine; N: L-asparagine; A: L-alanine; I: L-isoleucine; R: L-arginine; W: L-tryptophan; Y: L-tyrosine; V: L-valine; H: L-histidine; D: L-aspartic acid; E: L-glutamic acid; T: L-threonine; G: glycine; P: L-proline; C: L-cysteine; LA: L-leucyl-alanine; FA: L-phenylalanyl-alanine; LG_2: L-leucyl-glycil-glycine; G_3: glycil-glycil-glycine. Error bars represent standard deviation ($n = 3$).

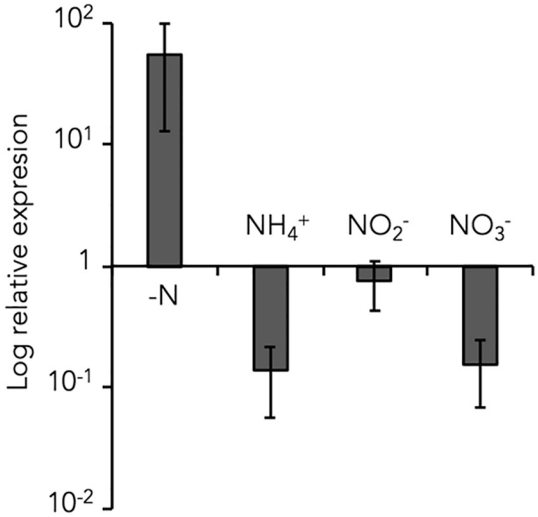

Fig. 4. *LAO1* gene expression in the presence of replete extracellular inorganic nitrogen. *C. reinhardtii* was cultured in ammonium-containing media, harvested, rinsed free of ammonium and transferred to medium supplemented with 4 mM of the inorganic nitrogen sources indicated and cultivated for 10 h. NH_4^+: as ammonium chloride; NO_2^-: as potassium nitrite; NO_3^-: as potassium nitrate; -N: without any nitrogen. Gene expression is shown relative to that of a housekeeping gene, ubiquitin ligase [39]. Error bars represent standard deviation ($n = 3$).

Likewise, LAO1 activity is inhibited by ammonium and induced by nitrogen starvation [13]. We examined the transcriptional activity of *the LAO1* gene after 10 h of incubation with inorganic nitrogen sources ammonium, nitrite, or and nitrate (Fig. 4). The presence of any of these nitrogen compounds repressed *LAO1* gene expression *C. reinhardtii* seems to prioritize the use of inorganic nitrogen sources over amino acids, the latter likely to be more energetically costly to assimilate.

3.3. LAAO genes occurrence in algal genomes

Two genes in the *C. reinhardtii* genome, *LAO1* and *LAO3*, encode for proteins that share 59% sequence identity. Other sequenced genomes of closely related green algae such as *Volvox* and *Ostreococcus* do not contain *LAAO* genes [14]. We queried several genome databases (see *Material and Methods* section) with the LAO1 protein sequence via reciprocal BLASTp to identify potential orthologs using the following criteria: (a) translated protein size of 400–600 amino acid residues; (b) sequence identity greater than 20%; and (c) sequence coverage greater than 60%. The resulting protein hits where subsequently used in tBLASTn queries against *C. reinhardtii* genome to identify reciprocal best-hits to confirm LAO1 orthology. No LAO1 orthologs satisfying these criteria were found in other Viridiplantae members, including other closely related green algae like *Dunaliella* and *Coccomyxa*, and land plants. LAO1 orthologs are also absent among five other currently available genomes in the *Chlamydomonas* genus (*C. eustigma, C. sphaeroides, C. debaryana, C. asymmetrica* and *C. applanata*) [50].

We identified LAO1 orthologs in 10 out of 27 algal species: *Porphyra umbilicalis, Gracilariopsis lemaneiformis* and *Chondrus crispus* (Rhodophyta), *Vitrella brassicaformis* (Alveolata), *Aureococcus anophagefferens* and *Pelagophyceae* sp. CCMP2097 (Heterokonta), *Pavlovales* sp. CCMP2436, *Emiliana huxleyi* and *Chrysochromulina* sp. CCMP291 (Haptophyta), and *Symbiodinium microadriaticum* (Dinophyta). These algae appear to have a single LAO1 ortholog, except for the diatom *Emiliana huxleyi* that seems to have two and the red alga *Porphyra umbilicalis* that has four.

To understand the evolutionary history of these algal LAO1 orthologs, we constructed a phylogenetic tree along with other LAAO proteins previously analyzed by Campillo-Brocal et al. [27] (Table S1). Fungal, gastropod and vertebrate LAAO members, as well as other distantly related amine oxidases were included: D-amino acid oxidases (DAAOs), LodA (L-Lysine ε-oxidase)-like proteins, L-aspartate oxidases (LASPOs) and other enzymes with experimentally reported amine oxidase activity [27]. Algal protein sequences identified as LAO1 orthologs cluster on the same branch that we named as "ALAAO" (Algal LAAO, Fig. 5). This ALAAO branch is distinct in lineage from fungal, gastropods and vertebrates LAAOs. In addition, two bacterial LAAOs from *Oceanobacter kriegii* and *Aquabacterium* sp. NJ1 were included in the phylogenetic analysis since they were found to be LAO1 orthologs according to our criteria. These two LAAOs did not cluster on the same branch as ALAAOs, however, suggesting that ALAAOs may have evolved separately instead of having been acquired through a recent lateral gene transfer event (Fig. 5).

Within this new ALAAO branch, the sequence identity between LAO1 and other ALAAOs was low (from 23 to 31% of identity), although this is consistent with previously reported data in LAAO proteins within similar groups [17]. Nevertheless, conserved motifs in the substrate- and FAD- binding domains are present [51] (Supplementary Fig. S1).

Although our data support LAO1 and LAO3 being grouped together within the ALAAOs, their origin is puzzling since we could find no other orthologs within the green algal lineage. We analyzed the genomic context of these genes in hopes of shedding light onto the possible evolutionary history of *C. reinhardtii ALAAO* genes. Immediately adjacent to *LAO1* is the *LAO2* gene [13] that codes for a putative RidA (Reactive intermediate/imine deaminase A) protein [52]. When LAO2/RidA was queried, the most closely related gene was found in cyanobacteria and interestingly, resides adjacent to a putative amine oxidase (*AO*) gene, as previously reported for other bacteria [53]. However, this putative *AO* gene shows low sequence identity to *LAO1* but is a better match to other putative amine oxidases in *C. reinhardtii* (e.g., *AOF2, Cre10.g447767.t1.1*) (Fig. 6). In contrast to LAO1 and LAO3, AOF2 shows orthologs in most genomes within the green and red algal lineages, as well as in cyanobacteria (indicated as AOs in Fig. 7) and did not cluster in the same ALAAO branch in our Maximum Likelihood analysis (Fig. 5), suggesting that the AOF/AO family may have given rise to ALAAOs.

No LAO2/RidA ortholog was found in any other algal genome. This gene may have been lost in most algal lineages but uniquely retained in *C. reinhardtii*, or *C. reinhardtii* may have acquired it via lateral gene transfer. Given the currently available genomes and genome assemblies, our phylogenetic analysis favours the idea that ALAAOs may have a common origin in an archaeplastidan ancestor that was the result of a primary endosymbiosis of a protist engulfing a free cyanobacterium. We propose that the presence of *ALAAO* genes in the red algal lineage and the thus far unique co-occurrence of *ALAAO-RidA* genes in *C. reinhardtii* (*LAO1-LAO2/RidA*) may be due to gene duplication and divergence events in the ancestral archaeplastida, with further loss of *RidA* gene in the red lineage and loss of the *ALAAO-RidA* cluster in the green algae (except for *C. reinhardtii*) (Supplementary Fig. S2). Alternatively, an ALAAO-RidA cluster from a microbe related to the ancestral archaeplastida may have been laterally transferred to *C. reinhardtii*. Nevertheless, future genome sequences and reassemblies are crucial to reveal the yet uncertain origin of LAAOs in algae.

