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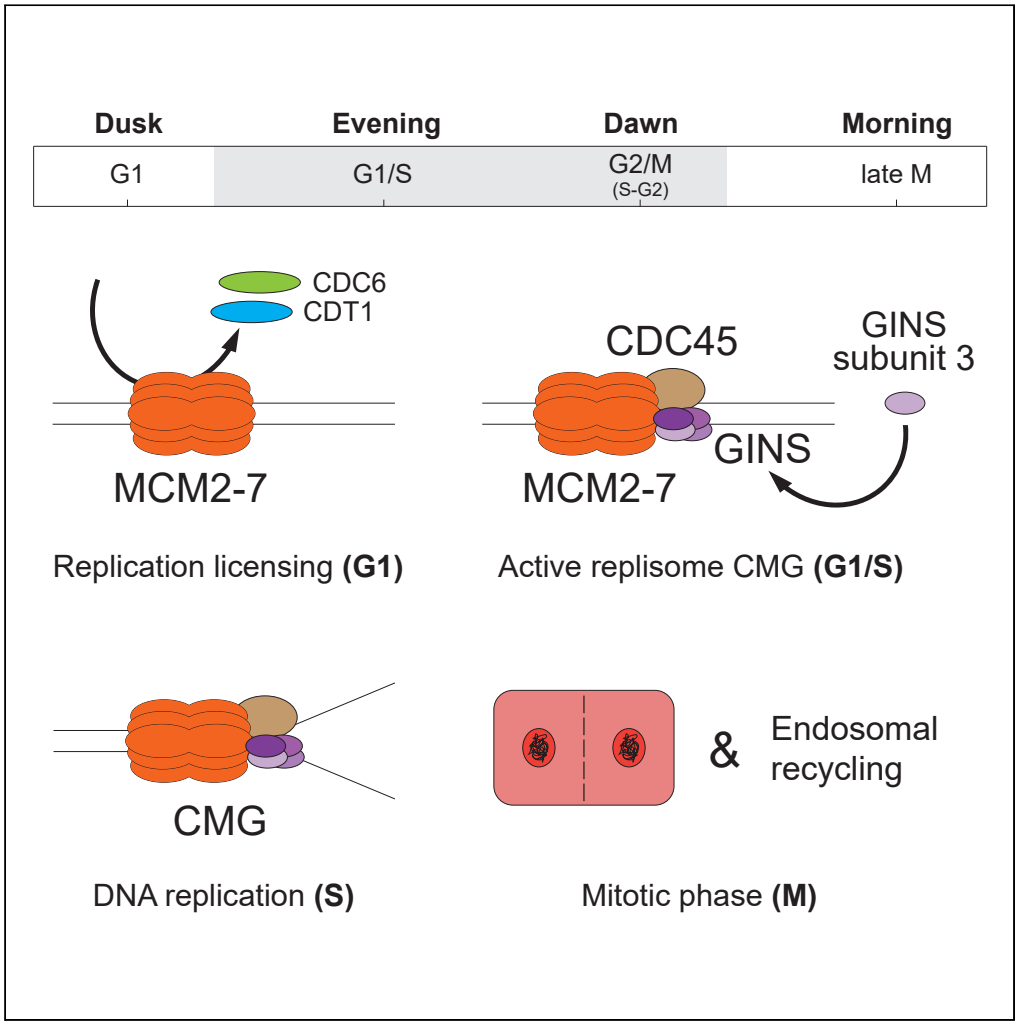

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Highlights

The G1-S transition is controlled by delayed transcription of *GINS subunit 3*

Cell cycle progression in red seaweeds does not rely on typical CDK inhibitors

Cell cycle transitions in red seaweeds primarily occur from dusk to dawn

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From dusk till dawn: cell cycle progression in the red seaweed *Gracilariopsis chorda* (Rhodophyta)

JunMo Lee,[1,2,7,*] Shin-ya Miyagishima,[3,4] Debashish Bhattacharya,[5] and Hwan Su Yoon[6,*]

SUMMARY

The conserved eukaryotic functions of cell cycle genes have primarily been studied using animal/plant models and unicellular algae. Cell cycle progression and its regulatory components in red (Rhodophyta) seaweeds are poorly understood. We analyzed diurnal gene expression data to investigate the cell cycle in the red seaweed *Gracilariopsis chorda*. We identified cell cycle progression and transitions in *G. chorda* which are induced by interactions of key regulators such as E2F/DP, RBR, cyclin-dependent kinases, and cyclins from dusk to dawn. However, several typical CDK inhibitor proteins are absent in red seaweeds. Interestingly, the G1-S transition in *G. chorda* is controlled by delayed transcription of *GINS subunit 3*. We propose that the delayed S phase entry in this seaweed may have evolved to minimize DNA damage (e.g., due to UV radiation) during replication. Our results provide important insights into cell cycle-associated physiology and its molecular mechanisms in red seaweeds.

INTRODUCTION

The eukaryotic cell cycle is generally divided into four phases: gap phase 1 (G1), S phase, gap phase 2 (G2), and mitosis (M). DNA replication (S phase) and chromosomal segregation (M phase) are separated by G1 and G2.[1,2] The primary role of the G1 phase is to prepare for DNA replication during S phase, thus the core replicative helicase, the mini-chromosome maintenance (MCM; six subunits MCM2-7) complex, is loaded in the G1 phase by Cell Division Cycle 6 (CDC6) and Cdc10-dependent transcript 1 (CDT1).[3] Proliferating Cell Nuclear Antigen (PCNA) is a DNA polymerase clamp that plays important roles in both DNA synthesis and repair, which are carried out by DNA polymerase epsilon (DNA pol-ε) in the presence of PCNA, Replication Protein A (RPA or Replication Factor A; RFA), and Replication Factor C (RFC).[4,5] The replisome complex for DNA replication is activated during S phase, but its prior accumulations, derived by the E2F transcription factors, are induced in the G1 phase.[3,6]

The active form of the replicative DNA helicase is required for the G1-S transition, which is constructed by the loaded MCM2-7 complex, Cell Division Cycle 45 (CDC45), and Go-Ichi-Ni-San (GINS; GINS1-3 and SLD5) complex.[3,7–9] Ataxia telangiectasia and Rad3-related (ATR) and Ataxia telangiectasia mutated (ATM) kinase support an efficient DNA replication response to DNA replication stress (e.g., DNA damage) before or at the early S phase.[3,10,11] Retinoblastoma-related (RBR) gene function is generally as a repressor of the G1-S transition at the G1 phase through inhibition of E2F/DP activity. Deactivation of RBR proteins is also required for the G1-S transition through phosphorylation by A-type cyclin-dependent kinase (CDK-A) and D-type cyclins (CycD).[1,12,13] Activation of the CDK-A and CycD complex could be involved in the CDK-activating kinase (CAK) pathway, which is induced by the CDK-D and CycH complex at G1 phase.[2,14–17] CDKs are major regulators of cell cycle progression through interactions with their corresponding cyclins.[12,18,19]

