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OPINION

# GCN1 Regulates Translation of Chloroplast-Encoded Genes in Response to Light via the Nuclear Gene-Encoded Protein RH39

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**Received:** 8 May 2025 | **Accepted:** 12 July 2025

**Funding:** This study was supported by National Natural Science Foundation of China to H.Z. (Nos. 31270309, 30900357).

## 1 | Introduction

Chloroplasts are semi-autonomous organelles in plants. Communication between chloroplasts and the nucleus is essential for coordinating developmental processes and stress responses in plants, enabling them to optimize growth and defense mechanisms in response to environmental stimuli, thereby enhancing their survival and adaptability. There are several thousand proteins in chloroplasts, most of which (~90%) are encoded by the nucleus and imported post-translationally. The chloroplast genome itself contains only approximately 120 genes, with many of these genes encoding vital elements of photosynthesis, such as the Rubisco large subunit (RbcL) and subunits of thylakoidal protein complexes crucial for light reactions (e.g., PsaB, PsaF, PsbA and PsbD). Many gene products encoded by the nuclear genes interact with chloroplast-encoded proteins within chloroplasts to form functional complexes, including ATP synthase, Photosystem I (PSI), and Photosystem II (PSII) (Daniell et al. 2016; Huo et al. 2019; Yagi and Shiina 2014). Meanwhile, other nuclear genes play regulatory roles within chloroplasts, contributing to processes such as transcription, RNA splicing and translation. This communication pathway from the nucleus to the chloroplast is referred to anterograde signaling (Barkan 2011; Jan et al. 2022). (Asakura et al. 2012; de Longevialle et al. 2010; Gu et al. 2014; Lee et al. 2013; Pogson et al. 2008; Robles et al. 2012). In contrast, chloroplasts also serve as environmental sensors that respond to various stress or environmental changes. They relay signals back to the nucleus, thereby optimizing the expression of nuclear-encoded genes through a process known as retrograde signaling (Jan et al. 2022; Pogson et al. 2008; Zhang et al. 2020). The signals involved in this communication include tetrapyrroles (Larkin 2016), phosphonucleotide (3,9-phosphoadenosine 5,9-phosphate [PAP]) (Estavillo et al. 2011), methylerythritol cyclodiphosphate (MEcPP) (Xiao et al. 2012), and reactive oxygen species (ROS) (Maruta et al. 2012). Currently, most studies of these signals mainly focused on the transcriptional regulation of nuclear genes; the mechanisms by which environmental signals are transmitted to the chloroplast to trigger gene expression at the translation level are not well understood. Additionally, whether anterograde and retrograde signaling can be effectively integrated under stress conditions remains unclear.

In mammals and yeast, the general control nonderepressible-2 kinase (GCN2) is activated by GCN1 under amino acid starvation (Harding et al. 2000; Hinnebusch 1988). Activated GCN2 phosphorylates the alpha subunit of eukaryotic translation initiation factor eIF2, leading to reduced global protein synthesis (Baird and Wek 2012). In plants, GCN2 phosphorylates eIF2α to inhibit protein translation during amino acid starvation (Lageix et al. 2008). GCN1 is conserved in *Arabidopsis* and interacts with GCN2 to regulate eIF2α phosphorylation under stress conditions, such as cold, amino acid deprivation or ultraviolet (UV) (Li et al. 2018; Sattlegger and Hinnebusch 2005; Wang et al. 2017). Recently, it is discovered that light activates

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GCN2 through ROS emanating from the chloroplast during light period. The phosphorylation of eIF2α by GCN2 is light-dependent and influenced by ROS levels (Lokdarshi et al. 2020). However, it is unclear whether this pathway will transmit the signal back to the chloroplast to modulate chloroplast function.

Chloroplast-encoded proteins are synthesized by the chloroplast 70S ribosome, which consists of a 50S large subunit and a 30S small subunit (Harris et al. 1994). Chloroplast rRNA is transcribed into a precursor RNA in the rrn operon, which is then processed into 16S rRNA, 5S rRNA, and 23S-4.5S rRNA intermediate before further cleavage into 23S and 4.5S rRNA (Olinares et al. 2010). The 23S rRNA needs to be processed by helicase and RNase (Bellaoui et al. 2003; Bisanz et al. 2003; Bollenbach 2005; Zenke et al. 1982). The processing includes the introduction of hidden break and removal of specified regions in the 23S rRNA. RH39, a member of the chloroplast DEAD box RNA helicase family, plays a critical role in introducing hidden breaks in 23S rRNA and is essential for the translation of chloroplast-encoded proteins (Nishimura et al. 2010). In this study, we demonstrate that light perceived by chloroplasts activates GCN1-GCN2, leading to the phosphorylation of eIF2α, subsequently activating the translation of RH39 and promoting the translation of chloroplast proteins. Our findings uncover that the integration between chloroplast anterograde and retrograde signals establishes a complete regulation loop for plant adaptation to light-dark variations.

## 2 | Materials and Methods

### 2.1 | Plant Growth Conditions

Columbia of *Arabidopsis thaliana* has been mutagenized using ethyl methane sulfonate (EMS), resulting in the *gl1* mutant, which is characterized by the absence of trichrome on the leaf surface. The *gl1* mutant serves as the wild type (WT) of *gcn1* (Wang et al. 2017). Both WT and *gcn1* mutants were sown on a half-strength MS medium containing 0.8% (w/v) agar, with or without the specified concentrations of inhibitors. The plants were then cultivated at 22°C under a light regimen of 16 h light and 8 h dark.