3.4. How do algae scavenge nitrogen from amino acids?

The use of amino acids by algae may be of eco-physiological importance, and the extracellular localization of ALAAOs such as LAO1 could be a strategy for scavenging from exogenous amino acids and peptides. To understand whether this could be a common feature for ALAAOs, we examined in silico predictions of cellular localization of these ALAAOs. Our analysis suggests that 5 out of 10 algal species contain putative extracellular LAAOs (Table 1), a pattern that indicates that ALAAOs could be a general strategy by algae to derive

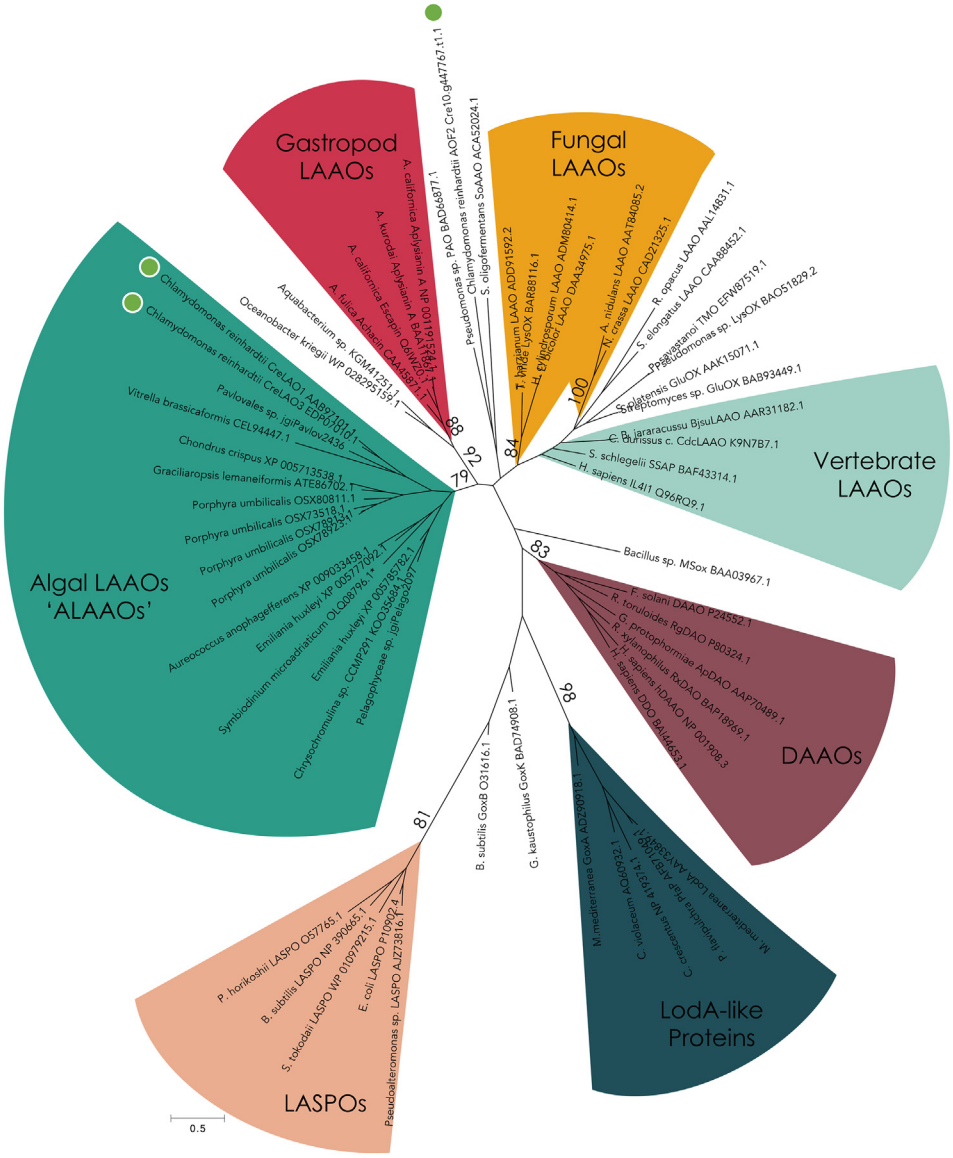

Fig. 5. Evolutionary relationships of LAAOs and other enzymes with amine oxidase activity. Tree was obtained using a maximum likelihood method with MEGA7 [41]. The alignment was performed using the Clustal method, and evolutionary distances were computed using the Poisson correction method [42]. Distances are in units of the amino acid substitutions per site. Numbers in branches show percent bootstrap values. Green circles correspond to *C. reinhardtii* protein sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