CDK-A and CDK-B in plants control the G2-M transition through the construction of a complex with CycA and CycB.[1,20,21] However, transcription of *CDK-A/B* and *CycA/B* is induced from S phase to mitosis, thus their inactive form is maintained before the G2-M transition through inhibitory phosphorylation by WEE1, indicating the inhibition of mitotic CDK activity.[1,22,23] However, the major function of WEE1 in plants is related to the DNA repair checkpoint under stress conditions, as validated by the *wee1* mutant.[24,25] Inhibition of CDK complexes is primarily done by CDK inhibitor proteins (CKIs), such as INHIBITOR of CDK4 (INK4) and CDK interacting protein/kinase inhibitory protein (CIP/KIP) families in animals and the INTERACTOR/INHIBITOR OF CDK/KIP-RELATED PROTEINs (ICK/KRPs) and the SIAMESE/SIAMESE-RELATED PROTEINs (SIM/SMRs) in plants.[13,25,26] The plant ICK/KRPs generally bind to the CDK-A and CycD complexes for the inhibition of M phase entry, but the interaction between ICK/KRPs and the CDK-B family is also reported.[13,17,27]

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Dephosphorylation of the mitotic CDK-cyclin complex for the G2-M transition is done by Cell Division Cycle 25 (CDC25) in metazoans and yeast.[28–32] However, in the plant *Arabidopsis thaliana* and the unicellular green alga *Chlamydomonas reinhardtii*, there is no CDC25 gene with a CDK-activating phosphatase function,[12,33–35] although CDC25 in the unicellular green alga *Ostreococcus tauri* possibly controls CDK-B activity.[36,37] In plants, inhibition of CKIs (e.g., ICK/KRPs) may be induced by ubiquitin-proteasome system (UPS)-mediated degradation through phosphorylation by CDK-B, and this process stimulates CDK-A activity and M phase.[13,38–42] The *CDK-B* family in plants is preferentially expressed in the G2 phase, but this enzyme is primarily activated at the G2/M phase.[21,43] The transcription of *CycA* and *CycB* peaks at the G2 and M phases, and CycB stimulates G2-M transition.[1,44,45]

Cell Division Cycle 20 (CDC20) is a coactivator of the anaphase promoting complex/cyclosome (APC/C; E3 ubiquitin ligase), and the APC/C^{CDC20} activates at the metaphase-anaphase transition in mitosis for chromosome segregation and cell division.[46–52] Transcripts of the *CDC20* gene accumulate from the S phase and peaks in mitosis, then rapidly drop after the end of mitosis.[47,49] In general, the CDC20 homolog 1 (CDH1; CELL CYCLE SWITCH 52, CCS52 in plants) also plays a role for APC/C coactivator, and the complex APC/C^{CDH1} mediates the degradation of mitotic proteins such as CDC20, primarily active during late mitosis and G1 phase.[47–49,51–56] Another function of CDH1/CCS52 is to act as a molecular switch between the mitotic phase and endoreduplication (or endocycling), which indicates genome duplication without mitotic cell division. This process is necessary in specialized cell differentiation (e.g., development and organogenesis in plants), including an increase of nuclear volume and cell size, directly related to the ploidy level.[12,48,57] However, the regulatory pathway of endoreduplication is regarded as a tissue-, organ-, species-specific mechanism.[57]

Endocytic metabolism is involved in diverse cellular processes such as nutrient uptake, cell adhesion, migration, and cytokinesis.[58] The small GTPase Rab and Arf (ADP ribosylation factor) family are key regulators of endosomal recycling and several different intracellular transport processes.[58–63] Particularly during cytokinesis, many types of cellular materials (e.g., ligands, proteins, and lipids) are reorganized by endosomal recycling pathways.[58,62,64,65]

Cell cycle progression in the unicellular red alga *Cyanidioschyzon merolae* has previously been studied.[18,66–69] This hot springs species has an extremophilic lifestyle (i.e., pH 0–5 and 35°C–63ºC) and the class Cyanidiophyceae to which it belongs, diverged ca. 1.5 billion years ago from the mesophilic clade of red algae.[70] The genomes of these highly specialized lineages contain many (often) adaptive horizontal gene transfers (HGTs) from prokaryote donors to enable life at the extremes.[70,71] In contrast, conspicuous red seaweeds in the class Florideophyceae generally have mesophilic lifestyles and the largest diversity within red algae (i.e., include ca. 98% of all species; https://www.algaebase.org) that has resulted from an explosive radiation ca. 781 Mya.[72,73] Consequently, Florideophyceae show evolutionary features defined by their more recent origin and adaptation to intertidal and subtidal habitats. These features include independent gene diversification of their photosynthetic machinery,[74] a cryptochrome-based photoreception system, and compact carbon metabolism.[75] However, cell cycle progression and its regulation in red seaweeds remain unclear. Here, we analyzed transcriptional data collected over the diurnal cycle, focusing on core components of cell cycle progression in the red seaweed *Gracilariopsis chorda*, Florideophyceae. We identified the G1, S, G2, and M phases and their transitions from dusk to dawn (or early morning) in this species and found that cell cycle progression in *G. chorda* is coupled with elevated transcription of genes involved in energy metabolism. These results are significant because this type of coordination is not present in *C. merolae*, which shows independent regulation of energy metabolism during the day and cell cycle during the night. Despite these differences, both species show synchronized cell division vis-à-vis the day-night cycle. Our results provide the foundation for future studies of cell cycle progression and its physiological impacts in red seaweeds.

RESULTS AND DISCUSSION

Cell cycle progression of the red seaweed *Gracilariopsis chorda*

In general, S phase progression genes are active in G1 phase for DNA replication during the S phase.[3] In *G. chorda*, most of the S phase progression genes, involving the transcriptional activator and DNA replication complex, are highly expressed at dusk (i.e., at 10 h in the cycle), then rapidly down-regulated through the day-night transition (Figure 1; Table 1). The *transcription factors E2F3-like* (K06620) and *E2F7/8* (or *E2Fe*; K09391) in *G. chorda* are highly expressed only in the dusk phase (Figure 1). The *E2F3-like* gene is a transcriptional activator of several core machineries for the DNA replication complex, such as MCM2-7 and PCNA from the G1 phase.[13,76,77] In *G. chorda*, except for GINS3, gene expressions of replisome components (i.e., CDC6, CDT1, MCM2-7, PCNA, DNA pol-ε, CDC45, GINS, RPA, and RFC) are high at dusk (Figure 1; Table 1). In addition, the ATR and ATM kinases are also highly induced at dusk (Figure 1), which are generally active before or at the early S phase to block S phase entry from DNA replication stress.[3,10,11] Therefore, G1 phase in *G. chorda* is active at dusk to allow the accumulation of DNA replication components before S phase (Figure 1; Table 1). However, we postulate that the G1-S transition is possibly active in the evening phase because *GINS3* is highly expressed in the evening phase (i.e., 16 h in the cycle), which is a core component of the active form of replicative DNA helicase.[3]

E2F transcription factors and dimerization partner (DP) proteins give rise to several different types of heterodimeric complexes, indicating transcriptional activators (E2F1, E2F2, and E2F3 in mammals; E2Fa and E2Fb in plants) and suppressors (E2F4, E2F5, E2F6, E2F7, and E2F8 in mammals; E2Fc, E2Fd, E2Fe, and E2Ff in plants) of cell cycle genes.[1,13,77–82] Red algal E2F families are divided into two phylogenetic groups (Figure S1; details in STAR methods). One includes a monophyletic clade of red algal E2F genes with a subclade of E2F1-6 (E2Fa, E2Fb, and E2Fc), which includes both activators and suppressors of cell cycle genes. Another clade of red algal E2F genes includes E2F7/8 (E2Fd, E2Fe, and E2Ff), indicating the transcriptional suppressors of cell cycle genes (Figure S1). Interestingly, a single copy gene from each clade is present in many red algal taxa [e.g., *G. chorda*, *Gracilaria domingensis*, *Chondrus crispus* (Florideophyceae), *Porphyridium purpureum* (Porphyridiophyceae), *Cyanidium caldarium*, *C. merolae* (Cyanidiophyceae)]. In *G. chorda*, *E2F3-like* (PXF50015.1; K06620) and *E2F7/8* (PXF48075.1; K09391)

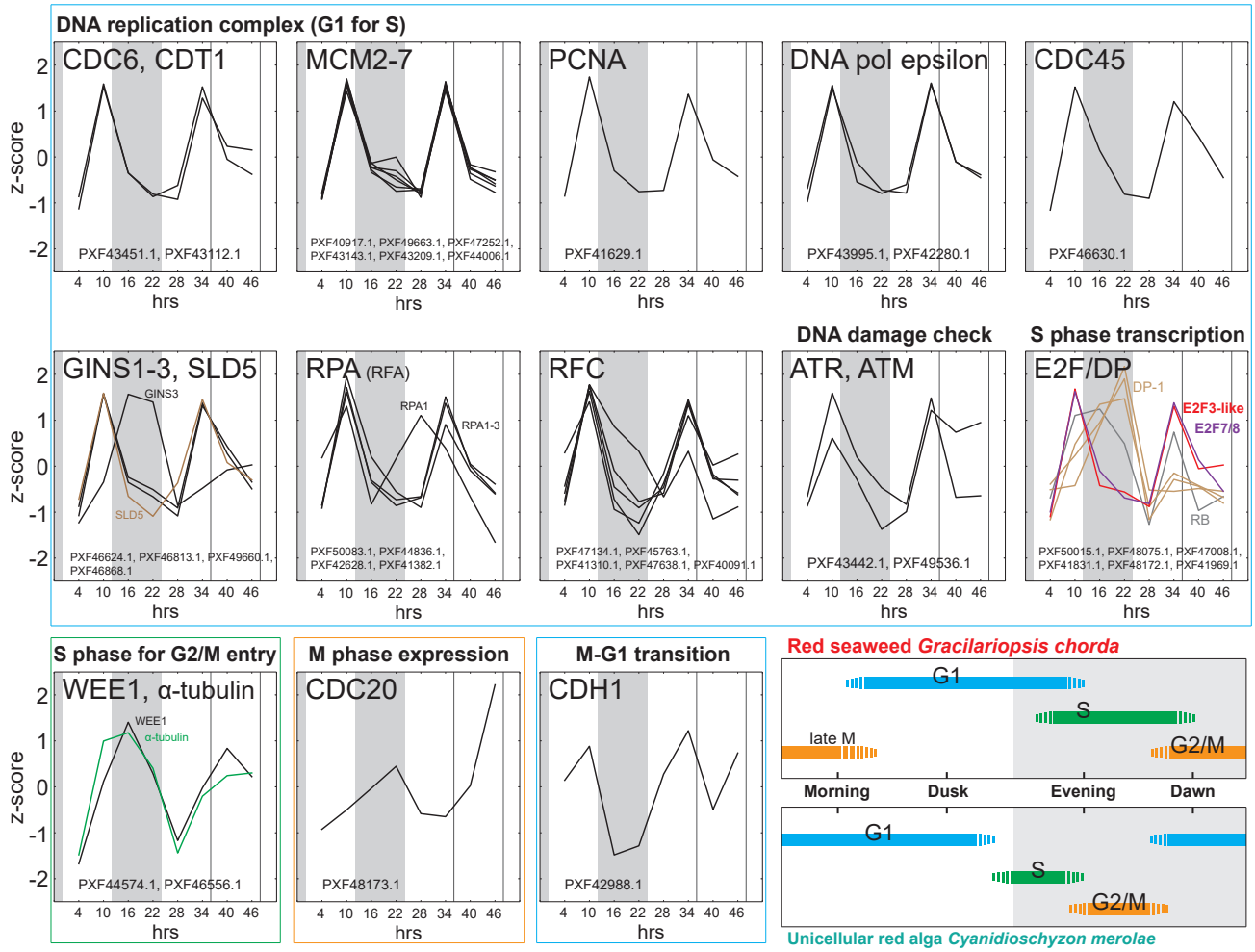

Figure 1. Gene expression patterns involved in cell cycle progression in the red seaweed *Gracilariopsis chorda*

Cell cycle progression of the unicellular red alga *Cyanidioschyzon merolae* is based on previous studies (Fujiwara et al. 2009, 2020). The gray-shaded areas in the plots represent dark conditions. NCBI accession numbers of the target genes are shown below the plots.

genes are highly expressed at the dusk phase (G1; Figures 1 and S1). In *C. merolae*, two E2F genes [XP_005539123.1 (CMT067C) in the E2F1-6 clade and XP_005539407.1 (CMT433C) in the E2F7/8 clade] are highly expressed at day-night transition (G1-S transition or S phase entry).[68] Although the sampling points of transcriptome analysis in *G. chorda* and *C. merolae* do not completely overlap, the results indicate that the transcription of both red algal E2F genes is highly induced for the G1-S transition. To determine whether these red algal E2F genes are activators or suppressors of cell cycle genes, analysis of protein-protein interactions of E2F-DP complexes and their ubiquitination and phosphorylation are needed. For example, the E2Fc-DP complex inhibits transcription of the S phase genes at the G1 phase, but when E2Fc in this complex is phosphorylated, destruction of E2Fc and activation of E2Fa/b-DP complex is induced by the CDK-A/CycD complex for the G1-S transition.[1,12]

Dimerization partners (DPs) participate in the transcriptional regulation of G1/S and G2/M genes.[83] In *G. chorda*, transcription of three DPs gradually increases at dusk and peaks at dawn (i.e., 22 h in the cycle, see Figure 1; Table 1). In *C. merolae*, among the three DP genes, one (XP_005536817.1; CML181C) is highly expressed at G1, whereas the others (XP_005539541.1 and XP_005536921.1; CMT601C and CML315C) are highly expressed at G1 and S, respectively, with a temporal transcriptional decrease at their transition.[68] Therefore, we postulate that the edge of the S phase (i.e., early G2/M) in *G. chorda* could extend to the dawn phase. However, the regulation of E2F-DP complexes for the cell cycle progression of *G. chorda* could be derived from protein-protein interactions and their post-translational modification (e.g., ubiquitination and phosphorylation) because two types of E2Fs are highly expressed together at the G1 phase, but their dimerization partner DPs are gradually induced from G1 to early G2/M phase (Figure 1). A key regulator of the transcriptional activator E2F-DP complex is retinoblastoma-related (RBR) protein, which binds to the E2F-DP complex and inactivates it until phosphorylation of RBR (by CDK-A/CycD) for the G1-S transition.[1,12,13,18] In *G. chorda*, the *RBR* (PXF41969.1; K06618) gene is highly expressed at the evening phase, thus the G1-S transition and S phase entry in *G. chorda* could start from the evening, supported by a transcriptional peak of *GINS3* in the evening (Figure 1). The G1-S transition of *G. chorda* (16 h in the dark period) is slightly later than in *C. merolae* (12 h in the day-night transition).[68] We postulate that the dark-phased

Table 1. Genes involving cell cycle progression in *Gracilariopsis chorda* genome

Accessions	KEGG descriptions	Transcriptional peak
PXF43451.1	cell division control protein 6 (K02213)	Dusk[a]
PXF43112.1	chromatin licensing and DNA replication factor 1 (K10727)	Dusk[a]
PXF40917.1	DNA replication licensing factor MCM2 (K02540)	Dusk[a]
PXF49663.1	DNA replication licensing factor MCM3 (K02541	Dusk[a]
PXF47252.1	DNA replication licensing factor MCM4 (K02212)	Dusk[a]
PXF43143.1	DNA replication licensing factor MCM5 (K02209)	Dusk[a]
PXF43209.1	DNA replication licensing factor MCM6 (K02542)	Dusk[a]
PXF44006.1	DNA replication licensing factor MCM7 (K02210)	Dusk
PXF41629.1	proliferating cell nuclear antigen (K04802)	Dusk[a]
PXF43995.1	DNA polymerase epsilon subunit 1 (K02324)	Dusk[a]
PXF42280.1	DNA polymerase epsilon subunit 2 (K02325)	Dusk[a]
PXF46630.1	cell division control protein 45 (K06628)	Dusk[a]
PXF46624.1	GINS complex subunit 1 (K10732)	Dusk[a]
PXF46813.1	GINS complex subunit 2 (K10733)	Dusk[a]
PXF49660.1	GINS complex subunit 3 (K10734)	Evening
PXF46868.1	SLD5 or GINS complex subunit 4 (K10735)	Dusk[a]
PXF50083.1	replication factor A1 (K07466)	Dusk[b]
PXF44836.1	replication factor A1 (K07466)	Dusk[a]
PXF42628.1	replication factor A2 (K10739)	Dusk[a]
PXF41382.1	replication factor A3 (K10740)	Dusk[a]
PXF47134.1	replication factor C subunit 1 (K10754)	Dusk
PXF45763.1	replication factor C subunit 2/4 (K10755)	Dusk[a]
PXF41310.1	replication factor C subunit 2/4 (K10755)	Dusk[a]
PXF47638.1	replication factor C subunit 3/5 (K10756)	Dusk[a]
PXF40091.1	replication factor C subunit 3/5 (K10756)	Dusk
PXF43442.1	serine/threonine-protein kinase ATR (K06640)	Dusk
PXF49536.1	serine-protein kinase ATM (K04728)	Dusk
PXF50015.1	transcription factor E2F3 (K06620)	Dusk
PXF48075.1	transcription factor E2F7/8 (K09391)	Dusk[a]
PXF47008.1	transcription factor Dp-1 (K04683)	Dawn
PXF41831.1	transcription factor Dp-1 (K04683)	Dawn
PXF48172.1	transcription factor Dp-1 (K04683)	Dawn
PXF41969.1	retinoblastoma-associated protein (K06618)	Evening
PXF46556.1	tubulin alpha (K07374)	Evening
PXF44574.1	wee1-like protein kinase (K06632)	Evening[a]
PXF48173.1	cell division cycle 20, cofactor of APC complex (K03363)	Dawn
PXF42947.1	cell division cycle 20-like protein 1 (CDH1; K03364)	Dusk[a]

[a]Rhythmic gene expression.
[b]Non-periodic gene expression.

G1-S transition in the seaweed can induce a stable DNA replication process without light stress (e.g., DNA damage from UV light). In addition, cell cycle progression and cell division in *G. chorda* are closely coupled with energy metabolism from dusk to dawn (Figure S2) because high activity of gene expression involving energy metabolism (i.e., TCA cycle and oxidative phosphorylation) has previously been reported from dusk to dark.[75] This result contrasts with the independent regulation of energy metabolism (daytime) and cell cycle progression (dark) in the unicellular red alga, *C. merolae*.[68]

A transcriptional peak of *WEE1* is present at the S phase in plants, however the plant protein acts from G2 for the G2/M checkpoint.[1,84,85] The *WEE1* gene (XP_005539533.1; CMT590C) in *C. merolae* has a transcriptional peak during the G1-S transition.[68] In *G. chorda*, the *WEE1*

gene is highly expressed at the evening phase (Figure 1). This result supports our idea that cell cycle progression in *G. chorda* at the evening phase includes the G1-S transition and the S phase. However, the post-transcriptional or translational regulation of WEE1 in *G. chorda* needs to be validated using experimental data. Transcription of α-tubulin (XP_005539467.1; CMT504C) in *C. merolae* is highly induced at the S phase and slightly decreases at M.[68] The α-tubulin gene (PXF46556.1) in *G. chorda* is highly expressed at the evening phase and its transcription slightly decreases at dawn (Figure 1). Therefore, S phase in *G. chorda* could begin during evening and G2/M initiates at dawn (Figure 1). Interestingly, the entry of the S and M phases and their transitions are delayed and extended in the red seaweed *G. chorda* compared to the unicellular red alga *C. merolae* (Figure 1).[66,68] These results indicate that physiological rhythms and their regulation, based on cell cycle progression, may be significantly different between multicellular red seaweeds and unicellular red algae.

Cell Division Cycle 20 (CDC20) is an essential regulatory gene for cell division, activating the anaphase promoting complex/cyclosome (APC/C) at the metaphase-anaphase transition of the mitotic phase.[46,47,49] Transcripts of *CDC20* accumulate from the S phase and peak during mitosis, and then rapidly decrease after the end of mitosis.[47,49] Expression of *CDC20* (K03363) in *G. chorda* increases from dusk and peaks at dawn although its expression level in the morning and dusk does not vary greatly, thus the M phase, including G2, in *G. chorda* occurs at dawn (Figure 1). The CDC20 homolog 1 (CDH1) induces inactivation of APC/C^{CDC20} by binding to the APC/C and mediates substrate degradation, including CDC20, from late M to G1 phase.[47,49,53–56] The *CDH1* gene (K03364) in *G. chorda* is highly expressed at dusk (Figure 1). Following mitosis, G1, or potentially the quiescent (G0) phase, is gradually induced in *G. chorda* from morning to dusk, derived by CDH1.[86] In summary, most cell cycle genes in *G. chorda* show rhythmic gene expression or the same transcriptional peak is observed between a day/night cycle and continuous light (Figure S2; Table 1; See STAR methods).[75] Periodicity of the cell cycle in this species could therefore be associated with physiological homeostasis and circadian rhythms.

Cell cycle-associated cyclin-dependent kinases and cyclins in *G. chorda*

Cyclin-dependent kinases (CDKs) regulate cell cycle transitions through interactions with cyclins (Cyc), thus these interactions are closely related to cell growth and development.[12,18,19] In this study, we identified *CDK-A*, *CDK-B*, *CDK-C*, *CDK-D*, *CDK-E*, and *CDK-G* (with *CDK-G*-like) genes in red algal genomes (Figure 2A). Most CDKs are conserved in red algae, although some show copy number variation. For cell cycle progression, CDKs are phosphorylated by CDK-activating kinase (CAK). The well-known vertebrate-type CAK is CDK-7, which interacts with H-type cyclin (CycH). The CDK-7-CycH complex activates CDK-1 and C-terminal domain (CTD) in the largest subunit of RNA polymerase II.[87,88] In plants, CDK-D, as a functional homolog of CDK-7, is needed for the CDK-D/CycH complex, which also activates A-type CDK (CDK-A) for cell cycle progression through phosphorylation in the G1 phase.[2,14–17] In *G. chorda*, *CDK-D* (K02202; PXF44578.1) and *CycH* (K06634; PXF40006.1) are highly expressed in the G1 phase (Figure 2B).

For S phase entry, deactivation (i.e., phosphorylation) of RBR is required, which is done by the CDK-A/CycD complex.[1,12,13] This mechanism is also suggested in *C. merolae*, in which the CDK-A and CycD/E complex phosphorylates RBR.[18] In this alga, transcription of *CDK-A* (XP_005535625.1; CME119C) and *CycD/E* (XP_005536848.1; CML219C) is elevated during G1 and S.[18,68] However, transcription of *CDK-A* in *G. chorda* gradually increases from G1 and peaks at G2/M (Figure 2B), but its activation for S phase entry requires construction of the complex with CycD. We identified *CycD* genes (PXF50028.1 and PXF42258.1) in the *G. chorda* genome that show a monophyletic relationship with the reference *CycD* genes in *C. merolae* (i.e., *CycD/E*, *cyclin 1*; XP_005536848.1; CML219C) (Figure S3). Two copies of *CycD* in *G. chorda* are highly expressed in G1 and S, respectively (Figure 2B). We postulate that the CDK-A/CycD complex in *G. chorda* deactivates RBR at the G1/S phase for S phase entry. The transcriptional peaks of *CycD* in *G. chorda* support G1-S transition in evening (Figures 1 and 2).

CDK-A and CDK-B play important roles in cell cycle progression in plants. CDK-A/B interact with A- and B-type cyclins (CycA/B), and the complexes lead to the G2/M transition.[12,13] Transcription of *CDK-A* (K02087) and *CDK-B* (K07760) in *G. chorda* are highly expressed at the G2/M and S phase, respectively (Figure 2B). However, *CDK-A* and *CDK-B* in other species (or lineages) show diverse gene expression patterns, as follows: 1) plants show relatively constant expression of *CDK-A* during the cell cycle, but transcription of *CDK-B* genes occur at G2 and M.[1,12,43,89] 2) The transcriptional peak of *CDK-A* (XP_005535625.1; CME119C) in *C. merolae* occurs at G1, but transcription of *CDK-B* (XP_005536088.1; CMH128C) peaks at S,[68] which is consistent with the *G. chorda* data. 3) Transcriptional peaks of *CDK-A* and *CDK-B* in *C. reinhardtii* occur at the S/M phase.[33,90] 4) *CDK-B* in *O. tauri* is highly expressed during the S/M phase, but expression of *CDK-A* is constant during the cell cycle without an M/G1 transition period.[91] Therefore, *CDK-A/B* show species- or lineage-specific transcription patterns. Furthermore, protein products of CDK-A/B are regulated by cyclins and several inhibitory pathways.[13,26,92] We postulate that red algal-specific regulatory mechanisms for CDK-A/B are possible because the plant-type CDK inhibitors, ICK/KRP and SIM/SMR, and mammalian-type CDK inhibitor INK4 and CIP/KIP are absent in red algal genomes (BLASTp search *e*-value cutoff = 1.*e*-05; details in STAR methods). Several *INK4* homologous genes were found, but these are ankyrin repeat domain-containing genes. In the *G. chorda* genome, *CDK-4* and *CDK-6* genes are also absent, which are regulatory targets of the INK4 family.[26,93] However, gene expression levels of *CycA* (K06627) and *CycB* (K21777) in this species gradually increase from S phase and peak at G2/M even though transcription of *CycB* is initially induced from G1 and *CycA* from S phase (Figure 2B). In *C. reinhardtii* and plants, transcription of *CycA* and *CycB* also starts from the G1/S (or S) phase, and their expression levels increase throughout S and G2 for the G2-M transition.[33,90,94–96] However, transcription of *CycA* and *CycB* in *C. merolae* and *O. tauri* shows sequential peaks, with *CycA* at G1/S (or S) and *CycB* at G2/M.[68,91] We postulate that the red algal CDK regulatory mechanism for cell cycle progression is primarily dependent on interactions with the corresponding cyclins.

In *G. chorda*, genes encoding CDK-E, CDK-C, and CDK-G are present, which are homologous with the mammalian CDK8/19, CDK9/12/13, and CDK10/11, respectively (Figure 2A). These mammalian CDKs are generally regarded as transcriptional CDKs that interact with the Mediator complex, C-terminal domain (CTD) of RNA polymerase II complex, and transcription factors.[87] The *CDK*s in *G. chorda* are highly

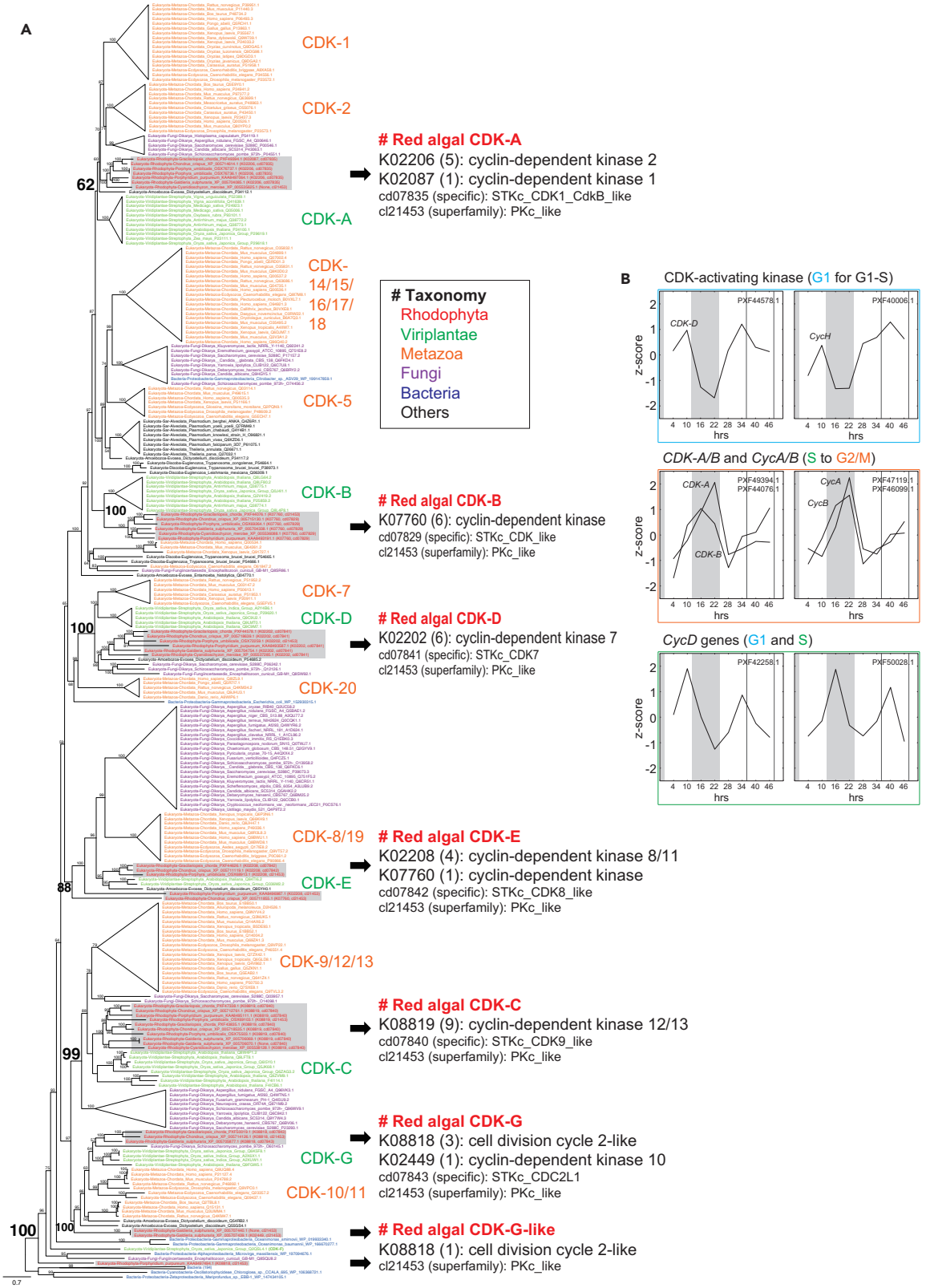

Figure 2. Cyclin-dependent kinases (CDK) in the *G. chorda* genome

(A) Phylogenetic analysis of red algal CDK genes.

(B) Gene expression patterns of *CAK*, *CDK*, and *CYC* genes in the *G. chorda* genome.

expressed in the morning (*CDK-C* and *CDK-G*) and dusk (*CDK-E*) (Figure S4). However, these transcriptional CDKs are generally controlled by protein-protein interactions or other mechanisms,[87] thus our transcriptome-based approaches are constrained when discussing the roles of these CDKs in *G. chorda*.

Endosomal recycling during mitosis in *G. chorda*

The Rab11 family is a well-known regulator with diverse functions (e.g., cytokinesis, cell adhesion, morphogenesis, and cell fusion) in endosomal recycling through the trans-Golgi network, post-Golgi vesicles, and recycling endosome during mitosis.[58,62,63] In the *G. chorda* genome, there are two copies of *Rab11a* (K07904) genes, which are highly expressed at G2/M (Figure 3). This gene family is typical because the red algal Rab11 gene family is closely related not only to photosynthetic eukaryotes but also to other eukaryotes (i.e., metazoan and fungi as outgroup taxa; Figure S5). Interestingly, the phylogenetic relationship of red algal *Rab11* shows a pattern observed in plastid gene trees comprising the primary (i.e., green lineage and red algae) and red alga-derived secondary (i.e., Stramenopiles) endosymbiosis groups, instead of the nuclear gene phylogeny.[97] The plastid gene phylogeny typically shows a monophyletic clade of red algae and Stramenopiles, positioned as a sister clade to the green lineages, similar to the phylogeny of *Rab11* genes (Figure S5). Therefore, this gene family in Stramenopiles could have originated from red algae via secondary endosymbiosis. We postulated that the eukaryotic Rab11 may contribute to mitotic progression in *G. chorda*, including mitotic spindle functions (e.g., organization and orientation) and cytokinesis.[61,98,99] Rab11 endosomal recycling is regulated by EPS15 homology domain-containing protein 1 (EHD1).[100–102] The *EHD1* gene in *G. chorda* is also highly expressed at the M phase (Figure 3).

The Arf family is also required for endosomal recycling during cytokinesis.[58–60] We found three types of *Arf* genes (class I: *Arf1*, *Arf3*, and class II: *Arf4*) in *G. chorda*. Class I genes (*Arf1* and *Arf3*) have the same gene expression pattern during the diurnal cycle (i.e., highly during the morning phase) but *Arf4* in *G. chorda* is highly expressed at dusk (Figure 3). The class I Arf1+Arf3 pair generally localizes to the trans-Golgi network to induce endosome recycling,[59] whereas the Arf1+Arf4 pair localizes to the trans-Golgi network to target primary cilia.[60,103] Both Arf pairs show similar localizations (i.e., trans-Golgi network) but are related to distinct transport pathways. In addition, the Arf1+Arf4 pair localizes not only to the trans-Golgi but also to the cis-Golgi and ER-Golgi intermediate compartment.[103] We postulate that Arf3 and Arf4 potentially have sequential functions in the endosomal recycling process during the cell cycle of *G. chorda*, consistent with their gene expression patterns (Figure 3).

The activation and deactivation of Arf proteins are induced by the GTP-bound active and GDP-bound inactive states, respectively.[65] GTPase-activating proteins, Arf GAPs, trigger GTP hydrolysis, inducing the inactive state of Arfs, whereas guanine nucleotide exchange factors, Arf GEFs, induce the GTP-bound active state of Arf proteins.[65,103] In the *G. chorda* genome, the genes encoding GAPs (SMAP, ArfGAP1, and ArfGAP2/3) are highly expressed from dawn to morning (Figure 3). The genes encoding GEFs (ArfGEF and GBF1) are also highly expressed during the mitotic phase (Figure 3). However, the interactive functions in the cell cycle of *G. chorda* remain unclear because gene expression peaks of both the Arf activator (GEP) and inactivator (GAP) protein groups are similar (Figure 3). In addition, Arf signaling and its regulatory mechanisms with GEFs and GAPs are still poorly understood.[65]

Putative model of cell cycle progression in *G. chorda* based on transcriptome data

We constructed a preliminary model of cell cycle progression in *G. chorda* based on the gene expression data described herein (Figure 4). In G1 (dusk), the pre-replication complex is induced by CDC6 and CDT1. In addition, most of the genes encoding the active replisome *CDC45/MCM2-7/GINS* (*CMG*) complex are highly expressed in G1, but transcription of the *GINS subunit 3*, as a core component of GINS, is highly induced in the evening. Therefore, the G1-S transition and S phase entry in *G. chorda* primarily occur in the evening phase (Figure 4A).

For S phase progression, the CDK-A and CycD complex is induced by CAK activity, including CDK-D and CycH in G1.[2,14–17] In *G. chorda*, *CDK-A* is gradually induced from dusk to dawn, but its corresponding cyclin *CycD* is highly expressed in the evening (Figure 4B). Therefore, S phase entry in *G. chorda* may be highly activated in the evening phase (G1/S). Transcription of S phase genes is stimulated by heterodimers of E2F transcription factors and their dimerization partner DPs, and the E2F/DP complex is inactivated by RBR until S phase entry.[1,12,13,18] Although *RBR* is highly induced in the evening in *G. chorda*, CycD is also highly induced in this phase (Figure 4C). We postulate that the highly induced CycD constructs the CDK-A and CycD complex to inactivate (phosphorylate) RBR, then S phase progression occurs by the active E2F/DPs. The gradual induction of CDK-A until the dawn phase in *G. chorda* could be explained by its role in the G2-M transition, together with CDK-B.[12,13]

The S phase in *G. chorda* progresses with the accumulation of E2F at G1, inactivation of RBR at G1/S, and continuous induction of DPs until around G2 (Figure 4C). The *Rab11* genes in *G. chorda* are highly induced during the dawn and morning phases, respectively, which potentially contribute to mitotic spindle functions and cytokinesis during mitosis (Figure 4D).[61,98,99] CDH1 mediates the degradation of mitotic proteins during late mitosis and G1,[47–49,51–56] thus M-G1 transition in *G. chorda* occurs in the morning (Figure 4D).

In *C. merolae*, the G1-S transition occurs during the day-night transition (12:12 h light and dark cycle; 12L:12D) and exit from M phase is induced in the dark period.[66,68] Similar patterns are observed in *C. reinhardtii* and *O. tauri*.[90,91] However, *G. chorda* has the G1-S transition during the dark period and remains in M phase until morning (Figure 4). Mitotic patterns in *G. chorda* resembles that of the green seaweed *Ulva pseudocurvata* and the red seaweed *Porphyra umbilicalis*, which show maximal cytokinesis 3 h after light exposure and at the night-day transition, respectively.[104] However, the highest karyokinesis activity (i.e., DNA replication), calculated using DAPI staining, in *U. pseudocurvata* and *P. umbilicalis* occurs 1 h before dark.[104] Nevertheless, the timing of maximal karyokinesis is potentially related to the G1/S transition

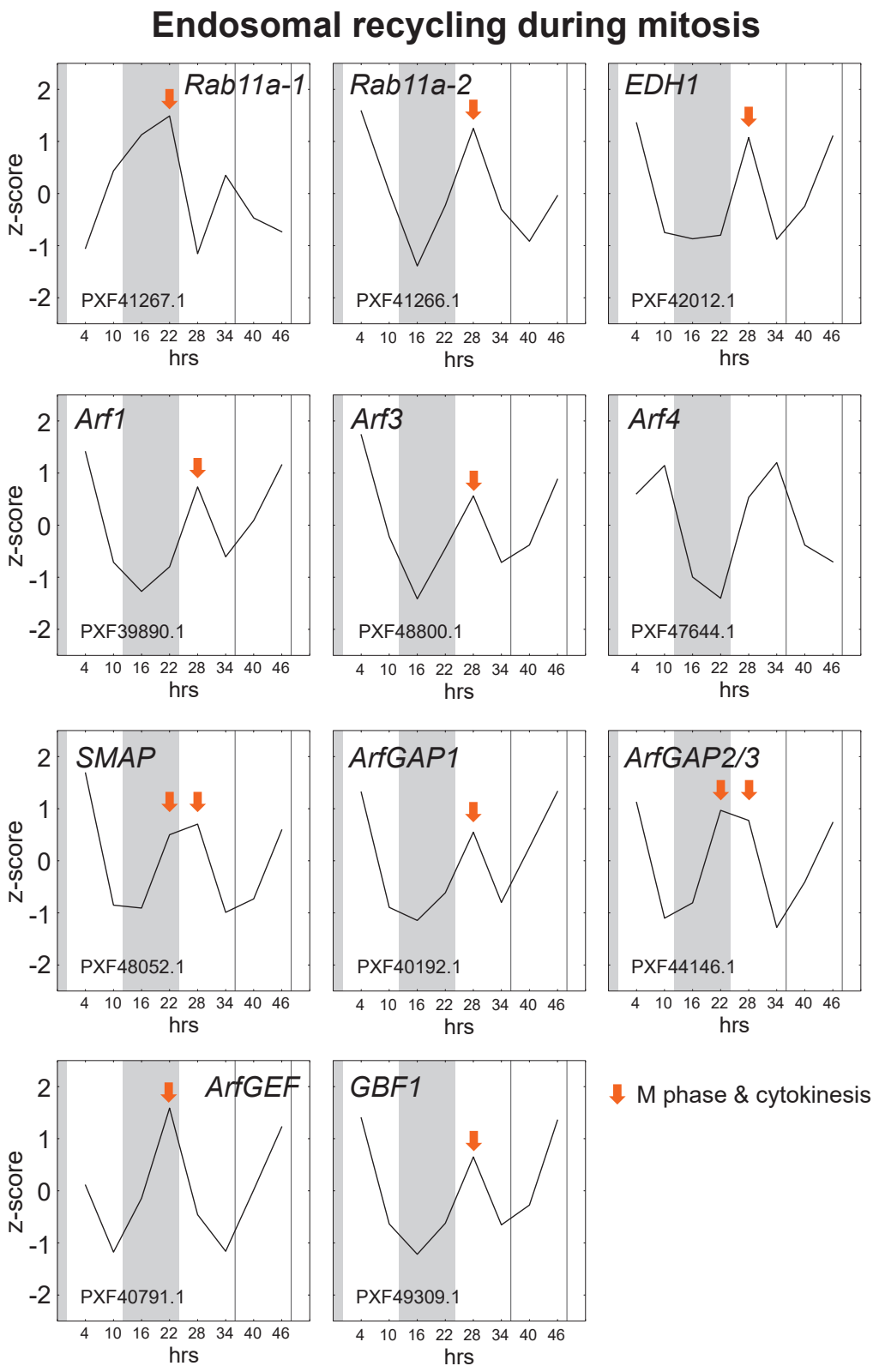

Figure 3. Gene expression patterns of endosomal recycling genes in *G. chorda*

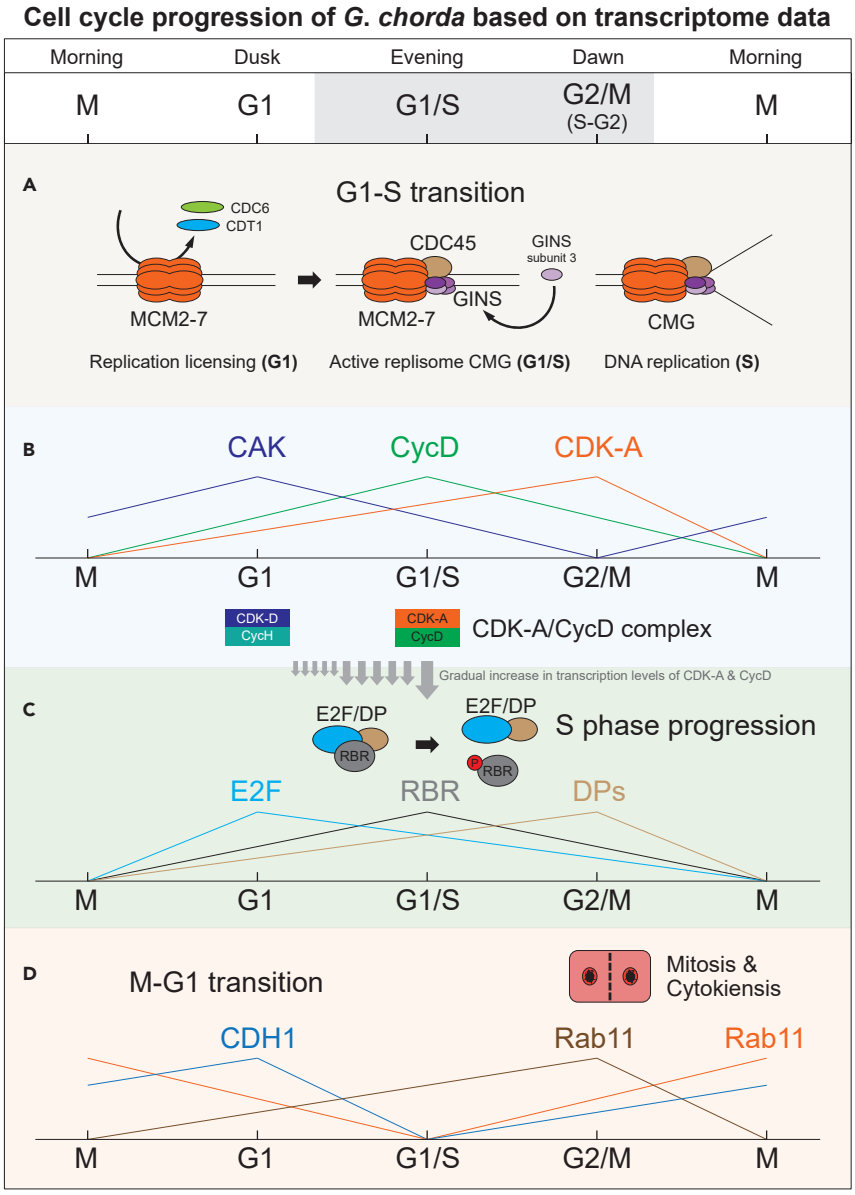

Figure 4. Putative model of cell cycle progression in *G. chorda*

(A) The G1-S transition.

(B) Gene expression patterns of *CAK*, *CycD*, and *CDK-A*.

(C) S phase progression.

(D) The M-G1 transition.

in *G. chorda*, which is highly activated during the evening phase (16 h after light exposure under a 12L:12D cycle), because the maximal karyokinesis point in *U. pseudocurvata* and *P. umbilicalis* is observed 15 h after light exposure under a 16L:8D cycle.[104] Therefore, we postulate that DNA replication in red seaweeds is controlled by rhythmic (or possibly circadian) regulation regardless of the timing of the day-night transition because most of the S phase genes in *G. chorda* show rhythmic gene expression (Table 1). The delayed entry into the S phase (15–16 h after light exposure) in red seaweeds, compared to unicellular algal species, could contribute to minimizing the risk of DNA replication stress from light (e.g., UV light) in natural environments.

Conclusions

In this study, using gene expression data we investigated cell cycle phases and their transitions in the red seaweed *G. chorda*. Our results provide important insights into cell cycle-associated physiology and its molecular mechanisms in red seaweeds.

Limitations of the study

Additional physiological studies are needed to address tissue and cell-specific cell cycle progression, cell differentiation, and their regulatory mechanism in red seaweeds. These studies should be based on tissue-specific observations during the cell cycle progression, focusing on protein-protein interactions and post-translational modifications (e.g., phosphorylation and ubiquitination).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- METHOD DETAILS
  - Analysis of gene expression patterns in *G. chorda*
  - Phylogenetic analysis of cell cycle-associated genes
  - Homologous gene search of CDK inhibitors
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110190.

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AUTHOR CONTRIBUTIONS

J.M.L. and H.S.Y. designed and supervised the project. J.M.L. led the transcriptome analysis including analysis of gene expression patterns, metabolic functions, and phylogeny. J.M.L. and H.S.Y. wrote the manuscript in collaboration. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Trimmomatic	Bolger et al.[105]	http://www.usadellab.org/cms/?page=trimmomatic
Salmon	Patro et al.[106]	https://github.com/COMBINE-lab/salmon
BLAST	National Library of Medicine	https://blast.ncbi.nlm.nih.gov/Blast.cgi
KEGG	the Kyoto Encyclopedia of Genes and Genomes	https://www.genome.jp/kegg/
Conserved Domain	Marchler-Bauer et al.[107]	https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml
MAFFT	Yamada et al.[108]	https://mafft.cbrc.jp/alignment/server/index.html
IQ-tree	Nguyen et al.[109]	http://www.iqtree.org
Rhythmic gene expression	Lee et al.[75]	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and data access should be directed to and will be fulfilled by the lead contact, JunMo Lee (junmolee@knu.ac.kr).

Materials availability

This paper did not generate new unique materials.

Data and code availability

- This paper analyses publicly available data. The RNA-seq data of *G. chorda* (SRR21594546 – SRR21594568; PRJNA872288) is available at the NCBI Sequence Read Archive (SRA) database.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS

Analysis of gene expression patterns in *G. chorda*

To analyze gene expression patterns, we utilized the published RNA-seq data of *G. chorda* (SRR21594546 – SRR21594568; PRJNA872288) from the NCBI Sequence Read Archive (SRA) database.[75] The RNA-seq data were prepared in triplicate, with one duplicate condition at each time point, but their consistent gene expressions were validated by quantitative real-time PCR analysis.[75] The RNA-seq reads were trimmed using Trimmomatic (v0.39; default options),[105] and the trimmed reads were mapped to coding sequences of the *G. chorda* genome (NCBI NBIV00000000.1)[110] using Salmon (v1.4.0; default options).[106] Based on the mapping results, we utilized the transcripts per million (TPM) values of each gene (cutoff: TPM <0.1) and calculated the average TPM values for each condition for further analyses. For comparative analyses of gene expression patterns in *G. chorda*, we calculated z-scores, which indicate relative gene expression, as follows: ('TPM value' - 'average TPM values of all conditions in each gene')/'Standard deviation of all conditions in each gene'. Rhythmic gene expression patterns, which indicate the same gene expression patterns between a day/night cycle (12:12 h light and dark) and continuous 24-h light, were identified based on the previous study.[75] The functions of protein-coding genes were predicted through metabolic pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/tools/blast/) and Conserved Domain (CD) searches.[107]

Phylogenetic analysis of cell cycle-associated genes

Red algal cyclin-dependent kinase (CDK), cyclin (Cyc), E2F, and Rab11 proteins were predicted based on their functional annotations (i.e., KEGG and CD search) and BLASTp search results (*e*-value cutoff = 1.e-05) using the UniProt database, which includes functionally validated proteins. To construct the phylogenetic tree of red algal CDKs, homologous proteins were collected from the top 100 BLASTp top hits using the Uniprot database (*e*-value cutoff = 1.e-05). For the analysis of the phylogenetic relationship of red algal Cyc proteins, we utilized a customized cyclin database, containing functionally validated *CycA/B/D/E* genes (as described in the KEGG and UniProt databases) from several well-known model species (*Chlamydomoans reinhardtii*, *Ostreococcus tauri*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Homo sapiens*, and *Mum musculus*). Representative reference E2F proteins were collected from *H. sapiens* (E2F1: Q01094, E2F2: Q14209, E2F3: O00716, E2F4: Q16254, E2F5: Q15329, E2F6: O75461, E2F7: Q96AV8, and E2F8: A0AVK6) and *A. thaliana* (E2Fa: Q9FNY0, E2Fb: Q9FV71,

E2Fc: Q9FV70, E2Fd: Q9LFQ9, E2Fe: Q8LSZ4, and E2Ff: Q8RWL0) for the phylogenetic analysis of E2F genes. Homologous proteins of red algal Rab11 were obtained from the top 100 BLASTp hits using the nr database (*e*-value cutoff = 1.*e*-05). Each set of red algal cell cycle-associated proteins and their homologous proteins were aligned using MAFFT (default option: –auto; v7.487).[108] Based on the alignments, maximum likelihood (ML) trees were constructed using IQ-tree v1.6.12[109] with ultrafast bootstrapping of 1,000 replications (-bb 1000) and model testing (-m TEST).

Homologous gene search of CDK inhibitors

To identify homologous proteins of CDK inhibitors in red algae, we conducted a BLASTp search (*e*-value cutoff = 1.*e*-05) using reference proteins of plant-type CDK inhibitors ICK/KRP (accessions of KRP1-7: Q67Y93, Q9SCR2, Q9FKB5, Q8GYJ3, Q9LRY0, Q0WNX9, and Q94CL9) and SIM/SMR (SIM: Q9LZ78 and SMRs: Q9SGE2, Q9LZ60, Q1JPP8, Q9LNX4, Q29Q81, Q9LVX6, Q9SAD3, Q3ECS5, Q9ZV27, Q9SKN7, O80930, F4IWB3, Q9LTJ2, Q1G3Y4, and Q9LN05), as well as mammalian-type CDK inhibitors INK4 (p15/CDN2B: P42772, p16/CDN2A: P42771, p18/CDN2C: P42773, and p19/CDN2D: P55273) and CIP/KIP (p21/CDN1A: P38936, p27/CDN1B: P46527, p57/CDN1C: P49918, CDKN3: Q16667).

QUANTIFICATION AND STATISTICAL ANALYSIS

The transcripts per million (TPM) values of each gene were used to compare gene expression levels. The z-scores [('TPM value' - 'average TPM values of all conditions in each gene')/'Standard deviation of all conditions in each gene'] were calculated to analyze the relative gene expression patterns. Rhythmic gene expression patterns were identified based on the previous study.[75] All bioinformatic analyses and their options are described in the method details section.