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Light Regulation of *LHCX* Genes in the Benthic Diatom *Seminavis robusta*

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Intertidal benthic diatoms experience a highly variable light regime, which especially challenges these organisms to cope with excess light energy during low tide. Non-photochemical quenching of chlorophyll fluorescence (NPQ) is one of the most rapid mechanisms diatoms possess to dissipate excess energy. Its capacity is mainly defined by the xanthophyll cycle (XC) and Light-Harvesting Complex X (LHCX) proteins. Whereas the XC and its relation to NPQ have been relatively well-studied in both planktonic and benthic diatoms, our current knowledge about LHCX proteins and their potential involvement in NPQ regulation is largely restricted to planktonic diatoms. While recent studies using immuno-blotting have revealed the presence of light regulated LHCX proteins in benthic diatom communities and isolates, nothing is as yet known about the diversity, identity and transcriptional regulation or function of these proteins. We identified *LHCX* genes in the draft genome of the model benthic diatom *Seminavis robusta* and followed their transcriptional regulation during a day/night cycle and during exposure to high light conditions. The *S. robusta* genome contains 17 LHCX sequences, which is much more than in the sequenced planktonic model diatoms (4–5), but similar to the number of *LHCX* genes in the sea ice associated diatom *Fragilariopsis cylindrus*. *LHCX* diversification in both species, however, appears to have occurred independently. Interestingly, the *S. robusta* genome contains *LHCX* genes that are related to the LHCX6 of the model centric diatom *Thalassiosira pseudonana*, which are lacking in the well-studied pennate model diatom *Phaeodactylum tricornutum*. All investigated *LHCX* genes, with exception of *SrLHCX6,* were upregulated during the daily dark-light transition. Exposure to 2,000 μmol photons m^{-2} s^{-1}, furthermore, increased transcription of all investigated *LHCX* genes. Our data suggest that the diversification and involvement of several light regulated *LHCX* genes in the photophysiology of *S. robusta* may represent an adaptation to the complex and highly variable light environment this benthic diatom species can be exposed to.

Keywords: diatom, microphytobenthos, light stress, LHCX, physiology

INTRODUCTION

Due to the complex interplay of diurnal and tidal cycles and weather conditions, the surface sediments of tidal flats experience highly variable light conditions. Nevertheless, they are very productive ecosystems thanks to the presence of biofilms dominated by benthic microalgae (microphytobenthos) and especially diatoms (Underwood and Kromkamp, 1999). The fluctuating light conditions challenge these diatoms to maximize light harvesting under low light (LL) conditions while avoiding oxidative damage to their photosynthetic apparatus under high light (HL), either by minimizing light absorbance or by the dissipation of excess light energy. Benthic diatoms possess two main strategies which are fast enough to track rapid fluctuations in light intensity, namely, vertical migration and dissipation of excess energy as heat (Lavaud and Goss, 2014; Laviale et al., 2016). Raphid pennate diatoms possess a special cell wall structure called the raphe through which mucilage is secreted, allowing the diatoms to move. Such motile diatoms, often referred to as epipelic diatoms, can form dense biofilms on fine-grained sediments (Sabbe, 1993; Ribeiro et al., 2013) and are able to position themselves within the sediment light gradient via vertical migration (Admiraal, 1984; Consalvey et al., 2004; Serôdio et al., 2006; Cartaxana et al., 2016). Dissipation of excess light energy as heat can be measured as Non-Photochemical Quenching of chlorophyll *a* fluorescence (NPQ). In diatoms, NPQ comprises a quickly and a slowly relaxing component (Lavaud and Goss, 2014). We will refer to the quickly relaxing component as "flexible NPQ" (Niyogi and Truong, 2013) and to the slowly relaxing component as "sustained NPQ" or NPQs (Lavaud and Goss, 2014). The capacity for flexible NPQ is mainly defined by the xanthophyll cycle (XC) pigment diatoxanthin (Dtx) produced via de-epoxidation of diadinoxanthin (Ddx) (Lavaud and Goss, 2014; Barnett et al., 2015; Goss and Lepetit, 2015; Blommaert et al., 2017), and the presence of Light-Harvesting Complex X (LHCX) proteins (Bailleul et al., 2010; Ghazaryan et al., 2016; Taddei et al., 2016, 2018; Lepetit et al., 2017). While the XC in benthic diatoms has been well-studied in natural communities (van Leeuwe et al., 2008; Jesus et al., 2009; Serôdio et al., 2012; Laviale et al., 2015) and more recently also using unialgal isolates (Barnett et al., 2015; Blommaert et al., 2017), our current knowledge about LHCX proteins as an NPQ regulator is mostly based on studies of planktonic diatoms (Nymark et al., 2009, 2013; Zhu and Green, 2010; Zhu et al., 2010; Büchel, 2014; Lavaud and Goss, 2014; Valle et al., 2014; Dong et al., 2015; Goss and Lepetit, 2015; Ghazaryan et al., 2016; Grouneva et al., 2016; Lepetit et al., 2017; Taddei et al., 2018, 2016). The latter includes studies on the pennate model diatom *Phaeodactylum tricornutum* which to date has only been isolated from water samples from various coastal environments, but may have a benthic growth phase as well (De Martino et al., 2007).

Light-Harvesting Complex X proteins are closely related to the Light-Harvesting Complex Stress-Related (LHCSR) proteins that are present in most eukaryotic algae and mosses, but absent in flowering plants (Niyogi and Truong, 2013; Goss and Lepetit, 2015). Even though LHCX/LHCSR proteins are Light Harvesting Proteins, they have an energy dissipating rather than a light harvesting function (Niyogi and Truong, 2013). LHCSR proteins appear to function both as excess light sensors and quenching sites (Bonente et al., 2011; Ballottari et al., 2016). Therefore, they possess protonatable amino-acid residues that sense a low luminal pH (ΔpH) (Ballottari et al., 2016). A similar function as NPQ regulators has been proposed for LHCX proteins in planktonic diatoms as HL induces LHCX transcription and enhances LHCX protein content (Oeltjen et al., 2002; Nymark et al., 2009, 2013; Bailleul et al., 2010; Park et al., 2010; Zhu and Green, 2010; Lepetit et al., 2013, 2017; Taddei et al., 2016; Hippmann et al., 2017). LHCX proteins are hypothesized to bind the XC pigments Ddx and Dtx (Beer et al., 2006; Lepetit et al., 2013) and change the supramolecular organization of antenna complexes (Ghazaryan et al., 2016). Furthermore, excess energy dissipation, mediated by LHCX proteins, was linked to a decrease in the functional absorption cross-section of photosystem II (Buck et al., 2019).

Light-Harvesting Complex X function and transcriptional regulation has been intensively studied in the diatom *P. tricornutum* (Nymark et al., 2009, 2013; Bailleul et al., 2010; Lepetit et al., 2013, 2017; Valle et al., 2014; Taddei et al., 2016; Buck et al., 2019). The *P. tricornutum* genome contains four *LHCX* genes (*LHCX*1-4). Of these four genes, *LHCX1* is highly expressed in non-stressful light conditions, whilst additional expression upon HL exposure is low (Nymark et al., 2009; Lepetit et al., 2013; Taddei et al., 2016, 2018). Its corresponding protein is consequently present in LL conditions, where it provides the diatom with a basal capacity for NPQ, localized mainly near the PSII core in *P. tricornutum*, to cope with sudden changes in light climate (Taddei et al., 2018). In addition, the different content in LHCX1 between different *P. tricornutum* ecotypes has been related to their natural variability in NPQ capacity (Bailleul et al., 2010). In (prolonged) HL conditions, both transcription of *LHCX*2 and *LHCX*3 is strongly induced (Nymark et al., 2009; Lepetit et al., 2013, 2017; Taddei et al., 2016). As both proteins accumulate in concert with the *de novo* synthesis of Ddx + Dtx, they may provide additional Ddx/Dtx binding sites to activate NPQ in the antenna to enhance the basal NPQ provided by LHCX1 (Lepetit et al., 2013, 2017; Taddei et al., 2018; Buck et al., 2019). Indeed, overexpression of both *LHCX*2&3 has been shown to rescue NPQ in a low-NPQ ecotype of *P. tricornutum* (Pt4) (Taddei et al., 2016).

LHCX4 gene expression is inhibited by light, whilst its transcript accumulates in prolonged darkness, questioning its role in photoprotection (Lepetit et al., 2013; Nymark et al., 2013; Taddei et al., 2016; Buck et al., 2019).

The findings for *P. tricornutum* may not be directly transferable to other pennate diatoms, as for instance 11 *LHCX* genes were discovered in the genome of the sea ice diatom *Fragilariopsis cylindrus*, none of which could readily be related to the four *LHCX* genes in *P. tricornutum* (Mock et al., 2017). In addition, the *F. cylindrus* genome contains an *LHCX* gene that is related to the *LHCX6* in *Thalassiosira pseudonana*, whereas a similar sequence is absent in the *P. tricornutum* genome. The *T. pseudonana* LHCX6 protein has been hypothesized to be associated with Dtx binding and as such play a direct role in excess energy dissipation via NPQs during acclimation to

prolonged HL stress (Zhu and Green, 2010). This protein, furthermore, is downregulated in fluctuating light conditions (Grouneva et al., 2016), underscoring its potential role in more prolonged oversaturating light conditions.

Recent studies using immuno-blotting revealed the presence of several light-regulated LHCX-proteins in natural communities and isolates of the microphytobenthic diatom *S. robusta*, of which some differ in size from *P. tricornutum* homologs (Laviale et al., 2015; Blommaert et al., 2017). To date, however, nothing is known about the diversity, organization and transcriptional regulation of these LHCX proteins in truly benthic diatoms. In the present study, we therefore identified *LHCX* genes in the *S. robusta* draft genome and followed their transcriptional regulation during a day/night cycle and during exposure to HL conditions. In addition, we investigated the conservation of potential ΔpH sensing amino-acid residues.

MATERIALS AND METHODS

Culture Conditions

Seminavis robusta strain 85A was obtained from the diatom culture collection (BCCM/DCG) of the Belgian Coordinated Collection of Micro-organisms[1], accession number (DCG 0105). Diatom cultures were grown at 20°C in semi-continuous batch culture in 1.8 L glass Fernbach flasks (Schott) under a day/night rhythm of 16/8 h with a light intensity of 20 μmol photons m^{-2} s^{-1}. Cells were cultured in Provasoli's enriched f/2 seawater medium (Guillard, 1975) using Tropic Marin artificial sea salt (34.5 g L^{-1}) enriched with $NaHCO_3$ (80 mg L^{-1} final concentration). Cultures were acclimated to these culturing conditions for at least 2 weeks.

Day/Night Cycle and Prolonged Darkness

After acclimation, *S. robusta* was grown in 650 mL culture flasks (Greiner bio-one) to monitor *LHCX* expression during a 16/8-h day/night cycle and in parallel during an extended dark cycle (during which cultures were kept in the dark for 24 h). Three biological replicates were sampled independently at 0:00, the start of the daily dark period, at 6:00, 2 h before the start of the light period, at 15 min, 1 h and 4 h after the start of the day period, and at the end of the light period (24:00). Gene expression was compared to gene expression in the samples at 6:00 h.