environmental nitrogen. Surveying the literature for reports of algal amino acid uptake, extracellular deamination activity and growth on amino acids (Table 2 and Supplementary Table S2), we synthesize different potential strategies for nitrogen scavenging below.

In the green alga *Dunaliella*, four species were recently reported to be unable to use proteinogenic amino acids as a sole nitrogen source, with the exception of L-histidine, which can be transported into the cell [56]. In *Volvox carteri*, only L-arginine is the only amino acid transported, although it is not catabolized and therefore not used as nitrogen source [67]. None of these algal genomes contains *ALAAO* [56]. This fact, together with the deficiency of transport systems could explain why these algae do not generally grow on most amino acids.

In *Chlorella*, growth on a broad range of amino acids and extensive amino acid uptake systems have been reported [57–59,68] (Table 2). Interestingly, amino acids have been shown to be supplied to this alga by symbiotic hosts [69,70]. Thus, amino acid uptake systems in algae may have been driven and/or retained by nutritional selective advantages provided in association with specific symbiotic partners.

Heterokonta algae show different abilities to assimilate nitrogen from amino acids and peptides. *Aureococcus anophagefferens* possesses similar abilities as *C. reinhardtii* to grow on amino acids, and show amino acids uptake as well as extracellular deaminase activities [64], consistent with a putative extracellular ALAAO encoded in its genome (Tables 1, 2). *Phaeodactylum tricornutum* grows on a wide range of amino acids, shows amino acid uptake activity for L-arginine and L-lysine, and exhibits extracellular deamination activity for several amino acids [19,20,63]; however, we did not find any LAO1 ortholog in the current version of the *P. tricornutum* genome. Whether the reported extracellular deaminase activity is due to another amine oxidase enzyme remains to be determined. *Thalassiosira pseudonana* is unable to grow on amino acids as sole nitrogen source [65,71] and lacks an *ALAAO* gene. Perhaps the inability of *T. pseudonana* (and *Dunaliella*) to grow amino acids can be complemented by interactions with bacteria that can mineralize these nitrogen sources, similar to what we recently reported for *C. reinhardtii* and *Methylobacterium* spp. [48].

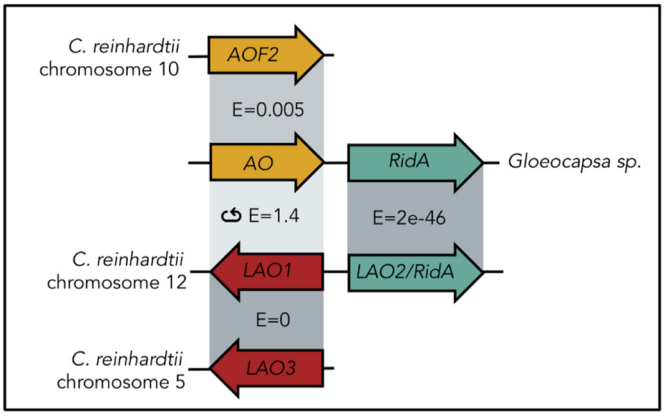

Fig. 6. Amine oxidase-*RidA* sinteny analysis between the green alga *C. reinhardtii* and the cyanobacterium *Gloeocapsa* sp. PCC7428. *AOF2* (Phytozome ID *Cre10.g447767.t1.1*); *AO*, putative amino acid oxidase (NCBI ID no. AFZ32287.1); *RidA*, Reactive intermediate/imine deaminase A family gene (NCBI ID no. AFZ32287.1); *LAO1* (NCBI ID no. AAB97101.1); *LAO2/RidA* (NCBI ID no. AAB97101.1); *LAO3* (NCBI ID no. EDP07010.1). 'E' refers to the expectation values obtained by pairwise alignment at the protein level. The circular arrow below the *AO* gene indicates a reversed orientation of the *C. reinhardtii LAO1* gene adjacent to the RidA ortholog relative to that seen in cyanobacteria.