### 2.2 | Total Protein Extraction and Western blot Analysis

Proteins were extracted from 10-day-old seedlings following previously established protocols (Wang et al. 2017). Both a complete protease inhibitor mixture (04693116001, Roche complete) and Phos STOP phosphatase inhibitor (PIA32959, Thermo Fisher Scientific; cat. no.) were added to the extraction buffer, which consisted of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 1 mM DTT, 1 mM PMSF. The extracted proteins were denaturized at 100°C for 5 min, subsequently separated using SDS-PAGE, and transferred onto PVDF membranes. Antibody targeting phospho-eIF2α (S51) (TA3087, Abmart, 1/2500 dilution) and Actin (M20009, Abmart, 1/10 000 dilution) were probed and visualized by ECL luminescence Kit (K22020, Abbkin).

### 2.3 | Chloroplast Protein Extraction and Western Blot Analysis

The fresh 14-day CP-HA transformed plant seedlings (1 g) were harvested and placed in a mortar on ice, and fully ground to homogenate with 1× CIB buffer (0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$, 20 mM HEPES, pH 8.0 adjusted with KOH). The solution was then filtered into a 1.5 mL centrifuge tube. Subsequently, the solution was mixed with 20 μL Anti-HA Magnetic Beads (VI307831, Thermo), and incubated with shaking for 15 min at 4°C. The beads were captured using a magnetic rack, and the supernatant was removed. Then the beads were washed for three times with 1 mL 1× CIB buffer. Finally, the beads were resuspended in 60 μL 1× CIB buffer and transferred to a new 1.5 mL centrifuge tube. 5× protein loading buffer was added, and the mixture was boiled for 10 min. The proteins were separated by 12% (w/v) SDS-PAGE. The antibodies used for western blot were obtained as follows. PsbA Ab (AS05084, Agrisera, 1/5000 dilution), PsbD Ab (AS06146, Agrisera, 1/5000 dilution), PsaB Ab (AS10695, Agrisera, 1/5000 dilution), RbcL Ab (AS03037, Agrisera, 1/5000 dilution), HA Ab (A02041, Abbkine, 1/10 000 dilution).

### 2.4 | Transient Expression of RH39 Protein in *Arabidopsis* Protoplasts

For the generation of construct which express Flag-tagged RH39 and Flag-tagged YFP, the pGRDR plasmid (Li et al. 2022b) which contains two individual 35S promoter and Nos terminator sequences, was utilized. Initially, the coding region of RH39 was amplified and ligated into the pS1300-FLAG vector using XmaI and KpnI restriction sites. Subsequently, the RH39-3×FLAG fragment (including the stop codon) was inserted into pGRDR via homologous recombination at the *BamH1* restriction site located downstream of the second 35S promoter. The Nos terminator sequence was then appended to the 3′ end of the FLAG tag. Additionally, another 3×FLAG fragment was integrated between the first 35 promoter and YFP, resulting in a new vector designated pGRDR-YFPF-RH39F. *Arabidopsis* protoplasts were isolated from 14-D-old seedlings following the method described by Zhai et al. (2009). Approximately 3 mL of protoplasts (~$2 \times 10^6$ cells/mL) from *gl1* or *gcn1* mutants were transfected with 100 μg of pGRDR-YFPF-RH39F. Total protein was extracted by macerating frozen tissue in a protein extraction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.25%[v/v] Nonidet P-40, 0.25% [v/v] Triton X-100, 0.05% [w/v] SDS, 1 mM DTT, 1×complete protease inhibitor mixture [4693132001, Sigma-Aldrich]). Proteins were boiled with 5×SDS loading buffer for immunoblot analysis using an anti-FLAG-HRP antibody (Biodragon, BDAA0197, 1/30 000 dilution).

### 2.5 | Northern Blot Analysis

Total RNA (5 μg) was extracted with Trizol solution (Invitrogen), electrophoresed in 1.2% agarose gel and transferred onto HyBond $N^+$ membranes as previously described (Gong et al. 1997). The transferred membranes were then fixed

at 80°C for 2 h, hybridized with probes labeling with digoxigenin-dUTP using a Prime-It II random primer labeling kit and detected enzyme immunoassay. The sequence information of probes used can be found in Supporting Information S1: Table S1.

### 2.6 | Ribosome Extraction, RNA Extraction and qPCR

Ribosomes were extracted following previously reported methods (Sormani et al. 2011) with minor modifications. Briefly, 200 mg of 10-day-old seedlings were ground in liquid nitrogen and resuspended in 1 mL of lysis buffer (100 mM Tris–HCl, pH 8.4, 50 mM KCl, 25 mM $MgCl_2$, 5 mM EGTA, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 0.5% Nonidet P40, RNase inhibitor). The samples were incubated at 4°C for 15 min, followed by centrifugation at 9000 g for 15 min, performed twice. The supernatant was then loaded onto 12 mL sucrose gradients (20%–50%) and subjected to centrifugation at 175 000 g using a Hitachi rotor P40ST (Figure 3c) or Beckman SW41 rotor (Figure 2a) for 165 min. Subsequently, the gradients were fractionized from the top using an ISCO gradient fractionator (BIOCOMP), while the OD value at 260 nm was recorded. RNA from each fraction was extracted using phenol extraction. A total of 10 μL of RNA was reverse-transcribed into cDNA using cDNA synthesis super mix (buffer, dNTP, Hifair III reverse transcriptase, RNase inhibitor and random primers/Oligo (dT)18 primer mix) (Yeasen, HB210629). The resulting cDNA was diluted five-fold for semi-quantitative PCR and quantitative real-time PCR. qPCR was performed using the ABI Stepone plus Real-time PCR system with the following parameters: 95°C for 15 min, 40 cycles of 95°C for 15 s, 60°C for 40 s, concluding with a melting curve analysis. The sequence information of primers used can be found in Supporting Information S1: Table S1.

## 3 | Results

### 3.1 | The Translation of Chloroplast-Encoded Proteins Is Downregulated in *gcn1*

GCN1 is a positive regulator of the GCN2 kinase, which phosphorylates eIF2α in mammals, yeast, and plants. This phosphorylation of eIF2α inhibits ribosome loading onto mRNA, resulting in a global suppression of protein translation (Lageix et al. 2008; Pereira et al. 2005; Sattlegger and Hinnebusch 2005; Wang et al. 2017). We previously found that *Arabidopsis* GCN1 also modulates the translation of chloroplast-encoded genes. In *gcn1* (referred from *atgcn1-1*) mutant, the translation of chloroplast-encoded genes was significantly reduced (Cui et al. 2021). To confirm this, we examined the differential impacts of cycloheximide (CHX) and Lincomycin (Linc) on *gcn1* mutants. CHX inhibits cytoplasmic translation, while Linc is a specific and highly effective inhibitor of chloroplast 70S ribosomal function and inhibits the chloroplast protein synthesis (Nott et al. 2006; Zhao et al. 2018).

Seeds of the wild type (WT) and the *gcn1* mutant were sown on MS medium with or without CHX supplementation, and green cotyledon ratios were counted at day 5 after the end of vernalization (Figure 1a). Results showed that *gcn1* exhibited reduced sensitivity to CHX compared to WT, as evidenced by a higher green cotyledon ratio in *gcn1* than in the WT (Figure 1b), suggesting that the translation efficiency of cytoplasmic proteins in *gcn1* was elevated relative to that in the WT.

Seedlings were also grown on MS medium with or without Linc under continuous high-intensity light (24 h/day) for 12 days, and the result showed a high ratio of *gcn1* seedlings with pale yellow or white leaves compared with the wild type, particularly in the presence of Linc. Notably, even in the absence of Linc treatment, *gcn1* leaves displayed a light-green coloration relative to wild-type counterparts. Further analysis of chlorophyll content in these seedlings indicated a lower level in *gcn1* compared to WT (Figure 1c,d). Therefore, we postulate that the hypersensitivity of *gcn1* to Linc may be related to altered chloroplast-encoded protein translation in *gcn1*.

To evaluate the accumulation levels of chloroplast-encoded proteins, we crossed *gcn1* with the OMCP-HA pants, which express a chloroplast outer membrane-anchored protein CP fused with a HA-tag (Bao et al. 2023). This approach enabled the rapid enrichment of intact chloroplasts using anti-HA beads. Subsequently, chloroplast-encoded proteins were analyzed via Western blot analysis with the corresponding antibodies. As shown in Figure 1e, while equal protein loading was confirmed by the comparable intensities of the HA bands, the levels of chloroplast-encoded proteins PsaB (ATCG00340), PsbA (ATCG00020), PsbD (ATCG00270), and RbcL (ATCG00490) were notably reduced in *gcn1* (Figure 1f). To rule out the possibility of transcriptional regulation, we examined the relative transcript levels of PsaB, PsbA, PsbD, and RbcL using quantitative PCR (qPCR) on cDNA synthesized from total RNA reverse-transcribed with random primers. The results indicated no significant differences in overall transcript levels between the wild type and *gcn1* (Figure S1). Collectively, these findings suggest that the reduction in chloroplast-encoded protein accumulation in *gcn1* is attributable to impaired translation.

### 3.2 | The Translation Level of *RH39* Is Down Regulated in *gcn1*

A previous study demonstrated that the RNA helicase RH39 mediates the introduction of hidden breaks in 23S rRNA, thereby regulating the translation elongation of chloroplast-encoded proteins (Nishimura et al. 2010). Additionally, our analysis of polysome RNA sequencing data revealed that the translation of RH39 is downregulated in *gcn1* (Cui et al. 2021). However, RH39 transcript levels showed no significant difference between the wild type and *gcn1* mutant (Supporting Information S1: Figure S4). We hypothesize that the translation suppression of chloroplast-encoded proteins may be resulted from the translation repression of *RH39* in *gcn1*.

To test this hypothesis, we analyzed the translation level of *RH39* in *gcn1* mutant. Total ribosomes were extracted and loaded onto a 20%–50% sucrose gradient. Ribosomes were subsequently separated via ultracentrifugation and the gradients were divided into 15 equal fractions from the top. RNA

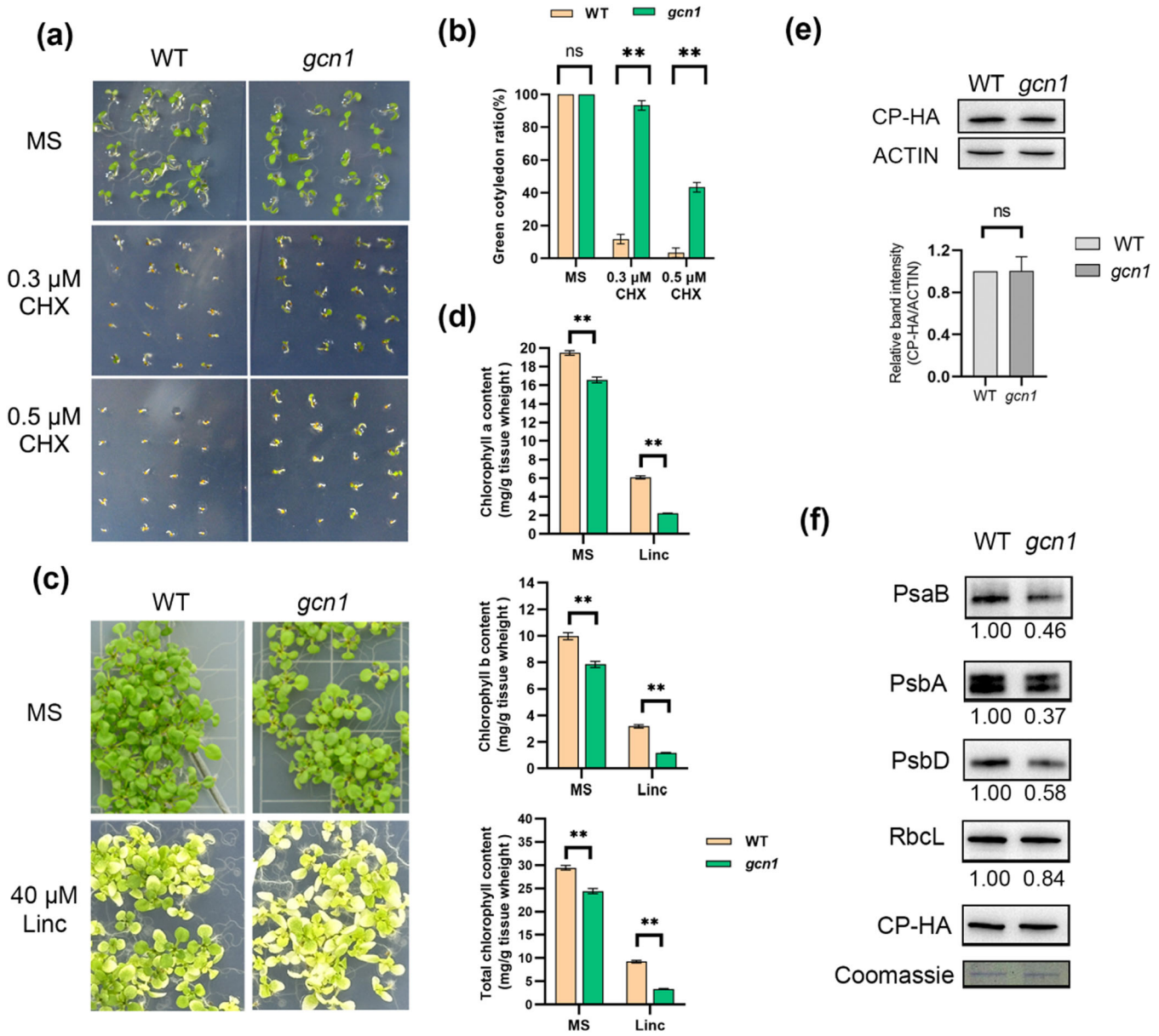


**FIGURE 1** | *gcn1* mutant is insensitive to CHX, but hypersensitive to Linc compared to the wild type. (a) WT and *gcn1* seeds germinated on MS ± CHX for 5 days. (b) Statistical analyses of the green cotyledon ratios in (a). Data represent mean ± SD ($n = 3$). (c) Seedlings were grown on MS medium ± 40 μM Linc under continuous strong light (15000 lux) at 22°C for 12 days. (d) The chlorophyll content of WT and *gcn1* mutant under MS medium with or without Linc, described as (c). Total chlorophyll means Chla+b. Data represent mean ± SD ($n = 3$). (e) Nuclear protein CP (localized to chloroplast membrane), fused to an HA tag, was transformed into WT plants. Transgenic plants were crossed with *gcn1*, and *gcn1* mutants carrying HA-tagged CP were selected from the F2 generation. Total protein extracts from WT and *gcn1* mutants were analyzed by Western blot using anti-HA antibody. ACTIN served as the loading control. Band intensities were quantified (*ImageJ*) and normalized to ACTIN. Data represent mean ±SD ($n = 3$). (f) Chloroplasts were extracted using anti-HA beads, and chloroplast-encoded proteins were detected by Western blot. CP-HA and Coomassie-stained bands were used as loading controls. Band intensities were quantified (*ImageJ*) and normalized to CP-HA. Data: mean ± SD ($n = 3$). $^{**}P$ value < 0.01 (Student's *t*-test). "ns" means no statistical significance ($P$ value > 0.05). [Color figure can be viewed at wileyonlinelibrary.com]

from each fraction was used to check the ribosome loading of various genes by quantitative PCR. The *alpha subunit of the eukaryotic translation elongation factor 1* (*eEF1α*) was identified as an ideal reference gene for studying the translation of other genes, as it is uniformly translated (Missra and von Arnim 2014). First, we assessed the translation levels of eEF1α in *gcn1* mutant and WT using semi-quantitative RT-PCR. Our findings indicated that the translation levels of eEF1α were consistent between WT and *gcn1* (Supporting Information S1: Figure S2). Subsequently, eEF1α was chosen as the reference gene for translation level detection using qPCR. The results revealed a significant reduction in RH39 transcripts in the *gcn1* mutant compared to WT in fractions 9, 10, 12, 13 and 14, where polysome accumulation was observed. Conversely, RH39 transcript levels were higher in *gcn1* than in the WT in fractions 7 and 8, where monosomes were present (Figure 2a), suggesting that the translation level of RH39 is repressed in the *gcn1* mutant.

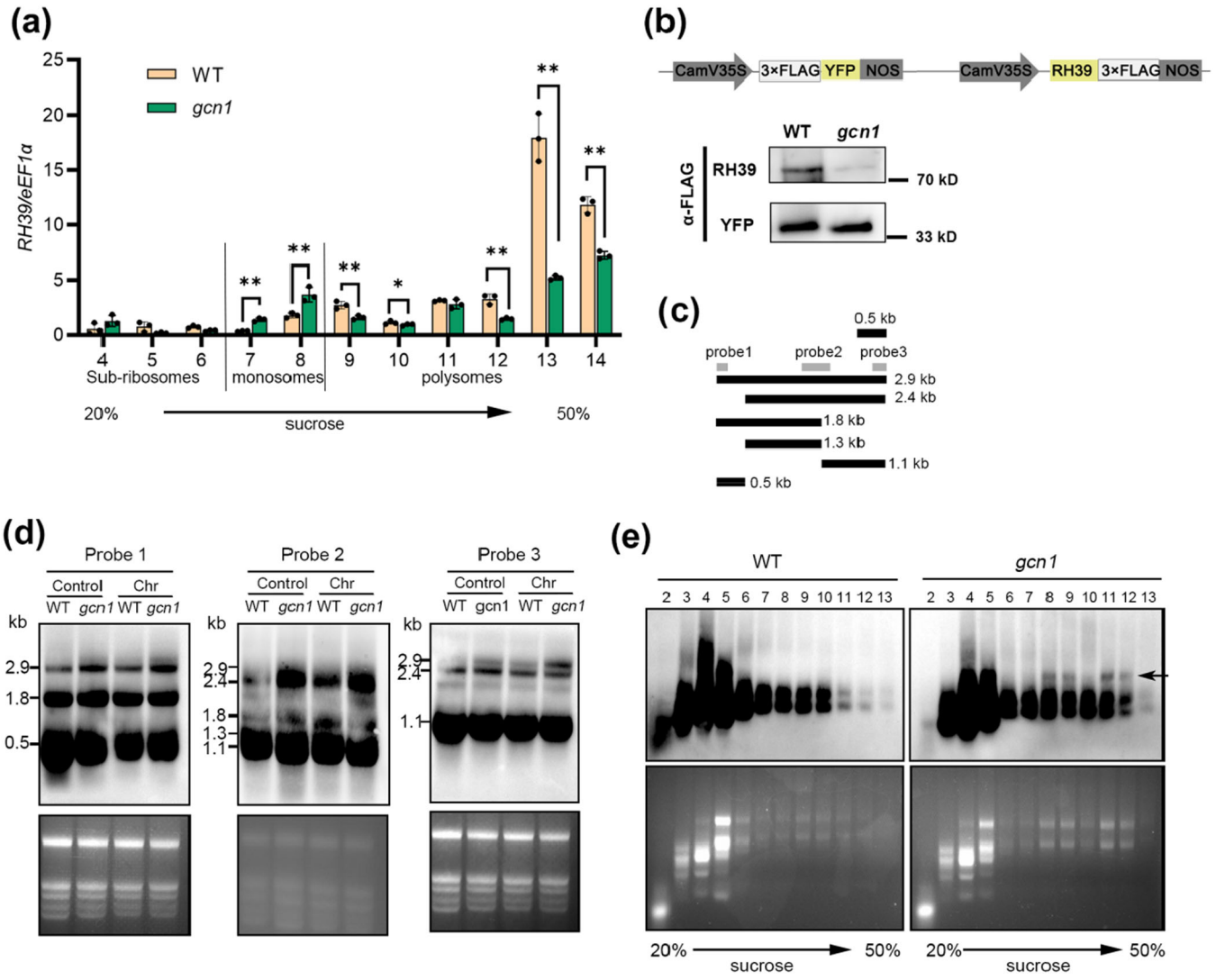


**FIGURE 2** | The reduction of RH39 in *gcn1* leads to the incomplete processing of 23S rRNA (a) Total ribosomes were separated by sucrose gradient ultracentrifugation. Gradients were fractionated into 15 equal fractions from the top; mRNA-depleted fractions 1–3 and 15 were discarded. RNAs were extracted from sub-ribosomal (4–6), monosomal (7–8), and polysomal (9–14) fractions. RH39 mRNA levels were quantified by qPCR (eEF1α reference gene). Data represent mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ (Student's *t*-test). (b) RH39 and YFP, fused with 3×FLAG tags and driven by independent 35S promoters, were transiently expressed in WT and *gcn1 Arabidopsis* protoplasts. Proteins were extracted for anti-FLAG Western blot. YFP served as transformation efficiency marker and loading control. (c) Three probes were designed according to the splicing sites of 23S rRNA following previous research (Nishimura et al. 2010). (d) Northern blot analysis of 23S rRNA fragments from 10-day seedlings using Probes 1–3. Ethidium bromide-stained rRNA served as loading control. Seedlings were treated with 0.5 mM chlorsulfuron for 2 h. (e) Total ribosomes were separated by sucrose gradient ultracentrifugation. RNAs from each fraction were extracted and hybridized with labeled probes specific to the full 23S rRNA sequence. Arrows mark enhanced 2.9-kb bands in *gcn1*. Ethidium bromide-stained rRNA served as the loading control. [Color figure can be viewed at wileyonlinelibrary.com]

Furthermore, we investigated the accumulation of RH39 protein in the *gcn1* mutant by transiently expressing RH39-FLAG in *Arabidopsis* protoplasts. We utilized the pGRDR vector, which contains two independent 35S promoter sequences and two Nos terminators (Li et al. 2022b). Both RH39 and YFP were fused with a 3×FLAG tag and expressed separately under the control of the two 35S promoters, with YFP serving as the reference gene and loading control. The results indicated that RH39 protein levels in the *gcn1* mutant were lower than those observed in the wild type, suggesting an inhibition of RH39 translation in the *gcn1* mutant (Figure 2b).

### 3.3 | Incomplete 23S rRNA Processing in *gcn1*

The full-length chloroplast 23S rRNA (2.9 kb) is cleaved into 0.5, 1.3 and 1.1 kb fragments through post-maturation processing, while RNA helicase RH39 is crucial during processing (Nishimura et al. 2010). Our findings indicated that the translation of RH39 was repressed in the *gcn1* mutant. Consequently, we investigated 23S rRNA processing in *gcn1*. Total RNA was extracted from 10-day-old seedlings, and 23S rRNA was analyzed using northern blot (Figure 2c,d). Based on previously identified splicing sites of 23S rRNA, we designed three probes

for this analysis (Figure 2c) (Nishimura et al. 2010). The northern blot results showed that the 2.9 kb full-length 23S rRNA were overstocked in *gcn1*, while the other fragments of 23S rRNA were not significantly influenced (Figure 2d). This finding indicates that the reduced translation of RH39 in *gcn1* leads to incomplete processing of 23S rRNA. Additionally, separated ribosomal fractions in a sucrose gradient were analyzed by northern blot, using the full-length 23S rRNA as a probe. Long fragments of 2.9 kb 23S rRNA were detected in fractions 8, 9, 11, and 12 from *gcn1*, but were absent in wild type (Figure 2e), further suggesting that the 23S rRNA processing is indeed incomplete in *gcn1*.

In *Arabidopsis*, GCN2 activation is induced by salicylic acid (SA), methyl jasmonate (JA), ultraviolet (UV), wounding, cold stress and amino acid deprivation, leading to the phosphorylation of eIF2α (Lageix et al. 2008; Wang et al. 2017; Zhang et al. 2008). Chlorsulfuron (Chr), a known inhibitor of amino acid biosynthesis (specifically isoleucine, leucine, and valine) serves as a tool for inducing amino acid starvation in plants. Notably, eIF2α phosphorylation levels were found to significantly increase following Chr treatment (Figure 3a) (Wang et al. 2017). To elucidate the impact of elevated eIF2α phosphorylation on the post-maturation processing of chloroplast 23S rRNA, various 23S rRNA fragments were analyzed under Chr conditions. Nonetheless, both the processing of 23S rRNA (Figure 2d) and the accumulation of chloroplast-encoded proteins (Supporting Information S1: Figure S3) did not exhibit significant alterations despite the marked enhancement in eIF2α phosphorylation following chlorsulfuron. We hypothesize that excessive phosphorylated eIF2α may have minimal influence on the splicing efficiency of 23S rRNA, highlighting the necessity of maintaining homeostatic levels of this modification.

### 3.4 | eIF2α Phosphorylation and RH39 Translation Were Controlled by Photoperiod and Down regulated Under Dark Conditions

A previous study demonstrated that GCN2 is activated to phosphorylate eIF2α in response to reactive oxygen species (ROS) derived from chloroplasts under light conditions, and the phosphorylation levels of eIF2α exhibited a circadian rhythm over a 24-h period (16 h of light/8 h of dark). (Lokdarshi et al. 2020). In this study, we also examined the phosphorylation levels of eIF2α throughout the light cycle by western blot with antibodies specific to phosphorylate eIF2α, and found that the peak phosphorylation level occurs at the 8th hour of the photo period during a 16-h light (from 7:00 to 23:00) and 8-h dark cycle (Figure 3a). Furthermore, the eIF2α phosphorylation was also detected under dark treatment. Ten-day-old seedlings were moved to darkness at 12:00 and 15:00 during the light phase and were harvested at 17:00. Control seedlings were also harvested at 17:00 under continuous light conditions. Our results revealed a significant decrease in eIF2α phosphorylation levels after 2 and 5 h of dark treatment compared to the control. Notably, eIF2α phosphorylation was completely absent in the *gcn1* mutant (Figure 3b), suggesting that light signal can modulate eIF2α phosphorylation, and that GCN1 is essential for eIF2α phosphorylation under light conditions.

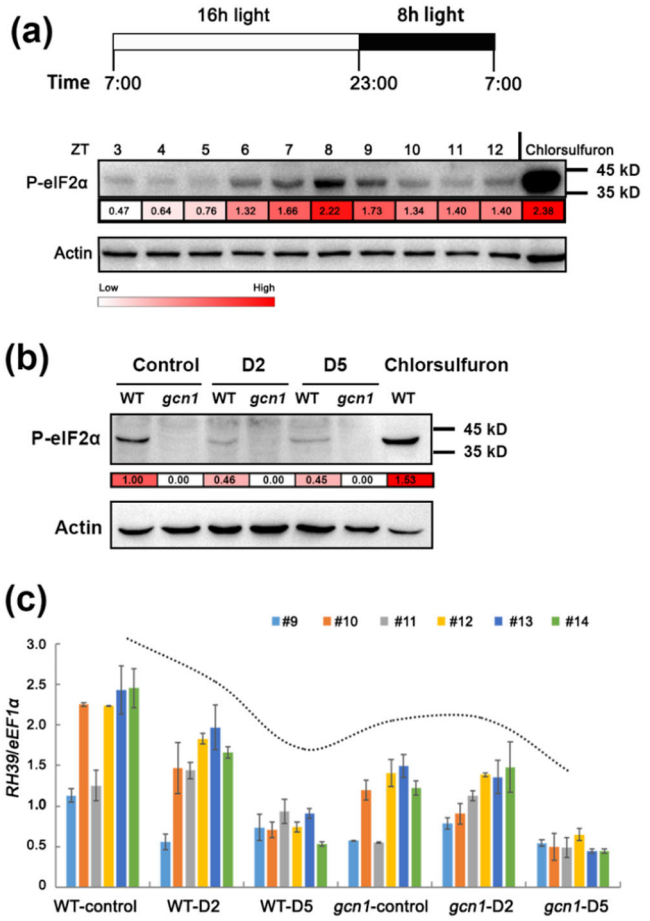


**FIGURE 3** | eIF2α phosphorylation and RH39 translation are down regulated during dark treatment (a) The temporal dynamics of eIF2α phosphorylation were assessed from 9:00 to 18:00 in 10-day-old wild-type seedlings grown under 16-h light/8-h dark cycles. Chlorsulfuron-treated seedlings (2-h exposure) served as positive controls for phosphorylated eIF2α. Phospho-eIF2α levels were assayed by Western blot using phospho-specific antibodies. ACTIN served as internal reference. Band intensities were quantified (*ImageJ*) and normalized to ACTIN. (b) Phosphorylation levels of eIF2α under dark treatment. Ten-day-old seedlings exposed to darkness starting at 12:00 (5-h dark/D5) or 15:00 (2-h dark/D2) were harvested at 17:00. Control seedlings were collected simultaneously (17:00) under continuous light conditions. (c) RH39 translation levels declined following dark treatment. Total ribosomes from 10-day seedlings (treatments in Figure 3b) were separated by sucrose gradient ultracentrifugation. RNAs from polysomal fractions (9–14) were quantified by qPCR (eEF1α reference gene). A dashed line was used to represent the trend line, calculated based on the average of six samples per group. Experiments were repeated three independent times. [Color figure can be viewed at wileyonlinelibrary.com]

Based on these findings, we hypothesize that the translation of RH39 is also modulated by light and dark signals, as its transcript levels show no significant difference following dark treatment (Supporting Information S1: Figure S4). We examined the translation levels of RH39 under dark conditions as outlined in Figure 3c. The polysome bound-RH39 transcripts were quantified using qPCR according to the protocols described in Figure 2a. Our findings indicated a decrease in the translation level of RH39 in the *gcn1* mutant, which were consistent with previous results (Figure 2a). Additionally, we

observed a downward trend in translation following dark treatment in both WT and *gcn1* mutant samples (Figure 3c). Since *eEF1α* was used as a reference gene, we also detected the ribosome loading of *eEF1α* mRNAs via semiquantitative RT-PCR. The results demonstrated that translation of eEF1α was synchronous between the WT and *gcn1*, but exhibited a decline under dark treatment (Supporting Information S1: Figure S2). Nonetheless, our data showed that the translation of RH39 was downregulated under dark condition, with eEF1α serving as a reference gene. This suggests that the translation of RH39 is indeed repressed during the dark period.

### 3.5 | 23S rRNA Processing Was Defective in the Dark

Together with the findings described above, we speculated that the post-maturation processing of chloroplast 23S rRNA may also be abnormal under darkness. Total RNA was extracted from 10-day-old seedlings subjected to 2-h and 5-h dark treatments. We next checked for 23S rRNA processing by northern blot using probes 1, 2 and 3 of 23S rRNA, as well as with the full-length 23S rRNA probe. The northern blot results indicated an increase in the 2.9 kb full-length 23S rRNA band in *gcn1* (Figure 4) (arrowheads denote the observed alterations), consistent with the data presented in Figure 2d. Furthermore, the 2.9 kb fragments of 23S rRNA exhibited significant accumulation in the northern blot analyses using probes 1, 2, 3, and the full-length 23S rRNA probe following dark treatment (Figure 4) (asterisks highlight the observed changes). We were unable to detect all changes due to the inadequate sensitivity of the nonradioactive DIG-based labeling method. Overall, exposure to darkness resulted in impaired splicing of 23S rRNA.

### 3.6 | The Accumulation of Chloroplast-Encoded Protein Decreased Under Dark Treatments

We observed a decrease in both the phosphorylation of eIF2α and the translation levels of RH39 under dark conditions, accompanied by a defect in 23S rRNA processing. Based on these findings, we subsequently investigated the accumulation of chloroplast-encoded proteins under dark treatment. For this experiment, 10-day-old seedlings were transferred to dark conditions at 12:00 and subjected to a 5-h treatment. Chloroplast proteins were extracted following the protocols described in Figure 1f. Western blot analysis revealed that the accumulation of chloroplast-encoded proteins, including PsaB, PsbA, PsbD, and RbcL, was significantly reduced following dark treatment. CP-HA was used as loading controls (Figure 5). Additionally, the transcript levels of these chloroplast-encoded genes did not exhibit any reduction after dark treatment compared to the control, thereby eliminating the possibility of a transcriptional effect (Supporting Information S1: Figure S1).

Collectively, we conclude that GCN1-GCN2-mediated eIF2α phosphorylation regulated the translation of nuclear gene *RH39*, thereby modulating the translation of chloroplast-encoded proteins. The light captured by chloroplasts activates this signaling pathway, which in turn regulates the synthesis of chloroplast proteins. This communication between chloroplast and nucleus enables plants to adapt to light-dark changes in the environment.

## 4 | Discussion

The interorganellar crosstalk ensures the coordination of nuclear and chloroplast gene expression to cope with environmental challenges and optimize growth and physiological processes. A critical aspect of this communication is regulation of chloroplast gene expression. The quantity of chloroplast proteins is controlled at various levels, such as transcription and translation. Previous research has indicated that chloroplast mRNA levels are not limiting factors for protein synthesis. For example, in response to light, protein levels can exhibit significant variation, while mRNA levels remain relatively constant (Eberhard et al. 2002; Malno et al. 1988; Nickelsen et al. 2014). This observation suggests that chloroplast translation plays a key role in determining the levels of photosynthetic proteins.

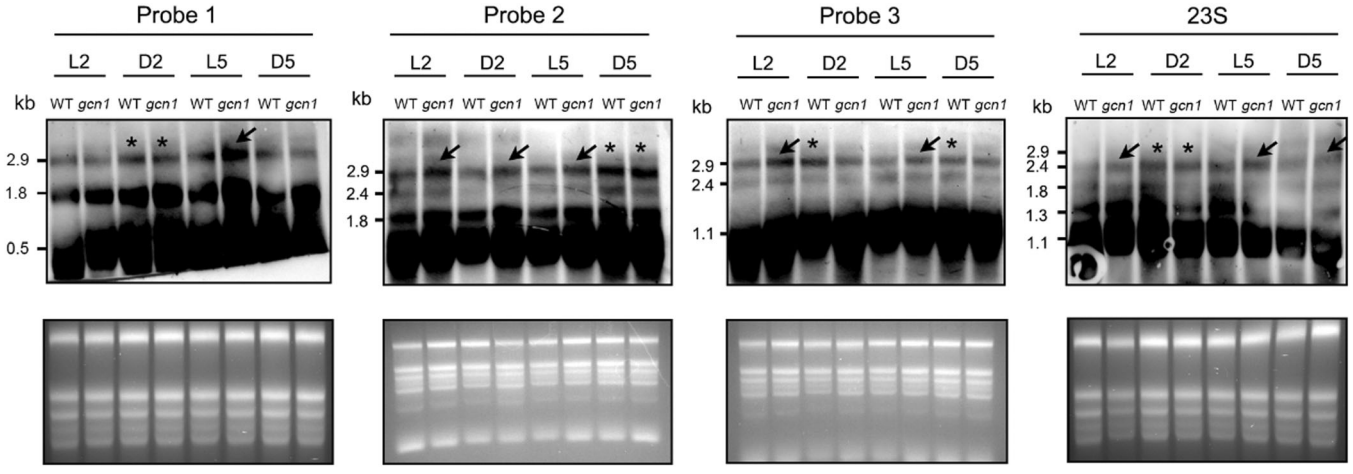


**FIGURE 4** | Defect of 23S-rRNA processing in dark treatments Seedlings were treated as in Figure 3b. Northern blot analysis of 23S rRNA fragments and full-length 23S rRNA was performed using probes 1-3 or labeled full-length 23S rRNA. Ethidium bromide staining of rRNA served as the loading control. Arrowheads: Indicate accumulated full-length 23S rRNA (2.9 kb) in *gcn1* mutants compared to wild-type (WT). Asterisks: Indicate accumulated full-length 23S rRNA (2.9 kb) under dark treatment versus light conditions.

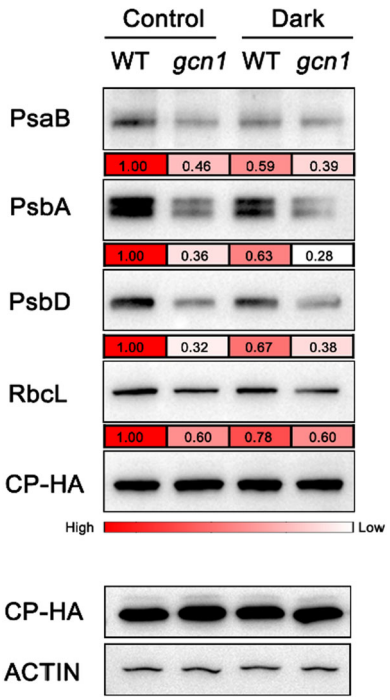


**FIGURE 5** | Analysis of chloroplast-encoded proteins accumulation under dark treatmentsSeedlings were treated with darkness for 5 h as in Figure 3b. Chloroplasts were isolated using anti-HA beads (as in Figure 1f). Chloroplast proteins were analyzed by Western blotting using CP-HA bands as loading controls. Band intensities were quantified with *ImageJ*, and relative protein amounts were normalized to CP-HA signals. CP-HA levels relative to ACTIN are shown below. [Color figure can be viewed at wileyonlinelibrary.com]

Consequently, exploring the regulation of chloroplast protein translation at the translation level holds important implications.

We have previously demonstrated that GCN1 is required for GCN2 activation to phosphorylate eIF2α, and the phosphorylated eIF2α selectively mediates the translation of specific genes (Cui et al. 2021; Wang et al. 2017). In *gcn1*, the phosphorylation of eIF2α was barely detectable (Figure 3b), resulting in suppressed translation of RH39 (Figure 2a,b), combined with incomplete 23S rRNA processing (Figure 2d,e) and a decrease in the accumulation of chloroplast proteins (Figure 1f), indicates that GCN1-GCN2-eIF2α regulates the translation of chloroplast genes through RH39. On the other hand, both the *gcn1* and *RH39* loss-of-function mutant *nara12* displayed a similar phenotype that the newly emerged leaves of *gcn1* and *nara12* mutants exhibited a yellow phenotype, suggesting that they are involved in the same signaling pathway (Nishimura et al. 2010; Wang et al. 2017; