The Fucoxanthin Chlorophyll a/c-Binding Protein in *Tisochrysis lutea*: Influence of Nitrogen and Light on Fucoxanthin and Chlorophyll a/c-Binding Protein Gene Expression and Fucoxanthin Synthesis

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We observed differences in lhc classification in Chromista. We proposed a classification of the lhcf family with two groups specific to haptophytes, one specific to diatoms, and one specific to seaweeds. Identification and characterization of the Fucoxanthin and Chlorophyll a/c-binding Protein (FCP) of the haptophyte microalga *Tisochrysis lutea* were performed by similarity analysis. The FCP family contains 52 lhc genes in *T. lutea*. FCP pigment binding site candidates were characterized on Lhcf protein monomers of *T. lutea*, which possesses at least nine chlorophylls and five fucoxanthin molecules, on average, per monomer. The expression of *T. lutea* lhc genes was assessed during turbidostat and chemostat experiments, one with constant light (CL) and changing nitrogen phases, the second with a 12 h:12 h sinusoidal photoperiod and changing nitrogen phases. RNA-seq analysis revealed a dynamic decrease in the expression of lhc genes with nitrogen depletion. We observed that *T. lutea* lhcx2 was only expressed at night, suggesting that its role is to protect cells from return of light after prolonged darkness exposure.

**Keywords:** carotenoids, fucoxanthin and chlorophyll a/c-binding proteins, Lhcf, Lhcr, Lhcx, *Tisochrysis lutea*, turbidostat culture, chemostat culture

**INTRODUCTION**

Marine planktonic photosynthetic organisms are responsible for approximately 50% of Earth’s primary production and fuel the global ocean biological carbon pump (Field et al., 1998). Among these producers, 50% are represented by Chromista (Cavalier-Smith, 2004), including haptophytes, which are abundant primary producers. Haptophytes are ubiquitous, and they play an important role in ocean carbon fluxes (Jordan and Chamberlain, 1997; Green and Jordan, 2000; Liu et al., 2009; Edvardsen et al., 2016). They are of great interest for aquaculture and human health...
applications (Custódio et al., 2014; Matos et al., 2019; Mohamadnia et al., 2019; Leal et al., 2020). Along with other Chromista, haptophytes contain the most abundant photosynthetic carotenoid pigment in oceans, fucoxanthin (Fx), which shows numerous pharmacological properties including antioxidant, anticancer, and anti-diabetes activities (Arora and Philippidis, 2021).

In Chromista, light-harvesting complexes (LHCs) are composed of pigments and fucoxanthin chlorophyll a/c-binding proteins (FCPs). FCPs belong to the superfamily of transmembrane LHC proteins currently well-described in diatoms (Büchel, 2020b). FCPs bind photosynthetic pigments such as Fx, chlorophylls a and c (Chl a/c), and diadinoxanthin (Ddx), which can be de-epoxidized in diatoxanthin (Dtx) for photoprotection purposes (Lavaud et al., 2002, 2004; Kuczynska et al., 2015). In particular, the spatial organization of FCPs and their carotenoids confer to Chromista a very strong absorption capacity in the blue-green spectral range of visible light, which are the most available radiations in the water column (Wang et al., 2019a,b).

Studies on FCP protein sequences characterized three main families (Bhaya and Grossman, 1993; Apt et al., 1994, 1995), and two families whose function is unknown, Lhcz and Lhcx in diatoms, with Lhcz being commonly found in red algal PSI (Nagao et al., 2020; Kumazawa et al., 2021). Lhcf proteins are dominant, and they are attached to PSII (Nagao et al., 2019a; Pi et al., 2019). Lhcr proteins are attached to PSI (Nagao et al., 2020; Xu et al., 2020). Both Lhcf and Lhcr proteins mostly bind photosynthetic pigments such as Fx and Chl a and c. Finally, Lhcx proteins play a role in photoprotection (Bailleul et al., 2010; Taddei et al., 2016; Lepetit et al., 2017; Buck et al., 2019). It is assumed that Lhcx proteins bind both Ddx and Dtx in diatoms (Beer et al., 2006; Zhu and Green, 2010; Lepetit et al., 2013). FCP genes encoding for the three families are referred to as lhcf, lhcr, and lhcx (i.e., lhca genes).

Studies on FCP structure and lhca genes have been mostly carried out on diatoms, for which the photosynthetic and photoprotective mechanisms are now well-described (Goss and Lepetit, 2015; Jensen et al., 2017). In Chaetoceros gracilis, two FCP supercomplexes were characterized and are related to both photosystems, PSI-FCPII and PSI-FCPI. By cryo-electron microscopy (cryo-EM) analyses, it was found that PSI-FCPI presents different binding patterns of pigments (Nagao et al., 2020; Xu et al., 2020) compared to PSII-FCPII (Nagao et al., 2019a; Pi et al., 2019). In the model diatom Phaeodactylum tricornutum, FCPs are composed of 17 Lhcf proteins, 14 Lhcr proteins, and 4 Lhcx proteins (Büchel, 2020b). In an in-depth description of the light-harvesting antenna of P. tricornutum by cryo-crystallography analysis (Wang et al., 2019a), 9 binding sites for Chl a and c have been identified along with 7 binding sites for Fx, among 17 Lhcf sequences. Furthermore, a study on these binding sites demonstrated that Chl a and Fx molecules are closely related (i.e., each Fx is related to 1 Chl a) (Wang et al., 2019a). This close spatial proximity allows for efficient energy transfer and dissipation (Papagiannakis et al., 2005; Nagao et al., 2013; Akimoto et al., 2014; Gelzinis et al., 2015).

Fucoxanthin molecules shift absorption properties according to their amino acid binding sites on Lhcf monomers (Wang et al., 2019a). There exists three types of Fx molecules named according to their absorption properties: “Fx-blue” strictly absorbs in the blue range of visible light spectrum ($\lambda_{max} = 463$ nm), “Fx-green” absorbs in a wider blue/green light range ($\lambda_{max} = 492$ nm), and “Fx-red” additionally absorbs in an even wider blue/green light called “red-shifted” range ($\lambda_{max} = 500–550$ nm) (Premvardhan et al., 2009). This specific shift in absorption properties explains the capacity of Fx to harvest photons over a wide light spectrum (Wang et al., 2019a), allowing Chromista to perform photosynthesis under a broad range of light conditions, including different habitats (water column, shallow sediments, sea-ice, etc.) and different depths (Di Valentini et al., 2012; Gelzinis et al., 2015; Büchel, 2020a).

Contrary to diatoms, FCPs of haptophytes have not been much described, except for Emiliania huxleyi. E. huxleyi FCPs are composed of 66 proteins (encoded by 75 genes), all named Lhcf and sub-divided into five groups: Lhcf group1, Lhcf group2, Lhcf (red), Lhcz-like, and LI818-like (Lefebvre et al., 2009; McKew et al., 2013). They are determined according to the classification of the LHC superfamily of Koziol et al. (2007) and have not been updated since. In Koziol et al. (2007) the LI818/LI818-like group is related to photoprotection, the Lhcr/Lhcc group is related to the LHC of red alge and cryptomonads, and the Lhcf group is related to FCPs; however, the specific function of the Lhcz group is still unknown. Because haptophytes are direct descendants of the cryptomonad lineage (Harper et al., 2005; Patron et al., 2007), the Lhcf (red) group of E. huxleyi was named after the Lhcr/Lhcc group.

In Chromista, light intensity and nitrogen availability are of a major influence on the photosynthetic process. Indeed, the concentration of pigments is changing with light wavelength and intensity (Marchetti et al., 2013; Nagao et al., 2019b; Yang and Wei, 2020; Duarte et al., 2021). It is known that low light increases photosynthetic pigment content (Brown et al., 1999; Anning et al., 2000; Mohamadnia et al., 2019; Gao et al., 2020a) and lhca expression (Lefebvre et al., 2010) while high light increases quenching of excess energy with photoprotective pigments (Lavaud et al., 2002; Demmig-Adams et al., 2014), increases lhcb expression (Blommaert et al., 2020), and decreases lhcb expression (Lefebvre et al., 2010). Furthermore, it was shown in the haptophyte Isochrysis galbana that the carotenoid content decreased with nitrogen (N) deprivation (Zarrinmehr et al., 2019), and that, in a reverse manner, in the diatom Odontella aurita, an initial high concentration of nitrate boosted the accumulation of Fx in chloroplast membranes, and even more when coupled with N supplement (Xia et al., 2018). Several studies showed increase in pigment content with increase in N availability (Geider et al., 1998; Wour et al., 2008; Dahmen-Ben Moussa et al., 2017; McClure et al., 2018; Zarrinmehr et al., 2019). In batch culturing mode, however, as an increase in N availability generates an increase in biomass, light availability decreases over time as a culture develops. With less access to light, photosynthetic pigment content is increased and the expression of photosynthesis-related genes such as lhca genes is impacted. Therefore, the direct impact of N availability is hard
to assess in the batch culturing mode. However, in continuous culturing mode with very limited variations in biomass, it is possible to measure pigment content per cell without the shading effect.

We selected *Tisochrysis lutea*, whose genome was sequenced (Berthelier et al., 2018), with the aim to update and complete *lhc* genes and FCP protein classification in haptothytes. This species contains high amounts of fucoxanthin and other carotenoids (Mohamadnia et al., 2019), whose biological activities are of great value in the field of aquaculture (i.e., bivalve hatcheries) (Delaunay et al., 1993; Rico-Villa et al., 2006) and biotechnologies (i.e., nutrition, pharmacology, etc.) (Delbrut et al., 2018; Cezar-Gomes et al., 2019). Our objectives were to identify for the first time *T. lutea* *lhc* genes, and classify them into the three families: *lhcf*, *lhcr* and *lhx*. Then, we relied on a crystallographic study performed with *P. tricornutum* (Wang et al., 2019a) to determine some of chlorophylls and fucoxanthin binding sites in *P. tricornutum* sequences (Mohamadnia et al., 2019), whose biological activities are of great value in the field of aquaculture (i.e., bivalve hatcheries) (Delaunay et al., 1993; Rico-Villa et al., 2006) and biotechnologies (i.e., nutrition, pharmacology, etc.) (Delbrut et al., 2018; Cezar-Gomes et al., 2019). With the Lhcf family representing the majority of FCP sequences, we additionally built a cladogram with the Lhcf sequences of *T. lutea*, of the four diatoms *Phaeodactylum tricornutum*, Fragilariopsis cylindrus, *Thalassiosira pseudonana*, and Chaetoceros gracilis, of Saccharina japonica and Saccharina latissima (brown seaweeds), and of *Emiliania huxleyi* (haptohtyte) (Supplementary Table 6). Using the software GENEIOUS®, we aligned 134 sequences with Clustal Omega and built a cladogram by neighbor-joining using the genetic distance model Jules-Cantor with 100 permutations. Bootstrap scores of the main nodes are displayed on the cladogram.

With the same method, we also built a cladogram representing the FCP sequences in *T. lutea* and another representing the Lhcr sequences of *T. lutea* and *E. huxleyi* in order to identify the subgroup Lhcz.

### Binding Sites of Chl a, Chl c, and Fx in *Tisochrysis lutea*

In a crystallographic study, binding sites of Chl a, Chl c, and Fx on Lhcf sequences of *P. tricornutum* were found (Wang et al., 2019a). We identified Lhcf proteins of *T. lutea* with those of *P. tricornutum*. Then, we identified amino acid binding sites in *T. lutea* by comparing with those in *P. tricornutum*. Binding sites are of two different kinds. They are either central ligands or H-bond ligands, as described in *P. tricornutum*. Furthermore, majority of pigments possess several binding sites (up to four) and are not necessarily present in all Lhcf sequences of a species. Therefore, for each amino acid binding site, the percentage of presence in all Lhcf sequences was calculated.

### Culture Conditions

All the experiments were performed with *T. lutea* strain CCAP 927/14. Inocula were maintained in Walne's medium (Walne, 1966) at 150 μmol m⁻² s⁻¹. Both experiments were conducted in a continuous 8-L flat panel photobioreactor (1,000 mm × 400 mm × 250 mm).

### Constant Light Experiment

To assess the effects of N availability on pigment content and levels of *lhc* genes, we used and completed the data of a continuous culture in a chemostat performed by Garnier et al. (2016). A chemostat is a continuous culture with a controlled chemical environment and for which the dilution rate (ratio of inflow rate over reactor volume) is constant. *T. lutea* was grown for 85 days with a 0.5-day⁻¹ dilution rate and illuminated with constant light (CL) (150 μmol m⁻² s⁻¹). The dilution rate was periodically checked by weighing the output culture. Modified Walne's medium containing 125/125 μM N:P ratio was used to induce limitation in N. The reactor was continuously aerated by bubbling and maintained at 27°C and pH of 7.3. Three nitrate (NO₃⁻) spikes consisting the injection of 3.5 mmol of NaNO₃...
in 10 L of culture were made on days 20, 43, and 83. The experiment was, therefore, run in consecutive triplicates. NO₃ spikes during cultivation aim to subject the algae to different N regimes monitored with N quota (qN), i.e., intracellular N/C ratio analysis: balanced limitation phase (steady phase, qN = 0.09 molN molC⁻¹), dynamic repletion phase (more dissolved N available in the culture medium, qN = 0.13molN molC⁻¹), and dynamic depletion phase (less dissolved N available in the culture medium, 0.013 > qN > 0.09).

Dynamic Light Experiment
We performed a second experiment in turbidostat to understand the impact of both diel cycle and N availability on pigment content and transcript levels of lhc genes expression. A turbidostat is also a continuous culture, but dilution is varied in order to maintain cell density at a constant level (i.e., turbidity). T. lutea was grown at 23°C for 15 days with Walne’s medium (Walne, 1966). The light regime was sinusoidal photoperiods of 12 h with maximal irradiance of 900 μmol m⁻² s⁻¹ at 12:00 h and darkness from 18:00 h to 00:00 h. Two NO₃ spikes of 3.5 mmol NaNO₃ were injected on days 1 and 8. The experiment was carried out two times under similar conditions (sequential duplicates). The culture was monitored in N repletion and N depletion phases (no dissolved N available in the culture medium) by sampling every 2 h. For transcriptomic analysis, the culture was harvested during the selected day at 8:00, 12:00, and 00:00 h for both N phases.

Intracellular Nitrogen Concentration and Cell Concentration
The number of cells per unit of culture medium and intracellular carbon (C) and N were measured once a day for the CL experiment and every 2 h for the Dynamic Light (DL) experiment. Intracellular C and N were measured on an elemental analyzer (Flash, 2000; Thermo Fisher). Cell counting was performed using a Malassez hemocytometer.

Pigment Extraction and High Performance Liquid Chromatography Analysis
For both experiments, we measured FCP pigment content: chlorophyll a (only in the DL experiment), Fx, Ddx, and Dtx. For the CL experiment, samples were centrifuged, and wet pellets were stored at Dtx. For the CL experiment, samples were centrifuged, and µa chlorophyll content and transcript levels of lhc genes was performed on R using the DESeq2 tool. The global influence of qN and light was determined with the fold change and adjusted p-value of the corresponding 52 transcripts in each culture condition. The resulting mean average plot (MA-plot) allows for visual identification of a significant difference in expression of all transcripts (Supplementary Figures 5–10).

Statistics
In order to analyze the influence of N status (qN) and light intensity on lhc gene transcript level in T. lutea, a differential expression (DE) analysis of lhc genes was performed on R using the DESeq2 tool. The global influence of qN and light was determined with the fold change and adjusted p-value (< 0.05) of the DESeq2 comparison of all transcripts in each culture condition. The resulting mean average plot (MA-plot) allows for visual identification of a significant difference in expression of all transcripts (Supplementary Figures 5–10).

The fold change and adjusted p-value of the corresponding 52 transcripts in the resulting table of the DESeq2 comparison were selected to observe if their expression was significantly influenced by qN and light intensity. In addition, an ANOVA was performed on the 52 transcript counts between each condition.

RESULTS
Homologous Fucoxanthin and Chlorophyll a/c-Binding Protein Sequences in Chromista
Our comparative analysis of sequences available in Chromista revealed that among all the sequences similar to those in P. tricornutum, the majority is classified as unknown protein sequences in Chromista.

RNA Sequencing
A total of 50 ml of algal culture was sampled for transcriptomic analysis. Total RNA was extracted and sequenced as described by Carrier et al. (2014). A library of RNA-seq was built for each sample. Poly(A) mRNA was isolated from total RNA using oligo (dT) magnetic beads [MicroPoly(A)Purist™ Kit; Ambion]. Two cDNA libraries were built according to the instructions of the manufacturer and sequenced with an Illumina HiSeq 3000 sequencer and with the GenoTool platform. We obtained an average of 2.617 and 3.217 reads per sample in the CL and DL experiments, respectively.

Raw reads of each sample were filtered using TrimGalore to remove known Illumina adapter sequences. Low-quality reads were excluded using a quality score threshold of 30 and a minimal length of 75 or 150 bases. The quality of reads was assessed using FastQC. Then, sequenced reads for each sample were aligned using Bowtie 2 in the QuasR package in R (Quantify and Annotate Short Reads in R). Each gene within the alignment was counted using the Rsparse package in R with the function feature Counts, based on T. lutea reference genome (Carrier et al., 2014). Gene counts were obtained for each sample and normalized with DESeq2.

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RESULTS
Homologous Fucoxanthin and Chlorophyll a/c-Binding Protein Sequences in Chromista
Our comparative analysis of sequences available in Chromista revealed that among all the sequences similar to those in P. tricornutum, the majority is classified as unknown protein
or as hypothetical light-harvesting proteins. In two diatoms (Fragilariopsis cylindrus and Thalassiosira pseudonana) and one haptophyte (Emiliania huxleyi), sequences are classified as LHC proteins (i.e., FCPs). These results lead us to study more specifically the FCP proteins of the FCP complex in T. lutea coded by the lhcf, lhcr, and lhcx genes.

**Lhcf, Lhcr, and Lhcx Annotation in the Proteome of *Tisochrysis lutea***

Our analysis revealed that 52 FCP proteins constitute the FCP in *T. lutea* (Figure 1 and Supplementary Table 7). All of them matched to either the Lhcf, Lhcr, or Lhcx -family of diatom FCPs. According to similarity scores obtained with *P. tricornutum*, *F. cylindrus*, and *T. pseudonana* (Supplementary Tables 3–5), among the sequences of *T. lutea*, 28 were Lhcf, 12 were Lhcr, and 12 were Lhcx (Figure 1). In *T. lutea*, Lhcf23 was been classified as Lhcr after one single match with low score in BLAST results, but the cladogram (Figure 1) clustered this sequence into the Lhcf family. It is likely that the algorithm did not display all lowest scores in the similarity search BLAST. Knowing it is classified as an Lhcf sequence with the cladogram, a new similarity search was performed between this sequence and the Lhcf sequences of all the three diatoms. We observed a very few matches of

![Cladogram of the 52 Lhcf sequences of *Tisochrysis lutea*. Orange: Lhcx. Blue: Lhcr. Green: Lhcf.](image-url)
Lhcf23 with Lhc diatom sequences, with scores in the same range compared to the previous match with the Lhcr sequence of *F. cylindrus*. We conclude, according to the BLAST results only, that the probability of Lhcf23 belonging to either the Lhcr or Lhcf family was the same, but according to the cladogram, it was decided to be classified as an Lhcf sequence. Furthermore, it was not possible to classify the Lhcx5 sequence with certitude, because although the BLAST results were slightly higher than the Lhcx of the three diatoms (Supplementary Tables 3–5), it did not appear as pertaining to the Lhcx family on the cladogram (Figure 1).

To further analyze the Lhcf proteins, major sequences of the FCP, we built a cladogram showing four main well-supported clades of Lhcf in haptophytes, diatoms, and brown seaweeds (Figure 2). Consequently, complementary to sequence classification in diatoms (Gundermann et al., 2013), we propose a new classification of FCPs for haptophytes (Table 1). Lhcf group A sequences are mainly found in haptophytes, Lhcf group B sequences are exclusively found in haptophytes, Lhcf group C sequences are exclusively found in diatoms, and Lhcf group D sequences are exclusively found in brown seaweeds. Based on this cladogram, we observed that the Lhcf groups I and II of *E. huxleyi* described in McKew et al. (2013) correspond to the Lhcf groups A and B, respectively. Furthermore, we aligned all the FCP sequences of *E. huxleyi* and found two other distinct groups: all sequences of the Lhcf (red) and Lhcx (green)
groups (Supplementary Table 6 and Supplementary Figure 1), respectively. It appeared that one subgroup of the Lhcr family corresponds to the Lhcz-like family described in McKew et al. (2013) By building a cladogram between the Lhcr of E. huxleyi and those of T. lutea (Supplementary Figure 2), we observed two Lhcr subgroups, one of which was composed of Lhcz-like of E. huxleyi along with three Lhcr proteins of T. lutea (Lhcr5, Lhcr6, and Lhcr10), which might, therefore, be Lhcz sequences. We propose a new annotation of the 75 E. huxleyi ilhc genes into the three lhcf, lhcr, and lhcx families (Supplementary Table 6), and we hypothesize the existence of a fourth family being that of Lhcz, both in E. huxleyi and T. lutea.

### Binding Sites of Chlorophylls and Fucoxanthin in Lhcf Proteins in *Tisochrysis lutea*

All Lhcf sequences of *P. tricornutum* and *T. lutea* were aligned, and pigment binding site candidates were identified in *T. lutea* (Figure 3 and Supplementary Figure 3).

Tables 2, 3 present the different types of ligands for Chl a, Chl c, and Fx in Lhcf sequences of *P. tricornutum* compared to those of *T. lutea* in same amino acid positions. A majority of pigments possess several binding sites (e.g., Chl a404, Fx302).

All Chl a- and c-conserved central ligands in *P. tricornutum* (excepted for c408) corresponded to homologous domains in *T. lutea*. All non-conserved central ligands (except for a405) were located in *T. lutea*. All H-bond sites (excepted for a401) were located in *T. lutea*. *T. lutea* conserved the same Fx binding sites compared with *P. tricornutum* (Table 3), except for 2 binding sites of Fx302. Among the 7 Fx of *P. tricornutum*, the comparison with *T. lutea* could only be done upon 5 which are Fx301, 302, 303, 304, 305. Among these five, four Fx binding sites in *P. tricornutum* corresponded completely to homologous amino acid domains in *T. lutea*. However, the Fx302 in *P. tricornutum* had four binding sites, R, T, Y, and M but only R and Y were found in *T. lutea*. More particularly, the Y binding site in *P. tricornutum* corresponded to a Y on a 3 as shifted position in *T. lutea*. This position corresponded to the T binding site of Fx302 in *P. tricornutum* (Supplementary Figure 2), highlighting the relevance of this protein section in binding an Fx molecule.

According to these results, 9 binding sites of Chl a and c, and 5 binding sites of Fx (for multiple sites, at least one is found) are found in *T. lutea* and described in Supplementary Figure 3. Another observation is that except for the Chl a404 central ligand and the Chl a407 H bond ligand, the percentages of the presence of binding sites among all *T. lutea* Lhcf sequences were very close to those in *P. tricornutum*.

### Effects of Nitrogen Availability and Light Intensity on the Expression of Lhc Genes

#### Constant Light Experiment: N Quota and Biomass

After NO₃ spike, qN (i.e., N quota, intracellular N/C ratio analysis performed to determine the N regime of the cultures) immediately increased, cells entered the depletion phase for 12 h. The biomass started to increase 10 h after the NO₃ spike. qN decreased within 24 h after reaching its maximum (0.13 mol N mol C⁻¹), and it is the depletion phase. The biomass increased for 24 h before stabilizing for another 24 h, and finally it decreased. Eventually, cells reached a new steady phase with constant qN and biomass (Figure 4A).

#### Constant Light Experiment: Pigments

Figures 4B,C s an increase in Fx, Ddx, and Dtx content in cells (expressed as Fx:C, Ddx:C, and Dtx:C ratio) concurrently with qN (Figure 4A) during the repletion phase. Fx and Ddx contents were tripled and were at maximum when qN was maximum, and then slightly decreased during the depletion phase. In contrast, Dtx content was doubled during depletion (Figure 4C). The Ddx + Dtx pool was up to five times higher during the dynamic phases of the experiment repletion and depletion phases (2.5e-03 mg mg⁻¹) than during the limitation phase (5.0e-04 mg mg⁻¹).

#### Constant Light Experiment: Expression of Lhc Genes

The evolution of FCP gene counts has been studied as a function of N availability in the same cultures. Transcriptomics analyses were performed on two replicates. We observed an increase in all lhcf, lhcr and lhcx transcript counts during the repletion phase compared to limitation, and a decrease in depletion phase compared to repletion (Figure 5 and Supplementary Tables 8–10). The global analysis of variance did not show significant differences in lhcx counts among all the N phases. However, specific analysis of genes revealed that two lhcx genes were regulated with significant differences. Along with one lhcf outlier (lhcf13), lhcx2, and lhcx5 gene counts were between 3 and 20 times higher than the median value of other lhcf and lhcx genes in each N condition (Figure 5). No expression was recorded for lhcf9.

The repletion phase was significantly correlated (DE p-value < 0.05) with an increase of the expression of several lhc genes. 11 lhcf over 28, 2 lhcr over 12 and 4 lhcx over 12 were 1.5 to 4 times more expressed than during the limitation phase (Supplementary Table 17). During the depletion phase, the expression was significantly lower than repletion, between 3 and 5.5 times for 22 lhcf over 28, 8 lhcr over 12, and 3 lhcx genes over 12 (Supplementary Table 17).

Looking at the CL experiment, it turned out that in parallel with the lower concentration of the pigments measured, the depletion in N corresponded to the lowest expression of the lhc genes.

#### Dynamic Light Experiment: N Quota and Biomass

In the DL experiment, the biomass was kept constant between 1.0 1⁶ and 1.5 1⁶ cells ml⁻¹ (Figure 6A). qN and Fx content (Fx:C) changed according to N bioavailability in the culture, following
Pattern similar to the one in the CL experiment. During the repletion phase, qN followed the light signal (day:night cycles), reaching a minimum and a maximum value, respectively, at 00:00 and 12:00 h (Figure 6B). During depletion, qN was not significantly impacted by light variation and decreased for 24 h before it stabilized at its minimum value. During the repletion phase, the biomass evolved in correlation with the day:night cycles. When the light intensity reached 10 µmol photons m⁻² s⁻¹ around 06:00 h, the algal biomass increased until the light intensity was back to 10 µmol photons m⁻² s⁻¹ at 18:00 h, and it further decreased during the night. However, during the depletion phase, a difference was observed. Biomass correlation with the day:night cycles was less distinct, and the difference in biomass concentration between day and night was reduced.

**Dynamic Light Experiment: Pigments**

Whatever the N phase of the cultures, Fx content (Figure 6B) and Chl a content (Supplementary Figure 4) increased during the night and decreased during the day. During N depletion, however, the influence of the day:night cycles on Fx, Chl a, and Ddx (Figure 6C) was minimized, as their content were two to three times lower than during repletion. On the contrary, during N repletion, the influence of the day:night cycles on Dtx content was barely noticeable, as it was two times lower than...
TABLE 3 | Binding sites of Fx amino acid (in capital letters) in T. lutea according to those identified in P. tricornutum (Wang et al., 2019a).

| Fx identified in P. tricornutum | % among Lhcf |
|--------------------------------|-------------|
| P. tricornutum                 |             |
| Fx 301                         |             |
| L                               | 77%         |
| P. tricornutum                 |             |
| T. lutea                       |             |
| R                               | 100%        |
| M                               | 24%         |
| T. lutea                       |             |
| T. lutea                       |             |
| F                               | 26%         |
| M                               | 26%         |
| T. lutea                       |             |
| F                               | 29%         |
| P. tricornutum                 |             |
| L                               | 68%         |
| T. lutea                       |             |
| K                               | 66%         |
| F                               |             |
| T. lutea                       |             |
| G                               | 68%         |
| T. lutea                       |             |
| K                               | 68%         |
| F                               |             |
| T. lutea                       |             |
| L                               | 24%         |
| T. lutea                       |             |
| L                               | 18%         |

Percentages represent the presence of binding sites among all the Lhcf sequences of each species.

During depletion. On the contrary, Dtx content increased during the day and drastically decreased during the night all along the experiment (Figure 6C). It was doubled in depletion compared to in repletion. However, the Ddx + Dtx pool decreased in depletion, mainly influenced by Ddx content.

Overall, these results corroborated those of CL experiment, apart from the Ddx + Dtx pool, which decreased during the N depletion phase.

Dynamic Light Experiment: Expression of Lhc Genes

In the DL experiment, Lhc expression varied according to both N bioavailability and light intensity (Figure 7). Similar to the CL experiment, gene counts were lower in the N depletion than in the repletion phase. It is especially highlighted with gene counts of replicate 1. Also, genes were more expressed in the repletion phase compared to the CL experiment. All the gene counts are available in supporting information (Supplementary Tables 11–16).

Most of the genes were upregulated during the day at 8:00 and 12:00 h and downregulated at night (00:00 h) (Figures 7A–C). Specifically, lhcf genes were significantly upregulated at 8:00 h rather than at 12:00 h, except for lhcx2, which was the only gene expressed exclusively during the night (Figure 7D and Supplementary Table 18). Also, lhcf21 and lhcr6 were the only lhcf and lhcr genes to be significantly upregulated at 8:00 h vs. 12:00 h (Supplementary Table 19).

The DE analysis reveals that the percentages of upregulation in the repletion phase compared to depletion were similar to those in the CL experiment. However, these results were for replicate 1 only. Indeed, in replicate 2, no lhc gene was significantly downregulated in depletion compared to repletion. We also confirmed that the great majority of the lhcf, lhcr and lhcx genes was significantly upregulated during the day (Table 4). Another observation was the high percentage of lhcx significantly upregulated at 8:00 h compared to 12:00 h (25%, lhcx3, lhcx11, and lhcx12).

DISCUSSION

Fucoxanthin and Chlorophyll a/c-Binding Protein Sequences in Chromista Species

Only a few annotated FCP sequences were available for haptophytes compared to the data available in literature for other groups of Chromista. The known classification in diatoms supported the annotation of the 52 FCP sequences in the haptophyte Tisochrysis lutea. Our results also emphasized the diversification of the Lhcf family: we propose the Lhcf group A mainly to be composed of haptophytes sequences, the Lhcf group B to be specific to haptophytes, the Lhcf group C to be specific to diatoms, and the Lhcf group D to be specific to brown seaweeds. These groups may indicate that the evolutionary distance between diatoms and haptophytes is also reflected in their light-harvesting complexes (Büchel, 2020a). As the novel group named Lhcq was proposed in diatoms (Nagao et al., 2020; Kumazawa et al., 2021), it is not excluded that some T. lutea sequences pertain to this group. However, there is not enough information, so far, to confirm it. Furthermore, our results suggested that three Lhcr proteins (Lhcr6, Lhcr10, and Lhcr11), in T. lutea belong to the Lhcz group, defined as a sister clade of Lhcr in Kumazawa et al. (2021). Overall, the lhcf genes in T. lutea are 54% lhcf, 23% lhcr (including lhcz), and 23% lhcx, while in E. huxleyi they are, respectively, 70, 9, and 21%. While the proportion of photoprotection-related Lhcx was similar between both species, T. lutea overall FCP composition is enriched in PSI-related Lhcr, while it is enriched in PSI-related Lhcf in E. huxleyi. In parallel, we know that the amount of light harvested by PSI and PSII is determined by the effective absorption cross-sections, σPSII and σPSI, respectively. In two strains of E. huxleyi, the ratio of σPSII/σPSI was found to be ~0.4–0.5 (Suggett et al., 2007), while in Isochrysis galbana, a species close to T. lutea, it was found to be ~0.25–0.35 (Dubinsky et al., 1986). As σPSII/σPSI is higher in E. huxleyi, this could mean that light harvesting is more effective in the PSII in E. huxleyi than with I. galbana, which is more effective in PSI. Supposing that the σPSII/σPSI ratio in T. lutea is similar to that in I. galbana, this observation is consistent with the FCP composition of both species.

Possibly, this distinction could be linked to a different adaptation to the in situ light climate with T. lutea originating from an environment with strong light variations and requires stronger balance between PSII and PSI photochemistry. In comparison, E. huxleyi is found in coastal, temperate continental, and cold regions with less harsh light intensity growing conditions (Brown and Yoder, 1993; Perrin, 2017) but still requires great ability to tolerate different light intensities (Cortès et al., 2001).
Binding Sites of Chlorophylls and Fucoxanthin in Lhcf Proteins in *Tisochrysis lutea*

In the Lhcf proteins of *P. tricornutum*, the most red-shifted, Fx306 and Fx307, were directly bound to Chl c (Wang et al., 2019a). Therefore, even if these two candidate sites were found on the Lhcf of *T. lutea*, it is not possible to certify that these Chls effectively bind Fx molecules. This could only be observed with a crystallographic or a cryo-EM study, as it was done on *P. tricornutum* (Wang et al., 2019a) and *C. gracilis* (Kumazawa et al., 2021). Amino acid homologies between *P. tricornutum* and *T. lutea* FCP sequences strongly suggested that *T. lutea* possesses at least 9 Chl *a* or *c* binding-sites among the majority of Lhcf monomers. Most of the Fx binding sites identified in *P. tricornutum* were also found in *T. lutea*, although none of the Lhcf proteins showed all the Fx binding sites identified in *P. tricornutum*. This result suggests the presence of Fx binding sites specific to haptophytes as compared to diatoms. Among the 28 *T. lutea* Lhcf proteins, three (Lhcf7, Lhcf11, and Lhcf13) showed a few of the identified Chl and Fx binding site candidates. It suggests that they rather...
have an important structural role in light-harvesting spatial organization. In particular, Lhcf13 was the most expressed in our experiments (Supplementary Tables 8, 11, 12). These results are to be carefully considered, because transcripts could be regulated before translation into proteins (Sonenberg and Hinnebusch, 2009). Over all, and although the exact oligomeric spatial organization of the light-harvesting system of *T. lutea* remains unknown, sequence comparison with *P. tricornutum* strongly suggested that the light-harvesting capacity of haptophytes is similar to that of diatoms.

**Lhcf and Lhcr Expression and Fucoxanthin Synthesis**

**Influence of Nitrogen**

In *P. tricornutum*, the FCP and general photosynthesis pathway are downregulated during N stress (Longworth et al., 2016). In *I. galbana*, a close strain of *T. lutea*, it was suggested that the FCP is a constant fraction of the total qN of about 6.8% (Falkowski et al., 1989). Both of our experiments showed that qN decreased by up to 1.6 times in N limitation and depletion compared to N repletion. It was, therefore, consistent to observe, from N limitation to N repletion phase, an increase in the expression of the photosynthetic genes *lhcf* and *lhcr*, an increase in Fx content, and a distinct decrease from repletion to depletion. In parallel, photosynthetic pigment content decreased during depletion. These results might indicate the correlation of the binding of Fx and Ddx on Lhcf and Lhcr. Furthermore, they demonstrate the positive influence of increase in qN on Fx and Ddx content and on the expression of the majority of *lhc* genes, significantly for 34 *lhc* genes including *lhcx*. The DL experiment showed that Chl a content varied similarly to the Fx content (Supplementary Figure 3). The positive linear correlation between Chl a and Fx in

**FIGURE 5** | CL experiment. Normalized transcript counts of (A) *lhcf* (green), (B) *lhcr* (blue), (C) *lhcx* (orange) genes according to N status (limitation, repletion, and depletion). Analysis of variance (ANOVA) test: *p*-value < 0.05, **p*-value < 0.01.
**FIGURE 6** Dynamic light (DL) experiment. The green curve represents photosynthetically active radiation (PAR) during the day/night cycle. (A) Evolution of the cell biomass expressed as total intracellular C and of the qN expressed as intracellular N:C ratio of the two culture replicates. (B) Evolution of the Fx:C ratio of the two culture replicates. (C) Evolution of the photoprotective pigment ratios: (Ddx + Dtx):C, Ddx:C, and Dtx:C of the two culture replicates. Vertical black dashed line marks the transition between the repletion and depletion phases in N.

*T. lutea* has already been reported in a study (Gao et al., 2020b). As chlorophylls contain N, contrary to carotenoids, they are positively influenced by N supplementation, which can explain the indirect dependence of Fx on N concentration in the culture. Furthermore, Chl a binds to Lhcf and Lhcr proteins. Therefore, there is a correlation between the N needed for Chl a synthesis and the Lhcf and Lhcr proteins needed for Chl a binding.

**Influence of Light**

Experiments with a photoperiodic cycle as our DL experiment can reveal the dynamic shift between photosynthesis and photoprotection. In our experiments, we observed that *lhcf* and *lhcr* genes were upregulated during the day, and were almost not expressed at night, contrary to photosynthetic pigments. On one hand, and as expected, Fx and Ddx content increased with low light intensity to perform photosynthesis even with a few photons. Furthermore, several studies hypothesized that Ddx is a precursor of Fx synthesis (Dambeck et al., 2012; Dautermann et al., 2020), enhancing the fact that both pigments evolve simultaneously. On the other hand, the fact that *lhcf* and *lhcr* genes were not upregulated during the night was unexpected. We supposed that cells synthesized the
whole FCP complex during the day, allowing for the binding of photoprotective pigments while preparing the need for photosynthetic pigments when the light decreases at night. This shift might explain a trade-off between the need for photoprotection during the day and the light-harvesting process mainly active at the beginning of the night. When comparing both experiments, \( lhc \) genes were more expressed in repletion with a day:night cycle than in repletion with constant light, which indicates that light intensity is a more impacting factor than N for FCP expression.

Finally, the qN varying according to NO\(_3\) concentration in the culture medium, along with light intensity, is partly responsible for the balance between the photosynthetic and photoprotective activities of \( T. \) lutea.
TABLE 4 | Percentages of lhcf, lhcr, or lhcx genes upregulated at 8:00 and 12:00 h in the dynamic light (DL) experiment.

|        | At 8:00 h vs. 00:00 h | At 8:00 h vs. 12:00 h | At 12:00 h vs. 00:00 h |
|--------|----------------------|----------------------|-----------------------|
| lhcf   | 3.6%                 | 82%                  | 68%                   |
| lhcr   | 8.3%                 | 82%                  | 92%                   |
| lhcx   | 25%                  | 75%                  | 75%                   |

**Lhcx Expression, Diadinoxanthin, and Diatoxanthin Synthesis**

The photopersiodic cycle of the DL experiment allowed to distinguish the photoprotection dynamic between the *lhcx* and the Ddx/Dtx content. At 8:00 h, after 2 h of illumination, cells expressed more *lhcx* than at 12:00 h. In parallel, we observed that Dtx content was higher during the day, corroborating its role in photoprotection. These results were in line with what was found in diatoms with increase, under high light, in Dtx content in *Cyclotella meneghiniana* (Beer et al., 2006), in both Dtx content and L1818-like proteins (also referred as Lhcx) in *T. pseudonana* (Zhu and Green, 2010), in the Ddx + Dtx pool and three *lhcx* in *P. tricornutum* (Lepetit et al., 2013), and in the Ddx + Dtx pool and synthesis of Lhcx2 and Lhcx3 in *P. tricornutum* (Blommaert et al., 2017; Lepetit et al., 2017). Furthermore, a cryptophycean residue located closely to the Ddx/Dtx binding site of Lhcx was characterized as involved in energy-dependent fluorescence quenching (qE) in *P. tricornutum*, a photoprotection mechanism (Buck et al., 2021). Dtx results from the de-epoxidation of Ddx; it was, therefore, consistent to observe that Dtx content was reversely symmetrical to Ddx content in both of our experiments. However, even under maximum light intensity, Ddx was not entirely de-epoxidized in Dtx, highlighting its double role as a photosynthetic and a photoprotective pigment (Lacour et al., 2020). Overall, the Ddx + Dtx pool increased with light intensity in repletion as was found in *Cyclotella meneghiniana* when applying high light (Guidermann and Büchel, 2008), supporting the main photoprotective role of these pigments. Finally, *T. lutea* cells seemed to activate their photoprotection mechanisms to endorse light onset without risking the deterioration of cellular mechanisms.

Similarly, in *C. meneghiniana*, proteins encoded by an *lhcx* named fcp6/7 were more present under HL conditions (Oeljekl et al., 2002; Beer et al., 2006; Guidermann and Büchel, 2008). In addition, *lhcx2* in *P. tricornutum* was strongly upregulated when exposed to a constant 48-h HL, whereas *lhcx3, lhcr6, and lhcr8* were strongly upregulated after 0.5 h of HL but slightly decreased during the last 47.5 h of HL (Nymark et al., 2009). It suggested a photoprotective role followed by acclimation to HL for *lhcx3* and for *lhcr6 and lhcr8*. According to our BLAST results, the corresponding Lhcr6 and Lhcr8 proteins in *P. tricornutum* had the highest similarity scores with Lhcr5 and Lhcr6 in *T. lutea*, respectively. The corresponding *lhcr6* gene in *T. lutea* was the only *lhcr* significantly upregulated at 8:00 h vs. 12:00 h, along with *lhcf21*. Other *lhcr* were not upregulated in HL either in *T. lutea* or in *P. tricornutum* (Nymark et al., 2009). The Lhcr6 protein in *T. lutea*, in addition to Lhcf21 and Lhcx, could therefore participate in binding of photoprotective pigments or play a structural role in FCP conformation for photoprotection under HL, like Lhcx1 in *T. pseudonana* (Zhu and Green, 2010; Ghazaryan et al., 2016). Furthermore, Lhcr6 and Lhcr10 could also be classified as Lhcz (Supplementary Table 7), which might highlight the potential photoprotective function of Lhcz. Indeed, although *lhcr10* was not significantly upregulated at 8:00 h, its p-value of 0.0512 was close to the 0.05 threshold.

In *T. lutea*, lhcx2 was the only gene upregulated at night. It is known that the Lhcz4 protein in *P. tricornutum* is also more synthesized at night (Lepetit et al., 2013; Nymark et al., 2013; Taddei et al., 2016; Buck et al., 2019). Furthermore, in the transcriptomics analysis, *lhcz4* was barely expressed under HL (Nymark et al., 2009), but there was no clear explanation about its function yet. A hypothesis is that Lhcz4 could protect from the return of light after prolonged darkness exposure. We can suppose that the Lhcx2 protein in *T. lutea* plays the same role.

**CONCLUSION**

We characterized the FCP of *Tisochrysis lutea* for the first time in three ways. Initially, 52 *lh* genes were annotated. We classified the genes into 3 families, *lhcf, lhcr, and lhcx*, as for diatoms. We also identified the Lhcz subgroup within the Lhcr clade of *T. lutea*. Furthermore, we did not exclude the existence of a novel group found in diatoms named Lhczq, whose function is unknown so far. Then, we evaluated the light-harvesting capacities of *T. lutea* by locating pigment-binding domains in Lhcz protein monomers, which are likely to be homologous to those of *P. tricornutum*. Finally, we investigated the expression of *lh* genes. The study on dynamic photopersiodic cycle and dynamic N intake within the culture of *T. lutea* demonstrated a very sensitive dynamics of both photosynthetic and photoprotective pigments, along with clear difference in *lh* gene expression.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA787725.

**AUTHOR CONTRIBUTIONS**

AP wrote the article, analyzed the pigment data and raw RNA-seq transcripts, and interpreted all the data of this study. EN and JL contributed greatly to the writing, ordering, and correction of Introduction, Results, and Discussion, and to the design of the graphics. EN and JL contributed greatly to the writing, ordering, and correction of Introduction, Results, and Discussion, and to the design of the graphics. EN and JL contributed greatly to the writing, ordering, and correction of Introduction, Results, and Discussion, and to the design of the graphics. EN and JL contributed greatly to the writing, ordering, and correction of Introduction, Results, and Discussion, and to the design of the graphics. EN and JL contributed greatly to the writing, ordering, and correction of Introduction, Results, and Discussion, and to the design of the graphics.

AP, BS-J, J-BB, CB, and MG designed, planned, realized, and analyzed the experiments. OB designed and analyzed the DL experiments and contributed to its funding. NR contributed to the creation of...
the R process for RNA-seq analysis. LM as thesis director of AP, contributed in supervising the writing of the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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