3.5. Models for nitrogen scavenging from amino acids and peptides in algae

The use of organic nitrogen by algal species may be a means of niche partitioning in habitats where these forms may be available and competition for inorganic nitrogen by other organisms is considerable. How algae use amino acids and peptides may be relevant for the dynamics of algal blooms and for biotechnological applications such as bioremediation. Here, we propose three different models based on our data for *C. reinhardtii* and other algae (Fig. 8).

In Model A, characterized by *C. reinhardtii*, growth on amino acids and peptides is supported by three different pathways to scavenge nitrogen (Fig. 8A). First, amino acids may be transported into the cell and catabolized to provide not only nitrogen but also carbon. For *C. reinhardtii*, this pathway seems to be restricted to a single proteinogenic amino acid, L-arginine [10]. Second, amino acids are deaminated extracellularly to produce ammonium that is then transported into the cell via ammonium transporters [12]. This pathway relies on LAO1 orthologs and is effective for most of the proteinogenic amino acids (including L-arginine) and also for di- and tri-peptides. Third, interactions with bacteria may allow metabolic complementation and facilitate algal growth on amino acids and peptides that are not transported or deaminated [48,65].

In Model B, characterized by *Chlorella* spp., many different amino acid transporters are responsible for growth on amino acids (Fig. 8 B). *Chlorella* spp. possess at least seven transport systems responsible for the uptake of most of amino acids [72,73]. In contrast, only single transporter systems have been reported for *Chlamydomonas*, *Volvox*, and *Dunaliella* [10,56,67]. This broad amino acid uptake strategy does not appear to be a common strategy among algae. In Model C, either deamination or uptake is effective for algal growth (Fig. 8C). Thus, metabolic complementation by nitrogen-mineralizing organisms may be the only effective pathway to grow on extracellular amino acids and peptides.

These different models for amino acid nitrogen scavenging likely reflect the evolutionary histories of adaptation of these algae to the natural environment and/or biotic partners. For example, strains of *Chlorella* that form symbioses with other organisms appear to be highly adapted for growth on amino acids and are unable to use nitrate; free-living strains, in contrast, appear to be better adapted for growth on

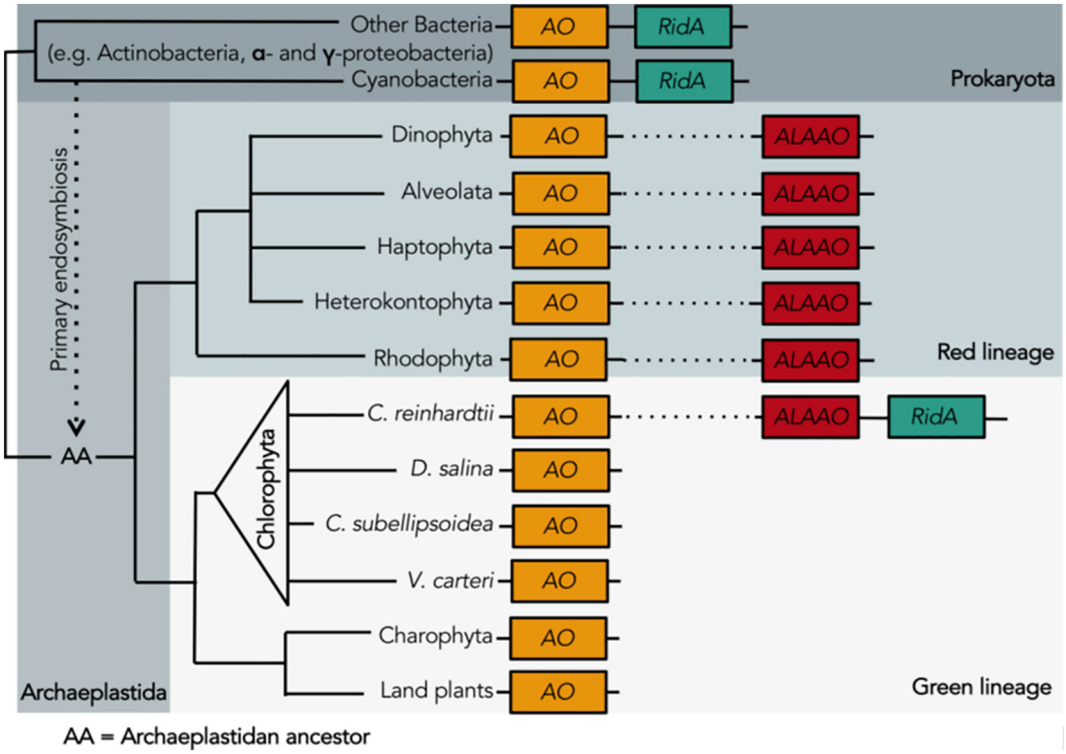

Fig. 7. Comparative genomic analysis of algal *LAAOs*. *ALAAO*, putative algal L-amino acid oxidase genes found in this work; *AO*, putative amine oxidase ortholog to *AOF2* family in *C. reinhardtii; RidA*, putative reactive intermediate/imine deaminase A family genes. Orthologs of the amine oxidase (*AO*) adjacent to *RidA* in the cyanobacterium *Gloeocapsa* sp. was included since this organism had the most closely matching *RidA* ortholog to *LAO2* of *C. reinhardtii*. The dotted arrow indicates the primary endosymbiosis event in which a protist engulfed a cyanobacterium and led to the archaeplastidan ancestor (AA). Dotted lines connecting gene boxes indicate that these genes are not adjacent to each other in currently available genome assemblies.

Table 1
ALAAO enzymes and their putative cellular locations.

Phylum	Species	Natural habitat	Protein ID	DB	Putative cellular location			
					Target	Score		
						Mit	Chl	SP
Chlorophyta	*Chlamydomonas reinhardtii*	Freshwater/ soil	AAB97101.1 (LAO1)	NCBI	SP	0.01	0.01	1.64
			Cre05.g246550.t1.2 (LAO3)	Phytozome	O	0.00	0.62	0.00
Rhodophyta	*Porphyra umbilicalis*	Marine waters	OSX78923.1	NCBI	SP	0.12	0.50	0.70
			OSX73518.1	NCBI	SP	0.00	0.10	1.16
			OSX78913.1	NCBI	Chl	0.01	1.85	0.21
			OSX80811.1	NCBI	SP	0.03	1.02	1.41
	Graciliaropsis lemaneiformis		ATE86702.1	NCBI	SP	0.03	0.21	0.21
	Chondrus crispus		XP_005713538.1	NCBI	SP	0.08	0.02	1.27
Heterokonta	*Pelagophyceae* sp. CCMP2097		jgiPelago2097	JGI	O	0.00	0.01	0.11
	Aureococcus anophagefferens		XP_009033458.1	NCBI	SP	0.17	0.02	2.02
Haptophyta	*Pavlovales* sp. CCMP2436		jgiPavlov2436	JGI	SP	0.17	0.08	1.51
	Emiliania huxleyi		XP_005785782.1	NCBI	Mit	0.50	0.11	0.07
			XP_005777092.1	NCBI	O	0.02	0.00	0.04
	Chrysochromulina sp. CCMP291		KOO35684.1	NCBI	O	0.18	0.02	0.05
Alveolata	*Vitrella brassicaformis*		CEL94447.1	NCBI	O	0.03	0.03	0.05
Dinophyta	*Symbiodinium microadriaticum*		OLQ08796.1[a]	NCBI	O	0.00	0.00	0.00

Putative subcellular location for ALAAO enzymes was determined using the algal-specific subcellular localization prediction tool, PredAlgo program, which computes a score for three cellular compartments: mitochondrion (Mit), chloroplast (Chl), and secretory pathway (SP). For scores below the following cutoffs, the target is indicated as 'other' (O): 0.42 for the mitochondrion, 0.41 for the chloroplast, and 0.14 or the secretion pathway [40]. Databases: NCBI (https://www.ncbi.nlm.nih.gov/), JGI (https://jgi.doe.gov/), and Phytozome (https://phytozome.jgi.doe.gov/).

[a] Partial protein sequence.

inorganic nitrogen sources [74]. The poor ability of *Dunaliella* spp. to use amino acids as a source of nitrogen may be a reflection of them occupying niches where inorganic nitrogen is commonly available [56] but free amino acids are not. The ability to scavenge nitrogen from amino acids may have been an ancestral trait that was lost due to relaxed selection. Having an extracellular LAAO is an unusual trait among green algae and suggests that it confers *C. reinhardtii* with an adaptive advantage in the nutrient-rich, disturbed soils this alga typically inhabits [75]. In such contexts, we surmise that extracellular deamination may be a better ecological strategy than relying on a broad amino acid uptake system. Since *C. reinhardtii* does not use resulting keto acids released by LAAO activity [12], these carbon skeletons may be key in cooperative C–N exchange interactions with bacteria that lead to enhanced mutual growth on amino acids [48]; keto acids may also play a

Table 2
Synthesis of literature findings on amino acid utilization by algae.

Algal species		Growth	Uptake	Extracellular LAAO activity	Extracellular LAO1 homolog	Ref.
Chlorophyta	*Chlamydomonas reinhardtii*	+	+	+	AAB97101.1	1, 2, 3, 4, 5
	Chlamydomonas inflexa	+	N.F.	N.F.	N.A.	6
	Chlamydomonas minuta	+	N.F.	N.F.	N.A.	6
	Chlamydomonas moewusii	+	N.F.	N.F.	N.A.	6
	Dunaliella salina	+	+	–	–	7
	Volvox carteri	N.F.	+	N.F.	–	1
	Chlorella pyrenoidosa	+	+	N.F.	N.A.	1, 8
	Chlorella vulgaris	+	+	N.F.	–	9, 10
	Ulva lactuca	N.F.	+	N.F.	N.A.	11
Rhodophyta	*Gymnogongrus flabelliformis*	N.F.	N.F.	+	N.A.	12
	Amphiora crassissima	N.F.	N.F.	+	N.A.	13
Alveolata	*Amphidinium carterae*	N.F.	N.F.	+	N.A.	14
	Amphidinium operculatum	+	N.F.	N.F.	N.A.	14
	Symbiodinium microadriaticum	N.F.	N.F.	–	– (intracellular)	14
Heterokonta	*Cyclotella cryptica*	N.F.	+	N.F.	N.A.	15
	Phaeodactylum tricornutum	+	+	+	–	16, 17
	Aureococcus anophagefferens	+	+	+	XP_009033458.1*	18
	Thalassiosira pseudonana	–	N.F.	N.F.	–	19
	Thalassiosira weissflogii	N.F.	N.F.	–	N.A.	14
Haptophyta	*Pleurochrysis carterae*	+	N.F.	+	N.A.	14
	Pleurochrysis scherfelli	N.F.	N.F.	+	N.A.	14
	Pleurochrysis sp.	N.F.	N.F.	+	N.A.	14
	Emiliana huxleyi	+	N.F.	–	– (intracellular)	10, 19
	Coccolitus pelagicus	+	N.F.	N.F.	N.A.	20
	Calcidiscus leptoporus	N.F.	N.F.	N.F.	N.A.	20
	Prymnesium parvum	+	N.F.	+	N.A.	14
	Pavlova gyrans	N.F.	N.F.	–	N.A.	14

(+) Detected; (–) Not detected; (N.F.) Not Found in the literature; (N.A.) Not Available genome; (*) Putative protein. References: 1, [10]; 2, [12]; 3, [31]; 4, [13]; 5, [54]. 6, [55]; 7, [56]; 8, [57]; 9, [58]; 10, [59]; 11, [60]; 12, [18]; 13, [61]; 14, [44]; 15, [62]; 16, [19,20]; 17, [63]; 18, [64]; 19, [65]; 20, [66]. Further information can be found in Supplementary Table S2.