High Light Exposure

High light exposure was identical to the conditions described in Blommaert et al. (2017) and imposed about 6–8 h after the onset of the daily light period. Cultures in exponential growth were concentrated to 10 mg L^{-1} Chl *a* (determined spectrophotometrically, Jeffrey and Humphrey, 1975) by centrifugation at 4,000 RCF for 5 min and were allowed to recover in growth conditions (20°C, 20 μmol photons m^{-2} s^{-1}) for 2 h. Immediately before the start of the experiment, $NaHCO_3$ (4 mM) was added from a 2 M stock to prevent carbon limitation during the experiment. Four 65 W white light energy-saving lamps (Lexman) were used to provide HL conditions (2,000 μmol photons m^{-2} s^{-1}) as used by Lepetit et al. (2013). Cells were continuously stirred in a glass test tube to obtain a homogenous cell suspension. The glass test tube was continuously cooled in a custom-made glass cooler by a water bath at 20°C. Three biological replicates were sampled immediately before the onset of 2,000 μmol photons m^{-2} s^{-1} and after 15, 30, and 60 min of HL. Gene expression in the treated samples was compared to the samples before HL (0 min).

RNA Extraction and cDNA Synthesis

Four milliliters of cell culture was sampled each time on 3 μm Versapor filters (PALL Corporation). The filter was washed with ice-cooled phosphate buffered saline (PBS) and immediately frozen in liquid nitrogen. Samples were stored at -80°C before RNA extraction. RNA extraction was based on Le Bail et al. (2008). Frozen samples were immediately incubated in 500 μL extraction buffer (100 mM Tris–HCl pH 7.5, 2% CTAB, 1.5 M NaCl, 50 mM EDTA, and 10% β-mercapto-ethanol) and subsequently beaten with carbid beads for 30 min in a bead-beater at 30 Hz. One hundred microliters of 10% Chelex-100 was added before the samples were incubated for 15 min at 56°C with occasional vortexing. One volume of chloroform:isoamyl alcohol (24:1, Vol/Vol) was subsequently added before shaking the samples for 25 min at 5 Hz. After centrifugation, the upper phase was transferred to a new tube and mixed with 0.3 volume of absolute ethanol to precipitate polysaccharides. One volume of chloroform was added and after centrifugation the upper phase was transferred to a fresh tube. RNA was precipitated overnight at −20°C, by adding 0.25 volumes of 12M LiCl and 1% (of final volume) β-mercapto-ethanol. The next day, the RNA was pelleted, dried and washed with 70% ethanol. Residual DNA was eliminated with DNAse I (Turbo DNAse, Ambion) according to the manufacturer's instructions. Extraction was performed with 1 volume Phenol-Chloroform (1:1, Vol/Vol). After centrifugation the upper phase was transferred to a fresh tube, extracted with one volume of chloroform:isoamylic alcohol (24:1, Vol/Vol) and centrifuged again. The upper phase was precipitated with 0.3 M NaOAc (pH 5.5) and 100% ice-cold ethanol by incubating for 1 h at −80°C. After the samples were centrifuged for 20 min at 4°C, the supernatant was discarded, and the pellet washed with 70% ethanol. The pellet was finally resuspended in RNAse-free water. The samples were reverse transcribed using Bio-Rad iScript cDNA kit.

Identification of *LHCX Genes* in the *S. robusta* Draft Genome

LHCX sequences from *T. pseudonana*, *P. tricornutum*, *F. cylindrus* and *Pseudo-nitzschia multiseries* (with kind permission of E. V. Armbrust) were obtained from the JGI database[2]. These LHCX sequences were used to build an amino-acid HMM (Hidden Markov Model) profile using HMMer (version 3.1b1; Mistry et al., 2013), which was used to search all genes (annotation version 1.0) predicted for the

[1] http://bccm.belspo.be/about-us/bccm-dcg

[2] http://genome.jgi.doe.gov/

TABLE 1 | Gene ID, GenBank accession number, Name, calculated molecular weight (Mw) and primer specificity. Name colours match the clade colours used in **Figure 1**.

Gene ID	GenBank accession	Name	Mw (kDa)	Primers: SrLHCX1	SrLHCX2	SrLHCX3	SrLHCX3h	SrLHCX4a	SrLHCX4b	SrLHCX6
Sro149_g068340	MN603019	SrLHCX1a	19.24							
Sro288_g108750	MN603018	SrLHCX1b	19.27							
Sro149_g068330	MN603017	SrLHCX2	19.51							
Sro656_g182410	MN603023	SrLHCX3a	20.83							
Sro235_g094670	MN603022	SrLHCX3b	20.78							
Sro2423_g327210	MN603015	SrLHCX3c	20.88							
Sro1506_g278310	MN603014	SrLHCX3d	20.87							
Sro1430_g271970	MN603013	SrLHCX3e	20.82							
Sro844_g209920	MN603012	SrLHCX3f	20.81							
Sro829_g208070	MN603011	SrLHCX3g	20.88							
Sro122_g059170	MN603010	SrLHCX3h	20.95							
Sro2297_g322430	MN603021	SrLHCX3i	20.81							
Sro412_g137900	MN603024	SrLHCX4a	19.27							
Sro652_g181650	MN603016	SrLHCX4b	19.34							
Sro1207_g252450	MN603020	SrLHCX4c	19.42							
Sro698_g189330	MN603025	SrLHCX6a	30.52							*
Sro698_g189300	MN603026	SrLHCX6b	27.01							*

Primer specificity was tested using FastPCR in silico PCR with default settings. Green shading represents an amplified PCR product, whereas orange represents the possibility of an amplified gene product, however, with one of both primers having a melting temperature 40°C < t_m < 50°C when binding on the corresponding LHCX transcript. As most LHCX3 sequences are rather similar in the primer regions, the primer LHCX3 picks up multiple related transcripts. The LHCX6 primer was designed on the SrLHCX6 RNA trailer and denoted with an "".*

in-house draft genome of the D6 strain of *S. robusta* (DCG 0489). Only hits having an E-value $\leq$ e_{-50} were retained. This way, 17 *S. robusta LHCX* (*SrLHCX*) genes were found. GenBank accession numbers are provided (**Table 1**). Similarly, LHCX/LHCSR were retrieved from *Thalassiosira oceanica, Cyclotella cryptica*, *Fistulifera solaris*, *Synedra acus*, *Pseudo-nitzschia multistriata*, the moss *Physcomitrella patens* and the brown alga *Ectocarpus siliculosus*.

A multiple alignment was made using MAFFT L-INS-i (version 7.187; Katoh and Standley, 2013) and visualized using ESPript (Robert and Gouet, 2014; **Supplementary Figure S1**; coding sequences are provided in **Supplementary Figure S2**). The signal peptide of each LHCX (between 17 AA and 22 AA) was found using ASAFind (Gruber et al., 2015) after running SignalP 3.0 (Bendtsen et al., 2004). It was removed to calculate the approximate molecular weight (Mw) using the Compute pI/Mw tool (ExPASy, Swiss Bioinformatics Institute).

This unedited alignment (383 amino-acid positions) was used for phylogenetic tree construction using IQ-tree (version 1.5.5; Nguyen et al., 2015) under the best amino-acid substitution model selected by the build-in model-selection function (ModelFinder) (Kalyaanamoorthy et al., 2017) allowing for the following set of potential models: JTT, LG, WAG, Blosum62, VT and Dayhoff. The FreeRate model (Yang, 1995) was chosen to account for rate-heterogeneity across sites and empirical amino-acid frequencies were calculated from the data. 10,000 ultra-fast bootstrapping cycles (Minh et al., 2013) were performed to validate clustering. LHCF sequences were used as an outgroup to root the tree, based on Nymark et al. (2013).

RT-qPCR

RT-qPCR was performed with a Light Cycler® 480II (ROCHE). Primer sets were designed using Primer3 (**Supplementary Table S1**). Primer specificity was tested *in silico* with FastPCR (PrimerDigital). Single nucleotide polymorphisms (SNPs) between the whole genome sequenced strain (D6) and the strain used in the experiments (85A) were identified using in-house RNAseq data (Bilcke et al., 2020) using Integrative Genomics Viewer (IGV, Broad Institute) and did not affect primer specificity. CDKA1, V4 and V1 (Moeys et al., 2016) were used for normalization as these were most stably expressed (Qbase + software). Qbase + normalized data is shown in **Supplementary Figure S3**. Log_2 expression ratios were compared with REST2013 software. The RT-qPCR program contained the following steps: pre-incubation: 95°C – 5 min, amplification: 95°C – 10 s, 58°C – 10 s, 72°C – 20 s (40 cycles), melting curve: 95°C – 5 min, 65°C – 1 min, 97°C.

RESULTS

LHCX Presence in the Genome of *S. robusta*

A HMMER search, with a profile based on annotated *LHCX* genes from *T. pseudonana*, *P. tricornutum*, *F. cylindrus* and *P. multiseries*, yielded 17 putative *LHCX* sequences in the draft *S. robusta* genome (*SrLHCX*), all with a calculated Mw of about 20 kDa (**Table 1**), with exception of SrLHCX6a and SrLHCX6b, the latter being truncated on the C-terminus and possibly being a pseudogene, see **Supplementary Figure S1**. The resulting

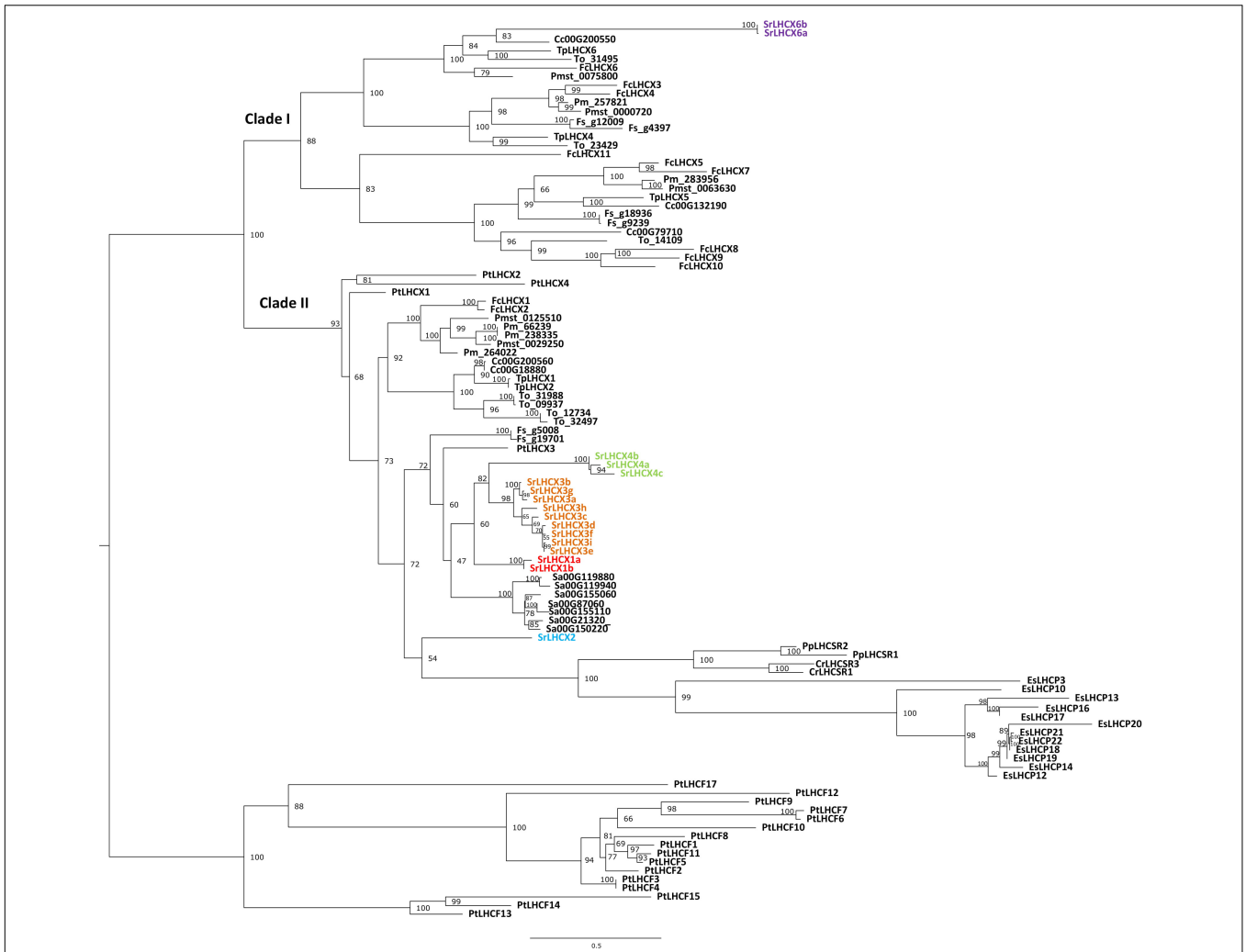

FIGURE 1 | Phylogenetic tree of *LHCX* genes in *Seminavis robusta* (Sr, colored), based on a multiple alignment made using MAFFT L-INS-i and constructed using IQ-tree. Total of 10,000 ultra-fast bootstrap approximation iterations were run (values are shown at the nodes). LHCX/LHCSR sequences are shown for the diatoms *Thalassiosira pseudonana* (Tp), *Thalassiosira oceanica* (To), *Cyclotella cryptica* (Cc), *Phaeodactylum tricornutum* (Pt), *Fragilariopsis cylindrus* (Fc), *Fistulifera solaris* (Fs), *Synedra acus* (Sa), *Pseudo-nitzschia multistriata* (Pmst) and *Pseudo-nitzschia multiseries* (Pm, with kind permission of E. V. Armbrust), the green alga *Chlamydomonas reinhardtii* (Cr), the moss *Physcomitrella patens* (Pp) and the brown alga *Ectocarpus siliculosus* (Es).

maximum-likelihood phylogenetic tree (**Figure 1**) shows two main well-supported clades, the uppermost diatom-specific clade comprising centric as well as pennate diatom sequences, but lacking sequences from the pennate model *P. tricornutum* and the araphid pennate *Synedra acus*. SrLHCX6a and b are found in a subcluster containing also TpLHCX6 and FcLHCX6.

The lower cluster contains all other SrLHCX proteins, all *P. tricornutum* proteins and proteins from the green alga *Chlamydomonas reinhardtii*, the moss *P. patens* and the brown algae *E. siliculosus*. Most SrLHCX proteins (apart from SrLHCX6a and b and LHCX2) cluster together with PtLHCX3 in one relatively well supported cluster. Within this cluster, three well-supported subclusters can be distinguished (grouping the SrLHCX1, three and four sequences). The seven *Synedra acus* LHCX proteins also cluster closely together and are related to SrLHCX1,3&4.

On a lower hierarchical level, the relatedness between both *Pseudo-nitzchia* species and *F. cylindrus* is evident, as is the case for both *Thalassiosira* species and *C. cryptica*.

LHCX Gene Expression

We studied *LHCX* expression in *S. robusta* during 24 h of a 16 h light (20 μmol photons m^2 s^{-1})–8 h dark cycle (**Figure 2A**). Cultures kept in prolonged darkness were sampled in parallel (**Figure 2B**). Note that for several primer sets multiple related transcripts can be amplified; the specificity of primer sets is given in **Table 1**. We will refer to the primer pair that amplifies multiple *SrLHCX3* transcripts as "LHCX3." Gene expression levels were compared to expression levels in samples 2 h before the light period (time point 6:00 in **Figures 2A,B**) and samples taken during the light period, compared to samples taken in parallel during the extended darkness treatment (**Figure 2C**). As in some

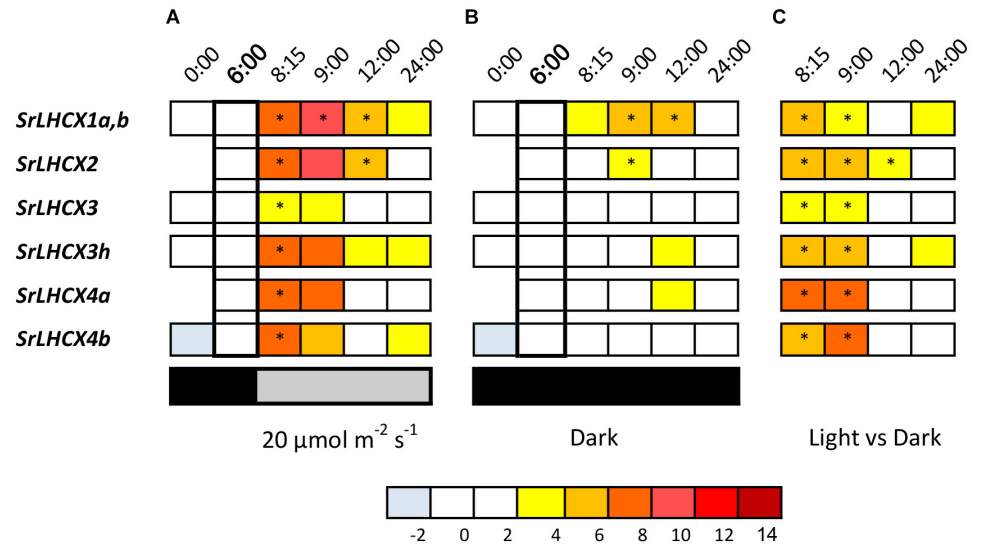

FIGURE 2 | *LHCX* transcription levels, compared to the 6:00 time point (in bold) during a 24-h 16/8 h day/night regime with light onset at 8:00 **(A)**, during extended darkness **(B)** and the transcript levels during the dark/light cycle compared to the extended dark period **(C)**. Expression ratios are $\log_2$ transformed and values are indicated by the color chart. Values are averages of three independent biological replicates. As for *SrLHCX2* and *SrLHCX4a* no transcripts were detected at (0:00) in some replicates, no values are shown. Significant changes ($p < 0.05$, Pairwise Fixed Reallocation Randomization Test performed by REST2006) are indicated with an asterisk.

replicates *SrLHCX2* and *SrLHCX4a* transcripts were not detected at 0:00, data for this timepoint is not shown. Due to considerable variance between technical replicates of *SrLHCX6* throughout the 24-h cycle, these data are not shown.

All investigated *SrLHCX* genes showed a significant upregulation 15 min after the dark/light transition compared to 2 h before light onset (**Figure 2B**), which was not the case for samples kept in prolonged darkness (**Figure 2B**). For samples kept in prolonged darkness only *SrLHCX1a,b* and *SrLHCX2* were significantly upregulated 1 h after the light period would have started, compared to 6:00. Transcript levels for all investigated *SrLHCX* genes were significantly higher in the cells sampled 15 and 60 min after the dark/light transition, compared to cells which were kept in prolonged darkness (**Figure 2C**).

In addition, we studied the expression of *SrLHCX* genes in *S. robusta* in response to HL (**Figure 3**). All investigated *SrLHCX* genes were highly upregulated after 15, 30, and 60 min of HL, compared to the LL before HL exposure, except for *LHCX3h* at 60 min. *SrLHCX4b* and *SrLHCX6* showed significantly higher expression at 30 min, compared to 15 min of HL, whereas *SrLHCX2* declined significantly in expression between 15 and 30 min of HL. Between 30 and 60 min of HL, *LHCX2*, *LHCX3*, and *LHCX3h* declined significantly in expression.

We investigated the presence of three amino-acid residues which are known to function as sensors of the thylakoid lumen pH in the LHCSR3 in *Chlamydomonas reinhardtii* and which are indispensable for NPQ functioning (Ballottari et al., 2016). Two of these are also present in *P. tricornutum* LHCX1-3 sequences, but only one in LHCX4 (**Figure 4**). SrLHCX6 contains none of the protonatable residues in *C. reinhardtii* as is the case for LHCX6 in *T. pseudonana*. The same residues are conserved in all SrLHCX sequences as in PtLHCX1-3, with the exception of SrLHCX4a,b,c which lack the same residue as PtLHCX4. However, unlike the PtLHCX4 sequence, the SrLHCX4a-c sequences contain a glutamate residue (E, highlighted in yellow), which may have a protonatable function.

DISCUSSION

As LHCX proteins play a central role in the NPQ mechanism of planktonic diatoms (Bailleul et al., 2010; Zhu and Green, 2010; Lepetit et al., 2013, 2017; Taddei et al., 2016) and light responsive LHCX-proteins have been observed in benthic diatom isolates and communities (Laviale et al., 2015; Blommaert et al., 2017), we investigated the presence of *LHCX* genes in the benthic diatom *S. robusta* and studied their transcriptional regulation during HL conditions and a darkness/LL transition.

We detected 17 *LHCX* genes in *S. robusta*, which is a high number compared to the model diatoms *P. tricornutum* (4) and *T. pseudonana* (5) but in the same range as in the psychrophilic sea ice diatom *F. cylindrus* (11) (Armbrust et al., 2004; Bowler et al., 2008; Mock et al., 2017), the brown alga *E. siliculosus* (13) and the haptophyte *Emiliania huxleyi* (15) (see Dittami et al., 2010 for an overview and a comprehensive phylogenetic tree). Even though a comparable number of *LHCX* genes was discovered in both *S. robusta* and *F. cylindrus*, *LHCX* diversification in both raphid diatoms seems to have occurred independently as *LHCX* genes of both species were found to belong to different clades, with most diversity in *S. robusta* being related to *PtLHCX3*. Interestingly, a diversification of *LHCX* genes related to *PtLHCX3* seems to have occurred as well in the araphid diatom *Synedra acus*. There seems, however, no general

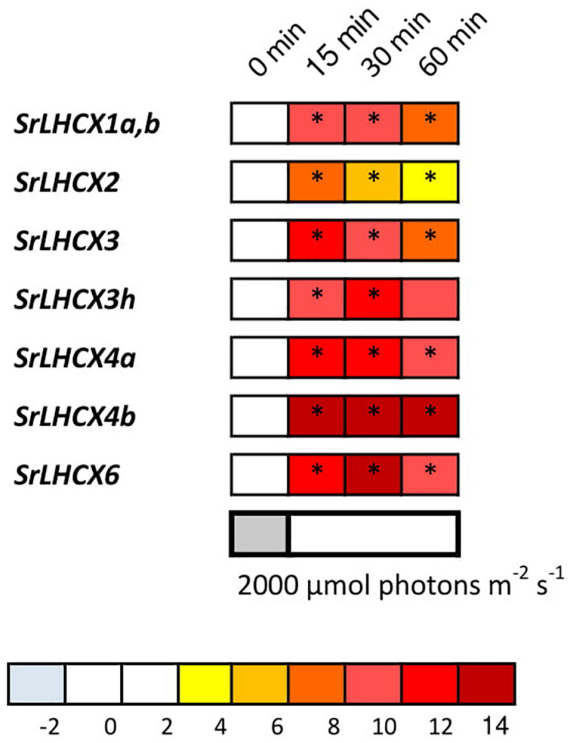

FIGURE 3 | *LHCX* transcription levels during exposure to high light (2,000 µmol photons m^{-2} s^{-1}). Expression ratios are log_2 transformed and indicated by the color chart. Values are averages of three independent biological replicates and relative to the respective initial values (LL). Significant changes at $p < 0.05$ (Pairwise Fixed Reallocation Randomization Test performed by REST2006) are indicated with an asterisk.

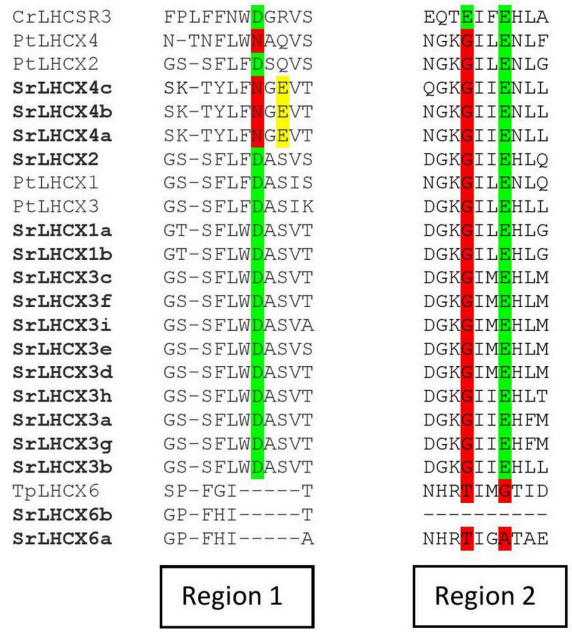

FIGURE 4 | Amino-acid alignment of regions 1 and 2 of *Chlamydomonas reinhardtii* CrLHCSR3, *P. tricornutum* LHCX1-4, *T. pseudonana* LHCX6, and all LHCX sequences of *S. robusta*. The highlights in green represent conserved pH-sensing residues, whereas red highlights represent the absence of a conserved pH-sensing residue. Glutamate residues (E) in SrLHCX4a-c are highlighted in yellow.

trend in the amount of *LHCX* genes in the genomes of centric and pennate diatoms.

Even though a certain degree of functional redundancy can be expected due to the high number of *LHCX* genes in *S. robusta*, transcription appears to be light regulated for all the studied genes and all investigated *LHCX* transcripts were strongly upregulated in HL conditions. However, the limited specificity of the LHCX3 primer combination makes it difficult to generalize about *LHCX* genes in this clade. Our results, as such, do not allow to conclude why *S. robusta* has such a high number of *LHCX* genes. The expansion of gene families seems to be a general feature of *S. robusta* and is not exclusive to the *LHCX* genes. The large genome size of S. *robusta* allows for duplication events which could in turn lead to adaptive evolution (Osuna-Cruz et al., 2020). A large set of *LHCX* genes could be required to cope with highly variable light conditions. However, *P. tricornutum* only possesses four *LHCX* genes, and these still enable the species to rapidly adjust to a highly fluctuating light climate (Lepetit et al., 2017). In addition, the ability of motile epipelic diatoms to rapidly migrate away from strong light conditions could minimize the need for strong physiological photoprotection (Serôdio et al., 2001; Barnett et al., 2015; Laviale et al., 2016; Blommaert et al., 2018) and hence also the need for so many functional genes in *S. robusta*. Lastly, the presence of many *LHCX* genes may allow to integrate various environmental signals and stresses to finely regulate LHCX content (Taddei et al., 2016; Buck et al., 2019).

LHCX transcripts are present during a LL day/night cycle in *S. robusta*. This is consistent with the presence of an LHCX protein in LL conditions, as reported by Blommaert et al. (2017). This protein, as *PtLHCX1,* could provide a basal NPQ capacity localized near the PSII core (Nymark et al., 2009; Bailleul et al., 2010; Lepetit et al., 2013, 2017; Taddei et al., 2018). As all *SrLHCX* transcripts (except *SrLHCX*6 for which data are lacking) were induced upon a dark/light transition, we cannot rule out that multiple SrLHCX proteins fulfill a similar role as PtLHCX1. Linking transcriptional data and immuno-blotting, in this case, is not straightforward as the used antibody (anti-LHCSR3, Bonente et al., 2011) was not specifically designed to recognize diatom LHCX isoforms. In addition, the large number of LHCX genes of similar sizes (**Table 1**) and differences in actual and predicted LHCX/LHCSR protein size, because of posttranslational modifications (Bonente et al., 2011), complicate the comparison of both datasets as was also observed for the discrepancies in transcriptional and translational regulation of LHCXs in *P. tricornutum* and *T. pseudonana* (Zhu and Green, 2010; Lepetit et al., 2017).

High light-induced transcription of *LHCX* genes as observed in *S. robusta* was reported for planktonic diatoms (Nymark et al., 2009; Zhu and Green, 2010; Lepetit et al., 2013; Taddei et al., 2018) and may enhance the basal NPQ level provided by the LHCX protein(s) already present in LL (Taddei

et al., 2018). HL induced transcription of *LHCX* genes in *S. robusta* is indeed reflected on the protein level after 1 h (Blommaert et al., 2017). Even though we did not study the effect of prolonged HL on LHCX accumulation, *S. robusta* is able to increase its NPQ capacity together with its XC features during long-term acclimation upon a shift from 20 to 75 μmol photons m^{-2} s^{-1} (Barnett et al., 2015). The accumulation of LHCX proteins during HL was also reported for natural benthic communities and in the benthic diatom *Navicula phyllepta* (Laviale et al., 2015) and may together with XC pigments allow epipelic species to acclimate to prolonged higher light conditions (Barnett et al., 2015; Ezequiel et al., 2015).

*SrLHCX*6, interestingly, is not closely related to the majority of *LHCX* genes in *S. robusta*: *SrLHCX*6 clusters in a clade containing *LHCX*6 of the centric diatom *T. pseudonana* and of the pennate diatom *F. cylindrus*. As *SrLHCX*6 was strongly upregulated in HL, it may play a role in sustained quenching (NPQs), as proposed for the LHCX6 protein in *T. pseudonana* (Zhu and Green, 2010; Grouneva et al., 2016). This matches the observation of sustained quenching and *de novo* Dtx synthesis in *S. robusta* under identical HL conditions (Blommaert et al., 2017). In *N. phyllepta*, which is phylogenetically closely related to *S. robusta* (Chepurnov et al., 2008), the anti-LHCX6 antibody raised against LHCX6 in *T. pseudonana* recognized a HL inducible LHCX protein, whose size (∼33 kDa) is similar to the calculated size of SrLHCX6 (**Table 1**; Laviale et al., 2015). The same antibody, nonetheless, failed to recognize a protein of any size in *S. robusta* (Blommaert et al., 2017).

*SrLHCX*4b was highly expressed upon HL exposure and remained highly expressed even after 60 min of HL exposure. This gene is closely related to *SrLHCX4a* and *SrLHCX4c* and seems to be differentiated from the majority of *SrLHCX3* sequences. A major difference between the SrLHCX4 proteins and the other SrLHCX proteins (except for SrLHCX6) is that only one instead of two protonatable amino-acid residues (compared to the three amino-acids responsible for the switch to energy-dissipating mode in *C. reinhardtii*) is conserved. A similar difference in amino-acid sequence has been reported for PtLHCX4, compared to the other *P. tricornutum* proteins, the former, however, being induced only in prolonged darkness (Nymark et al., 2013; Taddei et al., 2016). Even though NPQ regulation by a light-induced luminal pH change in diatoms is less clear than in green algae and plants, which relax NPQ immediately after ΔpH breakdown (Goss and Lepetit, 2015), the above findings may suggest that SrLHCX4a–c as PtLHCX4 are not, or only to a lesser degree, controlled by a *trans*-thylakoidal proton gradient. They could contribute to a more sustained NPQ component as was suggested for TpLHCX6 (Zhu and Green, 2010), which completely lacks these residues and may provide binding sites for Dtx.

In this study, we demonstrated the presence of multiple light-regulated *LHCX* genes, which may allow epipelic species to respond and/or acclimate to prolonged higher light conditions (Barnett et al., 2015; Ezequiel et al., 2015), either through an increase of the flexible NPQ component or through more sustained quenching (Lavaud and Goss, 2014). Our study paves the way for future detailed investigations with defined experimental approaches in order to dissect the specific regulation and function of individual *SrLHCX* genes and proteins, involving e.g. experiments including natural light simulation conditions such as gradual increasing fluctuating light (Grouneva et al., 2016; Lepetit et al., 2017) or nutrient starvation (Taddei et al., 2016; Hippmann et al., 2017), the localization of NPQ (Taddei et al., 2018) as well as reverse genetics approaches (Bailleul et al., 2010) as soon as *S. robusta* becomes transformable.

DATA AVAILABILITY STATEMENT

The datasets for this study can be found in GenBank: https://www.ncbi.nlm.nih.gov/genbank/. Accession numbers are provided in **Table 1**.

AUTHOR CONTRIBUTIONS

LB: experimental design, practical work, and manuscript writing. EV and MH: bioinformatics and manuscript writing. CO-C: bioinformatics. SD: molecular lab work. JL, BL, and KS: experimental design and manuscript writing. PW and AB: *S. robusta* sequencing, gene annotation, and bioinformatics. KV: supervision bioinformatics. WV: manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00192/full#supplementary-material

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