Early Stage Adaptation of a Mesophilic Green Alga to Antarctica: Systematic Increases in Abundance of Enzymes and LEA Proteins

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Associate editor: Irina Arkhipova

The Chlorella vulgaris NJ-7 Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number VATV000000000 (version VATV010000000). The C. vulgaris UTEX259 Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number VATW000000000 (version VATW010000000). Assembled transcripts ≥200 nt have been deposited in the NCBI Transcriptome Shotgun Assembly (TSA) sequence database with accession numbers GHLW00000000 for NJ-7 and GHLX00000000 for UTEX259. Raw sequence read data have been deposited in the NCBI Sequence Read Archive (SRA) with the following study identifiers: SRP198424 for NJ-7 transcriptome and RNA-seq; SRP198705 for UTEX259 transcriptome and RNA-seq. Organellar genome sequences have been deposited at the NCBI GenBank under accession numbers MK948100 (NJ-7 chloroplast genome), MK948101 (NJ-7 mitochondrial genome), MK948102 (UTEX259 chloroplast genome), and MK948103 (UTEX259 mitochondrial genome). The mass spectrometry proteomics raw data have been deposited to the ProteomeXchange Consortium via the MassIVE partner repository (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp; last accessed November 24, 2019) with the data set identifier PXD014018.

Abstract

It is known that adaptive evolution in permanently cold environments drives cold adaptation in enzymes. However, how the relatively high enzyme activities were achieved in cold environments prior to cold adaptation of enzymes is unclear. Here we report that an Antarctic strain of Chlorella vulgaris, called NJ-7, acquired the capability to grow at near 0 °C temperatures and greatly enhanced freezing tolerance after systematic increases in abundance of enzymes/proteins and positive selection of certain genes. Having diverged from the temperate strain UTEX259 of the same species 2.5 (1.1–4.1) to 2.6 (1.0–4.5) Ma, NJ-7 retained the basic mesophilic characteristics and genome structures. Nitrate reductases in the two strains are highly similar in amino acid sequence and optimal temperature, but the NJ-7 one showed significantly higher abundance and activity. Quantitative proteomic analyses indicated that several cryoprotective proteins (LEA), many enzymes involved in carbon metabolism and a large number of other enzymes/proteins, were more abundant in NJ-7 than in UTEX259. Like nitrate reductase, most of these enzymes were not upregulated in response to cold stress. Thus, compensation of low specific activities by increased enzyme abundance appears to be an important strategy for early stage cold adaptation to Antarctica, but such enzymes are mostly not involved in cold acclimation upon transfer from favorable temperatures to near 0 °C temperatures.

Key words: Antarctica, cold adaptation, intraspecies divergence, omics, enzyme activity, Chlorella vulgaris.

Introduction

Antarctica is the coldest continent on the earth, with 99.8% of the area (Burton-Johnson et al. 2016) covered by a sheet of ice that averages about 2 km thick (Fretwell et al. 2013). The lowest air temperature in Antarctica may reach −89.2 °C (National Polar Research Institute [Japan] 1991), but the ground surface temperatures on ice-free sites are in a range of −35 to 5 °C in most time of a year (Guglielmin 2006). In Antarctica, animal and plant communities are naturally separated from those on other continents (Convey and Stevens 2007; Fraser et al. 2012) and largely limited to ice-free sites that receive meltwater in warm seasons (Convey et al. 2014). In contrast, microalgae, bacteria, and fungi not only grow on these ice-free sites but also thrive in sea ice (Thomas and Dieckmann 2002), snow fields (Davey et al. 2019), and ice-covered lakes (Karl et al. 1999; Possmayer et al. 2016). Microbes from other continents could be transported to Antarctica by atmospheric circulation (Mayol et al. 2017; Cáliz et al. 2018), even though their contribution to Antarctic microbial communities is limited (Archer et al. 2019).

In the geological history, Antarctica was formed from the breakup of the supercontinent Gondwana. About 34–33 Ma,
the atmospheric CO2 level significantly dropped, and the ice cover expanded rapidly on the continent to near-modern dimensions or larger than present-day values; about 23 Ma, the Drake Passage opened between Antarctica and South America (Galeotti et al. 2016; Lear and Lunt 2016). Mesophilic microorganisms that experienced the quick cooling period might evolve into psychrophilic or psychrotrophic species; on the other hand, those microorganisms transported from other continents in later periods might also develop the cold-growth capability and freezing tolerance.

The main challenge faced by organisms in Antarctica is to keep cell activities at near 0 °C temperatures and maintain survivability under freezing conditions. The freezing tolerance depends on ice-binding proteins (Bar Dolev et al. 2016; Collins and Mangesin 2019) and LEA (late embryogenesis abundant) proteins (Shih et al. 2008; Liu et al. 2011; Wang et al. 2011). These proteins prevent the growth/recrystallization of ice or freeze/thaw-induced inactivation of enzymes. Cell activities depend on various biochemical reactions. Our understanding of biochemical reactions in the cold is largely based on cold-adapted or psychrophilic enzymes (Morgan-Kiss et al. 2006). Compared with their mesophilic homologs, such enzymes feature significantly lowered optimal temperatures and adaptive mutations that destabilize the structures bearing the active site or the overall structure (Feller and Gerday 2003; Santiago et al. 2016). It has been shown that the optimal temperature or thermal stability of a mesophilic enzyme can be downshifted by site-directed mutagenesis (Saavedra et al. 2018; Liao et al. 2019) and that the activity at a low temperature can be increased by directed evolution (Zhao and Feng 2018). However, because adaptive mutations that cause coding differences are relatively rare (Jones et al. 2012) and coding differences toward cold adaptation must be even rarer, it remains to be answered whether there is an alternative strategy to maintain relatively high enzyme activities at near 0 °C temperatures before enzymes are significantly cold-adapted.

Without cold-growing microorganisms generated from mesophiles by experimental evolution, natural intraspecies divergence in the capability to grow at low temperatures would provide very valuable materials for studies. Here, we analyzed an Antarctic strain and a temperate strain of Chlorella vulgaris (green alga) at genomic, transcriptomic, and proteomic levels. Our results indicate that systematic elevation in abundance of enzymes could allow a mesophilic newcomer to develop the capability to grow at near 0 °C temperatures, representing an early stage adaptive mechanism in Antarctic environments. Remarkably, most enzymes with higher levels in the Antarctic strain are not upregulated upon transfer from 20 to 4 °C.

Results
Physiological Divergence between C. vulgaris NJ-7 and UTEX259
Chlorella vulgaris NJ-7 was isolated from rock samples collected near a transitory pond 5 km away from the Zhongshan Station (69°22′S–76°22′E) in Antarctica (Hu et al. 2008). Its 18S rRNA-ITS1-5.8S rRNA region is identical to that of C. vulgaris UTEX259, a strain initially collected and isolated from Delft (52°00′N, 04°21′E), the Netherlands (https://utex.org; last accessed November 24, 2019); its ITS2-28S rRNA region differs from UTEX259 at five bases (supplementary fig. S1, Supplementary Material online). They show similar growth at 20 °C (fig. 1a), but only NJ-7 is able to grow at 4 °C (fig. 1b); after being frozen at −20 °C, the survivability of NJ-7 is much higher than that of UTEX259, and the survivability can be enhanced by preconditioning (48-h exposure) at 4 °C (fig. 1). When cultured at 20 °C, both strains show maximal photosynthetic activities at 30 °C (supplementary fig. S2, Supplementary Material online); NJ-7 maintains higher photosynthetic activities than UTEX259 at high temperatures. These physiological characteristics indicate that NJ-7 is a mesophilic algal strain evolving toward a psychrotrophic one. The higher photosynthetic activities of NJ-7 at high temperatures could be either associated with or independent of the enhanced freezing tolerance.

Nuclear Genome Assembly and Synteny Analyses
To analyze the relationship between the two strains of the same species and the mechanism for NJ-7 to develop the cold-growth capability and freezing tolerance, we sequenced the whole genomes of both strains. Transcriptomes were also sequenced to facilitate genome assembly and gene predictions. The assembled NJ-7 nuclear genome consisted of 39.1 Mb in 753 scaffolds with an N50 size of 938 kb; the UTEX259 nuclear genome consisted of 39.1 Mb in 780 scaffolds with an N50 size of 498 kb. A total of 9,412 protein-coding genes were predicted in NJ-7 and 9,439 in UTEX259; 13,390 transcripts (isotigs) were identified in NJ-7, and 13,880 identified in UTEX259 (features are summarized in table 1, and detailed information for sequencing, assembly and annotation in supplementary tables S1–S6, Supplementary Material online).

The quality and gene coverage of genome assemblies were then assessed. First, completeness of genome assemblies was assessed by BUSCO (Simao et al. 2015). Of the 2,168 universal single-copy orthologs of the chloroplast gene set, 90.0% were found in the NJ-7 genome assembly as complete genes, and 90.3% found in UTEX259; 4.6% and 4.3% were completely missing in NJ-7 and UTEX259 assemblies, respectively (supplementary table S7, Supplementary Material online). Numbers of predicted genes in C. vulgaris strains NJ-7 and UTEX259 are close to those of C. variabilis NC64A and Coccomyxa subellipsoidea C-169. Second, the genome assemblies were evaluated by aligning with assembled transcripts (Sanger-based fosmid-end sequences also used for assessing NJ-7). For NJ-7, about 97.3% of the fosmid-end sequences were mapped to the genome assembly, and 93.5% of the transcripts mapped over at least 90% of their length. Of 956 pairs of fosmid-end sequences, 790 pairs were mapped to the same scaffold, and the average distance was 34.7 kb, almost identical to the estimated average insert size of fosmid clones (35.0 kb). To the UTEX259 genome assembly, about 93.4% of the
transcripts were mapped over at least 90% of their length. Single-base mismatch and insertion/deletion frequencies of the two genome assemblies were <1.0 base/10 kb (compared with sequences generated by Sanger sequencing). These results indicated the high coverage and high quality of the genome assemblies.

Similarities between nuclear genomes of the two strains were evaluated with synteny and collinearity analyses. Scaffolds longer than 10 kb, which covered over 97% of each genome, were used to generate the syntenic dot plot (supplementary fig. S3, Supplementary Material online). The result indicated that syntenic regions covered 99.37% of the genome assemblies.

Comparison of Organelle Genomes and Estimation of Divergence Time

The relationship between the two strains was also shown with their organelle genomes and divergence time. Except for two genomic inversions and differences in psbA and psbC (with or without an intron that contains an endonuclease gene), the chloroplast genomes of NJ-7 and UTEX259 are identical in gene content and arrangement (supplementary fig. S5 and table S9, Supplementary Material online). The two chloroplast genomes closely resemble that of *C. vulgaris* C-27 [a strain isolated from Sendai (38°16′N, 141°02′E), Japan (NIES-2170, http://mcc.nies.go.jp; last accessed November 24, 2019)] but differ from those of other *Chlorella* strains in gene arrangement (supplementary fig. S6, Supplementary Material online). The mitochondrial genomes of NJ-7 and UTEX259 are even more similar to each other (supplementary fig. S7 and table S9, Supplementary Material online). Organelle genomes of NJ-7 are smaller than that of UTEX259 due to the reduced non-coding regions.

Divergence times between NJ-7, UTEX259, other green algae, and higher plants were estimated based on sequences of 36 chloroplast genes, with three fossil calibrations as previously reported (Herron et al. 2009). Broad-scale analyses were performed with four combinations of species/strains (fig. 2a; supplementary fig. S8a–c, Supplementary Material online). It is deduced that NJ-7 diverged from UTEX259 about 2.5 (95% confidence interval 1.1–4.1) Ma to 2.6 (1.0–4.5) Ma, much later than the divergence between NJ-7/UTEX259 and NC64A [153.4 (69.2–247.1) Ma], also later than the opening of Drake Passage (~23 Ma). The structure of the chronogram in figure 2a is consistent with the dendrograms based on amino acid and nucleotide sequences of 1,080 nuclear genes (supplementary fig. S9a and b, Supplementary Material online). In addition to the broad-scale chronograms, we also constructed a fine-scale chronogram for *Chlorella* species, in which the divergence time between UTEX259 and NJ-7 (and C-27) was estimated to be 2.9 (0.6–6.4) Ma (fig. 2b). In light of the mesophilic characteristics of NJ-7 and the estimated time for its divergence from UTEX259, the ancestor of
NJ-7 was probably transported to the Antarctic continent from a temperate region.

Genomic Evolution toward Cold Adaptation of NJ-7

Cold adaptation is an evolutionary process toward the capabilities to grow at near 0 °C temperatures and survive under freezing conditions. This process depends on accumulation of adaptive mutations and leads to divergence between strains.

With the genomic data, we first searched for genetic changes that may be associated with cold adaptation. NJ-7 shows much less nonsynonymous substitutions per site and gene duplications than UTEX259 (supplementary table S10, Supplementary Material online), but the two strains are comparable in numbers of positively selected genes (supplementary excel S1, Supplementary Material online) and alternative splicing events (supplementary excel S1, Supplementary Material online).

Fig. 2. Chronograms showing estimated divergence times for green algae and higher plants. Divergence times were estimated using BEAST based on 36 chloroplast genes. Branch lengths are proportional to the absolute ages of nodes (scale on x-axis in million years). Numbers to the right or left of nodes are the ages of nodes and 95% confidence intervals (in parentheses). (a) One of the broad-scale chronograms. A, B, and C indicate the nodes to which time constrains were applied based on fossil records. The other three chronograms (with different combinations of species/strains) are shown in supplementary figure S8, Supplementary Material online. (b) The fine-scale chronogram. The divergence time between Chlorella variabilis and C. vulgaris estimated from the broad-scale analysis, in particular 157.0 Ma (73.6–245.1 Ma), was used to calibrate the fine-scale analysis (indicated by an arrow) but slightly changed after the computations. Of the four divergence times between C. variabilis and C. vulgaris, 157.0 Ma (73.6–245.1 Ma) from (a) is close to the average.
conditions (Morgan-Kiss et al. 2006). It depends on the regulation of cold acclimation in plants, and the long chain base of sphingolipid, called sphinganine, can be desaturated by sphingolipid delta(4)-desaturase and sphingolipid delta(8)-desaturase (Luttgeharm et al. 2016). The delta(8)-desaturase plays a major role in desaturation of long chain base and cold tolerance in higher plants (Zhou et al. 2016) but is not found in Chlorella, Chlamydomonas, and Volvox. The positive selection of sphingolipid delta(4)-desaturase in NJ-7 probably reflects the critical role of sphingolipid desaturation in cold adaptation. Of genes with alternative splicing, there are seven related to DNA repair in NJ-7 but only one in UTEX259 (supplementary table S11, Supplementary Material online). These alternative splicing events in NJ-7 might have contributed to adaptation to the strong UV radiation in Antarctica (Liao and Frederick 2005).

Unique genes (supplementary excel S1, Supplementary Material online), high-copy-number genes (supplementary fig. S10 and table S12, Supplementary Material online), and expanded gene families (supplementary tables S13–S15, Supplementary Material online) were also analyzed, but no apparent connection to cold adaptation was identified. Gene duplication has been shown to be a rapid mechanism for adaptation to stressful or novel environmental conditions (Kondrashov 2012). We identified genes with multiple copies in NJ-7 and UTEX259 and analyzed the relationship between gene expression and copy numbers. Only one gene (MFS transporter) with higher copy number in UTEX259 showed increased expression relative to its homolog in NJ-7 (supplementary fig. S10 and table S12, Supplementary Material online).

Cold Adaptation and Cold Acclimation of NJ-7 Based on Altered Gene Expression

Unlike cold adaptation, cold acclimation is a physiological process that relieves the cold stress in temperature-fluctuating environments and prepares for the freezing conditions (Morgan-Kiss et al. 2006). It depends on the regulation of gene expression and metabolic pathways. To which extent genes/proteins regulated in cold acclimation are involved in cold adaptation has not been addressed.

To understand how NJ-7 adapted to the Antarctica, we further analyzed mRNA and protein profiles in NJ-7 and UTEX259 cultured at 20 °C with or without treatment at 4 °C, and identified differential gene expression in the same strain at mRNA (RNA-seq) (supplementary table S16 and excel S2, Supplementary Material online) and protein (proteomic analysis) levels (supplementary excel S3, Supplementary Material online). Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was conducted on eight genes in cells grown at 20 °C and exposed to 4 °C to evaluate the quality of RNA-seq data. Expression levels assessed with RT-qPCR correlated well ($R^2 = 0.903$) with those obtained from the RNA-seq analysis (supplementary fig. S11, Supplementary Material online). The RNA-seq and proteomic data were also used to analyze the differential expression between two strains at 20 or 4 °C (supplementary excels S2 and S3, Supplementary Material online), but the differences between protein abundance in two strains were analyzed by generating a new NJ-7/UTEX259 protein database, with which orthologous proteins were identified based on those identical trypsin-digested peptides. The RNA-seq and proteomic analyses were highly reproducible between biological replicates as shown with Pearson’s correlation coefficients (supplementary fig. S12, Supplementary Material online). Numbers of differentially expressed genes in four data sets are shown in supplementary table S17, Supplementary Material online. Genes up- or downregulated in NJ-7 or UTEX259 after transfer from 20 to 4 °C are related to cold acclimation, whereas those with altered expression in NJ-7 relative to their homologs in UTEX259 are related to strain divergence.

Using upregulated genes as the example, we first analyzed overlaps between different data sets. The results are summarized in figure 3, from which four implications can be derived: 1) Strain divergence is less dependent on regulation at mRNA level than cold acclimation. During cold acclimation, over 70% of genes [215/(215 + 84)] upregulated in NJ-7 at the protein level are concurrently upregulated at the mRNA level (see fig. 3a–I); relative to their homologs in UTEX259, only 28.1% of genes [113/(113 + 299)] with higher protein levels in NJ-7 also show higher mRNA levels (at 4 °C, see fig. 3b–II); 2) most proteins [193/(193 + 106)] upregulated in NJ-7 during cold acclimation are also upregulated in UTEX259 (see fig. 3c–I); 3) most proteins [312/(312 + 100)] with higher abundance in NJ-7 than in UTEX259 at 4 °C show higher abundance at 20 °C (see fig. 3c–III); and 4) most proteins [374/(374 + 38)] with higher abundance in NJ-7 than in UTEX259 at 4 °C are not upregulated during cold acclimation (see fig. 3c–II).

For downregulated genes, overlaps between data sets of "NJ-7/UTEX259" exhibit a similar pattern (supplementary fig. S13b, c-II, and c-III, Supplementary Material online) to those for upregulated genes (fig. 3b, c-II, and c-III), but the number of proteins downregulated during cold acclimation (supplementary fig. S13a, Supplementary Material online) is much less than that of proteins upregulated (fig. 3a), and most proteins downregulated in NJ-7 and UTEX259 during cold acclimation do not overlap (supplementary fig. S13c-I, Supplementary Material online).

Divergence between the two strains was driven by adaptation to environments and genetic drifts. Adaptation of NJ-7 to the cold environment is one of the most important factors. Of the 38 upregulated proteins (fig. 3; supplementary table S18, Supplementary Material online) involved in both strain divergence and cold acclimation, sphingolipid delta(4)-desaturase was positively selected (supplementary excel S1, Supplementary Material online). Other proteins in the list are involved in freezing tolerance (cytoprotective protein), cold stress response and tolerance, cell division, carbon metabolism, etc. As we pointed out above, many more proteins with
higher abundance in NJ-7 are not upregulated during cold acclimation. Increases in abundance of these proteins may play an important role in the maintenance of cellular activities at near 0 °C temperatures and the greatly enhanced freezing tolerance.

To identify functions enhanced in NJ-7 relative to UTEX259, we performed gene set enrichment analysis (GSEA) of differentially expressed genes based on Gene Ontology (GO) categories [supplementary fig. S14, Supplementary Material online]. NJ-7 (supplementary fig. S14a and b, Supplementary Material online) and UTEX259 (supplementary fig. S14c and d, Supplementary Material online) show positive enrichments for many GO terms in cold acclimation but only one to several GO terms in strain divergence [supplementary fig. S15, Supplementary Material online].

At 20 °C, 138 genes show increased expression at both levels in NJ-7 relative to UTEX259; at 4 °C, 113 genes. (c) Overlaps between proteomic data sets showing 193 genes upregulated at the protein level in both NJ-7 and UTEX259 during cold acclimation (I), 312 genes upregulated in NJ-7 relative to UTEX259 at both 20 and 4 °C (III), but only 38 genes upregulated in NJ-7 in both cold acclimation and strain divergence (II).

Elevated Abundance of LEA Proteins in NJ-7

LEA proteins were first identified in cotton and so named because they accumulate during the late maturation stages of seed development (Dure et al. 1981). Proteins in this family play important roles in stress tolerance in bacteria, fungi, plants, and animals (Shih et al. 2008). In particular, they can enhance freezing tolerance as cryoprotectants (Honjoh et al. 2001; Sasaki et al. 2014). In this study, we systematically extracted predicted NJ-7/UTEX259 proteins based on their similarities to gene products of the two GO categories in AmiGO 2 (http://amigo.geneontology.org/amigo; last accessed November 24, 2019). GSEA showed positive enrichments for these two groups of proteins in cold acclimation (supplementary fig. S15, Supplementary Material online).

We also identified 152 transcription factors (TFs) in NJ-7 and 163 TFs in UTEX259 and analyzed their differential expression across temperatures and strains (supplementary excel S4, Supplementary Material online). The differential expression of TFs may lead to the differences in transcription of target genes. Several TFs with identified recognition motifs (for orthologs) in C. variabilis NC64A were used to test this possibility, but none of them showed a change in expression correlated with that of a potential target gene.
identified LEA protein genes in NJ-7 and UTEX259 by motif and secondary structure searches, Pfam domain searches, and similarity (to known LEA proteins) searches (supplementary table S19, Supplementary Material online). Compared with other green algae, *C. vulgaris* strains possess more LEA proteins (supplementary table S20, Supplementary Material online). Except hiC6-5 that is not found in UTEX259, all LEA protein genes are very similarly arranged in the two genomes. In *C. variabilis* NC64A, there are genes homologous to *CvLEA1* (1 copy) and *hiC6* (1 copy), but no homologs to *CvLEA7*, *CvLEA8* or *Ccor1/Ccor2*. Homologs to *Chlorella* LEA genes are not found in other green algae. Apparently, these gene clusters in *C. vulgaris*, except for the occurrence of hiC6-5, had been formed by gene duplication before the divergence between NJ-7 and UTEX259. The encoded LEA proteins are predicted to be located in the cytoplasm, nucleus, or organelles (fig. 4b) to provide cryoprotection of enzymes/proteins in different cellular compartments, but the predicted locations need to be confirmed with experiments in the future. Most LEA proteins in the two strains differ from each other by <3% amino acid residues (supplementary table S21, Supplementary Material online).

Based on proteomic analyses, we identified seven LEA proteins (five isoforms of hiC6 and two versions of Ccor2 were respectively treated as one protein, plus *CvLEA3*, *CvLEA5*, *CvLEA10*, *CvLEA11*, and *CvLEA14*) with higher abundance in NJ-7 than in UTEX259 (fold change ≥ 1.3, P-value < 0.05) at both 20 and 4 °C, three additional LEA proteins (*CvLEA2*, *CvLEA13*, and *hiC12*) with higher abundance in NJ-7 at 20 °C (fig. 5). All of their encoding genes, except *CvLEA2* and *CvLEA14*, also showed higher mRNA levels in NJ-7 (fold change ≥ 2.0, P-value < 0.05) under the corresponding conditions. GSEA of LEA proteins differentially expressed between NJ-7 and UTEX259 showed significant positive enrichments in NJ-7 at both 20 and 4 °C (supplementary fig. S16a, Supplementary Material online). Because LEA proteins are not listed as a GO category, and only one from NJ-7 (*NJ-7.evm.TU.scaffold00034.28*) shows similarity to an LEA protein in the GO category “response to cold” (including cold acclimation), the positive enrichment would not be identified by the GSEA based on GO (supplementary fig. S14, Supplementary Material online). The increased abundance of so many LEA proteins would greatly enhance the freezing tolerance of NJ-7. On the other hand, because LEA proteins can reduce cellular peroxides and protect enzyme activities under stresses, they may promote tolerance against high temperature and other stresses (Zhang et al. 2014; Wang et al. 2017). In NJ-7, such effects of LEA proteins may indirectly promote photosynthetic activities at high temperatures (supplementary fig. S2, Supplementary Material online).
exposure to cold treatment (3 °C) (Honjoh et al. 1995; Joh et al. 1995) and shows cold-induced freezing tolerance (Hatano et al. 1976). It is deduced that the ancestor of NJ-7 had possessed cold-inducible freezing tolerance before arrival at Antarctica, but the tolerance of NJ-7 was further developed to a higher level with much less dependence on cold induction (fig. 1). Even so, many LEA protein genes in NJ-7 remained to be cold-regulated. hiC6, hiC12, CvLEA1, CvLEA2, CvLEA7, and CvLEA13 in NJ-7 showed weaker responses to cold than in UTEX259; CvLEA14 showed similar responses in the two strains; the two Ccor2 genes and CvLEA3, CvLEA5, CvLEA9, and CvLEA11 even showed stronger responses in NJ-7 (supplementary fig. S17, Supplementary Material online).

Systematic Increases in Abundance of Metabolic Enzymes in NJ-7

In addition to the greatly enhanced freezing tolerance, NJ-7 acquired the capability to grow at 4 °C. How are metabolic activities maintained in NJ-7 at such a low temperature? Nitrate reductase (NR) is the most often used enzyme in studies of cold adaptation of green algae (Loppes et al. 1996; di Rigano et al. 2006). We first compared the amino acid sequences of NR (877-aa) in NJ-7 and UTEX259 but found only 19 substitutions (fig. 6a). Then, we assayed the NADH:NiR activities in NJ-7 and UTEX259 at different temperatures. The two curves of NR activity versus temperature were similar to each other in shape, with the optimal temperature slightly shifted from 25 to 30 °C in UTEX259 to 25 °C in NJ-7 (fig. 6b). However, the NR activity in NJ-7 was significantly higher than that in UTEX259 at all the temperatures tested. The activity of NJ-7 NR at 4 °C was approximately equal to that of UTEX259 NR at 25–30 °C. This implied that NJ-7 probably has higher abundance of NR than UTEX259. Western blot analysis confirmed this idea (fig. 6b). The ratio of NR abundance in NJ-7 and UTEX259 (cultured at 20 °C) was 2.54 ± 0.62, very close to the ratio of NR activity, 2.70 ± 0.89. In the proteomic analysis data, the abundance of NR in NJ-7 was shown to be 1.36 ± 0.07-fold of that in UTEX259 at 4 °C (fig. 6c). Compared with Western blot and enzyme activity analyses, proteomic analysis produced lower values for fold changes. This supported the use of fold change ≥ 1.3 (P-value < 0.05) as the criterion for upregulation of protein abundance in the proteomic analysis. In addition to NR, proteomic analysis data also showed a higher abundance of nitrite reductase (NiR) in NJ-7 than in UTEX259 at 20 and 4 °C (fig. 6c). The higher abundance of NR and NiR in NJ-7 may not be necessarily associated with increased mRNA level (fig. 6c). In algal cells, NR converts NO3⁻ into NO2⁻, then NiR converts NO2⁻ into NH₄⁺ for synthesis of glutamine. Increased abundance of NR and NiR in NJ-7 could significantly enhance the metabolism of nitrate. In other words, before NR and NiR are cold adapted in NJ-7, their concentrations are elevated to compensate for the low specific activities, so that nitrate can be utilized actively at low temperatures.

Many enzymes involved in other aspects of cell activities are also upregulated in NJ-7 relative to UTEX259.
especially some involved in carbon metabolism: for two critical steps in Calvin cycle, RuBisco activase/small subunit of RuBP carboxylase and sedoheptulose-1,7-bisphosphatase (supplementary fig. S18, Supplementary Material online); for glycolysis, the bifunctional enzyme phosphofructokinase (PFK-2)/fructose-2,6-bisphosphatase, phosphoglycerate kinase, phosphoglycerate mutase, and phosphate dikinase (fig. 7); linking glycolysis to TCA cycle or fatty acid synthesis, phosphoenolpyruvate carboxylase and pyruvate dehydrogenase E1 (α and β subunits) (fig. 7); for degradation of polysaccharides or oligosaccharides (including those covalently linked to proteins and lipids), α-mannosidase and seven other enzymes (fig. 7); for reutilization of monosaccharides in polysaccharide or oligosaccharide synthesis, UDP-N-acetylglucosamine pyrophosphorylase and three other enzymes (fig. 7); for reutilization of monosaccharides in polysaccharide or oligosaccharide synthesis, UDP-N-acetylglucosamine pyrophosphorylase and three other enzymes (fig. 7). Differences in protein and mRNA levels of these genes between NJ-7 and UTEX259 are shown in supplementary figure S19, Supplementary Material online. No enzymes shown in figure 7 are downregulated in NJ-7. Taking genes for the enzymes in figure 7 as a gene set, we also performed GSEA of the differential expression between two strains at the protein level and found significant positive enrichments in NJ-7 at both temperatures (supplementary fig. S16b, Supplementary Material online). Systematic increases in abundance of critical enzymes would significantly accelerate carbon metabolisms at low temperatures. However, the early stage cold adaptation is still underway in NJ-7. For example, RuBisco activase and the small subunit of RuBP carboxylase increased in abundance, whereas the large subunit did not. Like LEA proteins, most of these enzymes from the two strains differ from each other by <3% amino acid residues (supplementary table S22, Supplementary Material online).

**Discussion**

Typical mesophilic microorganisms do not grow at near 0 °C temperatures, those transported by atmospheric circulation to Antarctica need to develop the cold-growth capability. To survive the deeper freezing conditions than encountered before, these species also need significantly higher levels of ice-binding proteins or LEA proteins. *Chlorella vulgaris* NJ-7 is such an example. Based on comparative omics data, we systematically analyzed the genetic divergence between NJ-7 and the temperate strain UTEX259 and deduced the underlying mechanism for the early stage cold adaptation of *C. vulgaris*.

The cold-growth capability may be developed in two steps: elevation of protein abundance and cold adaptation of enzymes. Adaptation of NJ-7 to the Antarctic cold environment is basically at the early stage—elevation of protein abundance. Positive selection of genes (leading to cold-adapted enzymes/proteins) should have contributed to the cold adaptation of NJ-7 (supplementary excel S1, Supplementary Material online) but is apparently not the dominant contributor. Instead, most homologous proteins in NJ-7 and UTEX259 are nearly identical to each other. Some of them, such as NR, NiR, and many enzymes involved in other metabolic pathways, showed higher abundance in NJ-7 than in UTEX259.
irrespective of temperature (supplementary fig. S19 and excel S3, Supplementary Material online). The capability to grow at near 0 °C temperatures may depend on almost all aspects of cellular activities and involves a large number of enzymes/proteins. Relative to positive selection of enzymes for higher specific activities, increases in cellular concentrations would be a quick adaptation pathway for promoting biochemical reactions at low temperatures. Presumptively, the increase in cellular concentration is only necessary for enzymes whose activities are not sufficient to support cell proliferation and preferentially occurs at rate-limiting steps in metabolic pathways (such as fructose-2,6-bisphosphatase/phosphofructokinase in glycolysis shown in fig. 7 and sedoheptulose-1,7-bisphosphatase in Calvin cycle shown in supplementary fig. S18, Supplementary Material online).

There are different mechanisms for increasing the expression of enzymes/proteins. Gene duplication is one of the rapid mechanisms (Kondrashov 2012). In an Antarctic fish, augmented gene expression for cold adaptation is largely associated with gene duplication and family expansion (Chen et al. 2008). In microbes, experimentally evolved phenotypes (Wenger et al. 2011) or development of drug resistance (Sandegren and Andersson 2009) may also be associated with gene amplifications. In NJ-7, however, the elevated abundance of enzymes is apparently dependent on the upregulation at transcription, posttranscription (RNA stability), translation, or posttranslational (protein stability) levels rather than increased gene copy numbers.

In coping with seasonally changing temperatures, mesophilic microorganisms developed the capability to relieve the cold stress through cold acclimation (Morgan-Kiss et al. 2006); based on “anticipation and associative learning” (Bleuven and Landry 2016), the cold acclimation also prepares for the freezing condition that may follow. In cold acclimation of \( \text{C. vulgaris} \), sphingolipid delta(4)-desaturase, RNA helicases, and other enzymes are upregulated for resumption of cellular activities at 4 °C (predominantly for relief of cold stress), LEA proteins are upregulated to provide cryoprotection of cells at the “anticipated” subzero temperatures. Presumptively, the expression of some of these proteins/enzymes could have been further enhanced in NJ-7 after its arrival in Antarctica. A small proportion of cold-inducible proteins in NJ-7 indeed showed higher abundance than their orthologs in UTEX259 (fig. 3 and supplementary table S17, Supplementary Material online). However, most enzymes/proteins with higher abundance in NJ-7, such as those in figures 6 and 7, are not inducible in response to cold stress, because increases in their expression are not essential for cold stress relief but are required for cell proliferation at near 0 °C temperatures. The enhanced expression of “chromatin” proteins in NJ-7 (supplementary fig. S14e, Supplementary Material online) may also contribute to the cold-growth capability.
Relatively speaking, Antarctica is a permanently cold environment. In coastal ice-free regions, the ground surface temperature fluctuates at large amplitudes in December and January (Guglielmin 2006). These sites could allow newcomer mesophilic species to grow shortly every year. Some species may evolve to acquire the capability to grow at near 0 °C temperatures in a way like NJ-7. Because of the energy burden for keeping higher abundance of enzymes, selective pressure in permanently cold environments would continue to drive the cold adaptation in enzymes in subsequent evolutionary processes.

**Materials and Methods**

A short form of "Materials and methods" is presented as follows. More detailed descriptions are provided in supplementary text S1, Supplementary Material online.

Algal Strains, Culture Conditions, and Physiological Analyses

*Chlorella vulgaris* NJ-7 was isolated from rock samples collected (January 1999) near a transitory pond 5 km away from the Zhongshan Station (69°22′S–76°22′E) in Antarctica (Hu et al. 2008) and deposited at the Freshwater Algal Culture Collection of the Institute of Hydrobiology (FACHB2411). *Chlorella vulgaris* UTEX259 was purchased from the Culture Collection of Algae at The University of Texas at Austin (https://utex.org; last accessed November 24, 2019). Algal strains were purified by repeated streaking of single colonies on agar plates, and the axenicity was confirmed by microscopic examination and culture on solid Luria-Bertani medium and BG11 (Stanier et al. 1971) supplemented with glucose.

*Chlorella* strains were cultivated in BG11 under the light of 30 μE m⁻² s⁻¹ at 20 °C with aeration. Algal cells grown at 20 °C were rapidly cooled to 4 °C and exposed to the same temperature with aeration in a refrigerator with illumination of 30 μE m⁻² s⁻¹. For comparison of their growth at 20 and 28 °C, cell density was monitored turbidimetrically at 730 nm. For evaluation of antifreeze capability, algal cells grown at 20 °C with a saturating light of 2,000 μmol photons m⁻² s⁻¹ were used. Rates of photosynthesis were measured using a saturated light of 2,000 μmol photons m⁻² s⁻¹ with a temperature fluctuates at large amplitudes in December and January (Guglielmin 2006). These sites could allow newcomer mesophilic species to grow shortly every year. Some species may evolve to acquire the capability to grow at near 0 °C temperatures in a way like NJ-7. Because of the energy burden for keeping higher abundance of enzymes, selective pressure in permanently cold environments would continue to drive the cold adaptation in enzymes in subsequent evolutionary processes.

For extraction of genomic DNA, cells cultured at 20 °C were used. For transcriptome analysis, RNA was extracted from a variety of culture conditions to increase the number of expressed genes. These conditions included different growth temperatures (20 and 28 °C), exposure to cold (4 °C for 24 h), salt (0.3 M NaCl for 24 h), or oxidation (0.2 mM H₂O₂ for 5 h) stress, exposure to N, P, C, Fe, Ca, Mg, K, or trace element starvation (48 h). For differential expression analyses, total RNA and proteins were extracted from algal cells cultured at 20 °C with or without exposure to 4 °C, harvested and frozen in liquid nitrogen. Considering the different rates for accumulation of mRNA and proteins of cold-induced genes (Liu et al. 2011), cells exposed to 4 °C were collected at 6 h for RNA extraction and at 24 h for protein extraction.

All physiological and differential expression (transcriptomic, proteomic, RT-qPCR) data were calculated from results of three biological replicates.

### Genome Assembly and Annotation

High-molecular-weight genomic DNA was extracted using the cetyltrimethyl ammonium bromide method (Murray and Thompson 1980) with modifications. For 454 sequencing, shotgun and paired-end libraries were constructed. For Illumina sequencing, two short-insert paired-end genomic DNA libraries and a long-insert mate-pair library were constructed. Total RNA was extracted using Trizol reagent (Invitrogen) from cells cultured/treated under a variety of conditions to increase the number of expressed genes, and the pooled total RNA was used to construct the cDNA library for 454 sequencing.

The NJ-7 genome assembly was generated from 454 shotgun, 454 paired-end and Illumina GAIIx paired-end reads, using Newbler (GS De Novo Assembler, Roche) and Velvet (Zerbino and Birney 2008). The UTEX259 genome assembly was generated from 454 shotgun, 454 paired-end, Illumina GAIIx-paired-end, Illumina MiSeq-paired-end and mate pair reads, using Newbler, Velvet, ALLPATHS-LG assembler (Gnerre et al. 2011) and the scaffold SSPACE (Boetzer et al. 2011).

De novo transcriptome assembly was performed using Newbler with parameters -cdna -urt -tr, generating isotigs that represent transcripts. Genome assemblies were further improved by L_RNA_scaffold (Xue et al. 2013), which uses the transcripts to order and join genome sequences into larger scaffolds, and GapCloser tool in SOAPdenovo (Li et al. 2010), which makes use of the information of paired-end Illumina reads to fill gaps within scaffolds. Short scaffolds (<500 bp) and organelle sequences were excluded from the assemblies of nuclear genomes.

The quality of NJ-7 and UTEX259 genome assemblies was assessed in multiple ways, including BUSCO (Benchmarking Universal Single-Copy Orthologs) completeness assessment (Simao et al. 2015), mapping of fosmid-end sequences, and assembled transcripts onto the genome sequences.

Scaffolds and contigs corresponding to organelle genomes were extracted from the genome assemblies using BlastN searches against the *C. vulgaris* C-27 chloroplast genome (GenBank accession number AB001684) or *Prototheca wickerhamii* mitochondrial genome (GenBank accession number NC_001613). The gaps were filled by sequencing of PCR fragments, producing circular organelle genomes.

Prediction of protein-coding genes was performed using both ab initio gene predictions and transcript-based approaches, followed by integrating them using EVidenceModeler (EVM) (Haas et al. 2008). Protein motifs
and domains of predicted gene models were annotated using InterProScan search (Zdobnov and Apweiler 2001) against InterPro databases (Hunter et al. 2009). Gene functions were assigned based on BLASTP searches (E-value ≤ 1e-5). tRNA and rRNA genes were identified using tRNAscan-SE v1.3 (Lowe and Eddy 1997) and RNAmmer 1.2 (http://www.cbs.dtu.dk/services/RNAmmer/; last accessed November 24, 2019). Organelle genomes were annotated using DOGMA (http://dogma.ccbb.utexas.edu; last accessed November 24, 2019), ORF-Finder (https://www.ncbi.nlm.nih.gov/orffinder/; last accessed November 24, 2019), and BLASTX searches based on sequence similarity to genes in other annotated organelle genomes.

Synteny and Comparative Analyses
A BLASTP alignment (E-value ≤ 1e-5) was performed between all protein sequences of the two strains. OrthoMCL (Li et al. 2003) was applied to identify orthologous protein pairs based on the BLASTP results, with the criteria E-value ≤ 1e-5, identity ≥ 80%, and length coverage ≥ 70%.

Synteny between genomes was identified by aligning genomic regions using orthologous gene pairs as anchors. Using MCScanX (Wang et al. 2012), pairwise synteny segments were identified, grouped into blocks, and displayed by a dot plot graph. A dual synteny plot was generated with the longest 12 scaffolds of NJ-7 and the corresponding scaffolds of UTEX259. Nine chlorophyte chloroplast genomes were compared using the Progressive Mauve algorithm implemented in Mauve 2.4.0 (Darling et al. 2010).

Phylogenetic and Evolutionary Analyses

i. Phylogenetic analysis. Phylogenetic trees were constructed based on 1,080 single-copy genes shared by NJ-7, UTEX259, and other nine chlorophytes, with the land plant Arabidopsis thaliana as the outgroup. Using the MUSCLE program (Edgar 2004), 1,080 genes from these species/strains were aligned. Poorly aligned regions were removed from multiple-alignments using Gblocks implemented in TranslatorX (Abascal et al. 2010). The cleaned multiple alignments were concatenated and subjected to ML (maximum likelihood) tree reconstruction using PhyML (Guindon et al. 2010).

ii. Divergence time estimates. Divergence times between lineages were estimated using the Bayesian MCMC approach as implemented in the program BEAST v1.7.5 (Drummond et al. 2012) based on 36 chloroplast genes (atpA, atpF, atpF, atpH, atpI, petA, petB, petG, psaA, psaB, psaC, psaI, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbL, psbN, rcL, rpl2, rpl14, rpl16, rpl20, rpl23, rps7, rps8, rps11, rps12, rps14, rps18, and rps19). Two sets of analyses were performed: broad-scale analyses with 12–19 species/strains of green algae (in four combinations) and four species of higher plants; a fine-scale analysis with nine species/strains of Chlorella. BEAST used concatenated nucleotide alignments as input and estimated phylogeny and divergence times simultaneously. To calibrate nodes in the broad-scale tree, three reliable fossil dates were used: the divergence between angiosperms and gymnosperms (290–320 Ma) (Goremykin et al. 1997; Doyle 1998), the divergence between Nymphaeales and eudicots (115 Ma minimum) (Friis et al. 2001), and the divergence between monocots and dicots (90–130 Ma) (Crane et al. 1995). The maximum clade credibility tree was generated using TreeAnnotator v1.7.5 (Drummond et al. 2012) and visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree; last accessed November 24, 2019). The estimated divergence time between C. variabilis and C. vulgaris was further used to calibrate the fine-scale analysis.

iii. Evolutionary rate estimates. The number of nonsynonymous substitutions per non synonymous site (dN) and the number of synonymous substitutions per synonymous site (dS) were estimated based on the 1,080 nuclear genes, using codeml program in the PAML package v4.6 (Yang 2007).

Detection of Genes with Positive Selection
The codeml program in the PAML package was applied to all the 5,899 core genes shared by NJ-7, UTEX259, and NC64A. Codeml analysis with branch-site model was conducted on either NJ-7 or UTEX259 branch. The null hypothesis (assuming ω = 1 or ω < 1) and the alternative hypothesis (assuming ω > 1) were compared to identify significantly higher likelihood values for the alternative hypothesis. Those genes with significance (χ² P-value < 0.01) were deemed to be under positive selection.

Analyses of Alternative Splicing Events, Repetitive Elements, Gene Duplication, and Gene Families
Alternative splicing events were detected by comparing the predicted genes on genome and the assembled iso-tigs and processing the clusters of aligned iso-tigs with at least one splice site.

Repetitive elements were identified with both homology-based methods and de novo repeat finding programs. The library of identified repeats was used to estimate repeat contents of each genome.

Duplicated genes were identified by performing self-versus-self BLASTP on protein sequences. The copy number of recent duplicated genes was deduced based on highly similar genes that were detected by performing self-versus-self BlastN, or roughly estimated by aligning trimmed illumina reads onto gene sequences to calculate the relative depth of coverage.

Homologous protein families were constructed based on genome sequences of 11 species/strains of green algae, and all predicted protein sequences were compared using all-against-all BLASTP. Protein families were annotated according to Pfam domains or InterPro descriptions. The significance of family expansion or reduction was analyzed by chi-square test in R.

Identification of LEA Protein Genes
In addition to Ccor1/Ccor2 identified by experimental analyses (Liu et al. 2011) and genes similar to Ccor1/Ccor2, LEA protein genes were identified by searching for LEA motifs and
secondary structures (Tunnacliffe and Wise 2007; Battaglia et al. 2008) using the fuzzipro program in EMBOSS package (Rice et al. 2000) and the PSIPRED Server (http://bioinf.cs.ucl.ac.uk/psipred; last accessed November 24, 2019), searching for Pfam domains using hmmssearch (Eddy 2011) against LEA HMM profiles, searching for homologs using BLASTP (E-value ≤ 1e-5) against LEA proteins in the Late Embryogenesis Abundant Proteins DataBase (http://forge.info.univ-angers.fr/~gh/Leadb/index.php; last accessed November 24, 2019). Subcellular locations were predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP; last accessed November 24, 2019), MitoProt (https://ihg.gsf.de/ihg/mitoprot.html; last accessed November 24, 2019), and PSORT (http://psort1.hgc.jp/form.html; last accessed November 24, 2019).

Analyses of Differential Expression at mRNA Level
Differential expression at mRNA level was analyzed based on RNA-seq. Processed sequencing reads were aligned to exonic regions of predicted genes using Bowtie (Langmead et al. 2009) and Tophat (Trappnell et al. 2009) allowing one mismatch. Raw count data and RPKM (reads per kilobase of exon per million mapped reads) values were generated from the alignment files using bam2rpkm (http://bam2rpkm.sourceforge.net/; last accessed November 24, 2019). Raw count data were then used as input into DESeq (Anders and Huber 2010) for differential expression analyses (three biological replicates). Differentially expressed genes were identified with criteria: fold-change ≥ 2 (either up- or downregulated), FDR (false discovery rate) adjusted P-value < 0.05, and RPKM ≥ 10 under at least one condition.

Real-Time Quantitative Polymerase Chain Reactions
DNA-free RNA was used to synthesize the first strand of cDNA using PrimeScript RT reagent Kit (Takara) and oligo (dT)15 primer (Promega). SYBR Green I (Takara) was added to the PCR reaction mixture according to manufacturers’ protocol, and RT-qPCR was performed on Applied Biosystems ABI 7500. β-Actin was used as the internal standard. Primers were designed based on identical sequences of orthologous genes (supplementary table S23, Supplementary Material online).

Analyses of Differential Expression at Protein Level
Differential expression at the protein level was analyzed using the quantitative proteomics approach. Algal cells were sonicated and centrifuged. Proteins in the supernatant were precipitated and subjected to trypsin digestion. After labelling with Tandem Mass Tags/Isobaric Tag for Relative Absolute Quantitation (TMT Kit/iTRAQ), peptides were analyzed by liquid chromatography coupled with tandem mass spectrometry (MS/MS). The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8) (Cox and Mann 2008). Peptide and protein ratios were obtained by direct comparison of signals of “light” and “heavy” isotope in the same liquid chromatography run. Cross-strain comparison of expression of orthologous proteins was performed using identical peptides instead of whole protein sequences. In cases where multiple copies of proteins could not be distinguished, the multiple copies were treated as one in the quantitative analyses. Differential expression with ratio ≥ 1.3 (P-value < 0.05) was defined as upregulation, that with ratio ≤ 0.77 (P-value < 0.05) as downregulation.

GO Enrichment Analysis
GSEA software version 3.0 (Subramanian et al. 2005) was used to identify significantly enriched GO gene sets. GSEA was run with 1,000 permutations, and functional gene sets with FDR-adjusted q-value < 0.05 were considered significant.

Analyses of Regulation Networks
TFs were predicted by searching for Pfam domains using hmmssearch against the HMM profiles for TF families at PlantTFDB (Jin et al. 2014). DNA-binding motifs for orthologous TFs in C. variabilis NC64A were used to scan for recognition sites upstream of protein-coding genes in NJ-7 and UTEX259 by FIMO (Grant et al. 2011). The potential role of a TF in gene regulation was defined based on the correlation between its own expression and the expression of genes with its recognition site.

NR Assays and Western Blot Analysis
NR activity in crude extracts was assayed as described by di Rigano et al. (2006) using NADH as electron donor, with a few modifications. Reactions (three technical repeats) were carried out for 30 min at temperatures from 5 to 45 °C. Western blot analysis of total soluble proteins was performed using the rabbit antiserum against the recombinant NR.

Data Availability
Annotations of NJ-7 and UTEX259 genomes are available in the Figshare repository (DOI: 10.6084/m9.figshare.c.4678916; https://figshare.com/s/66909bf96c4a06159c94).

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

Acknowledgments
The authors express great gratitude to Guoxiang Liu for his advice on the taxonomic analysis of strains of Chlorella vulgaris based on the ribosomal RNA region. This research was supported by the Knowledge Innovation Project of Hubei Province (2017CFA021), the STS Project of Chinese Academy of Sciences (KJF-SW-STS-163), the national special support program “Wan-Ren-Ji-Hua” of China and the State Key Laboratory of Freshwater Ecology and Biotechnology at IHB, CAS (2019FBZ06).

Author Contributions
XX, J.X., and A.-Y.G. designed the project. Y.W. performed bioinformatic analyses. H.-M.Z. contributed to genome assembly/annotation, multicopy gene analyses, and identification of LEA protein genes. X.L. performed experimental analyses of the expression and activity of nitrate reductase,
Y.W. and H.G. analyzed the physiological characteristics of algal strains; Y.W. prepared DNA and RNA samples, performed PCR and RT-qPCR analyses. XX, Y.W., XL, H.G., A.-Y.G., and J.X. interpreted the results. XX, J.X., and A.-Y.G. edited the manuscript. XX and Y.W. wrote the manuscript. All authors read and approved the content of the manuscript.

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