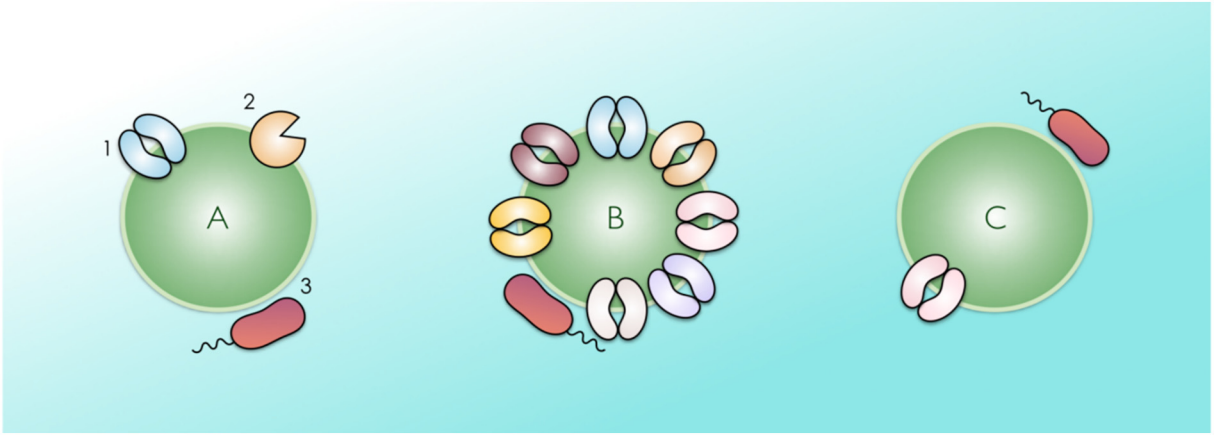

Fig. 8. Models for how algae acquire nitrogen from amino acids and/or peptides. A, *Chlamydomonas*-like model with: (1) amino acid-specific transport systems, (2) extracellular L-amino acid oxidase(s), and (3) metabolic complementation with bacteria. B, *Chlorella*-like model whereby extracellular ALAAO orthologs are missing but there are several amino acid transporter systems. C, *Dunaliella*-like model whereby ALAAOs are missing but there is a single amino acid transporter. For all three systems, metabolic complementation by nitrogen mineralizing bacteria may support algal growth.

role in chelating/solubilizing iron and/or buffering the effects of hydrogen peroxide [76,77].

Given the prevalence of algae-bacteria co-ocurrences and reported mutualistic interactions in nature [78–83], it seems likely that algal-bacterial complementation may be the most common pathway in algae to assimilate nitrogen from amino acids and peptides. It is well-known that many algae are difficult to cultivate under laboratory conditions, which is most likely due to a failure to recapitulate the requirements provided by other mutualistic microorganisms like bacteria [84]. Our data suggest that a distinct lineage of extracellular LAAO proteins (ALAAOs) is shared among algae in the Chlorophyta, Alveolata, Heterokonta, Haptophyta and Dinophyta. Understanding the function, substrate specificity, localization, occurrence, and evolutionary history of LAAO enzymes will provide a fuller picture of the role of algae and amino acids/peptide substrates in the cycling of nitrogen in natural environments, and may provide useful information for LAAOs biotechnological applications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.101395.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors thank the financial support by MINECO (Grant BFU2015-70649-P), the European Regional Development Fund program, Junta de Andalucía (BIO-502), the Plan Propio de la Universidad de Córdoba, and the U.E.INTERREG VA POCTEP-055_ALGARED_PLUS5_E.

We thank María Isabel Macías for performing genetic cross, and Aitor Gómez for assistance with experiments.

Author contributions

VC, AG and EH conceived the project and designed the experiments; VC performed the experiments; VC, EH, AL, AG and EF analyzed and discussed the data. VC, AG and EH wrote the paper.

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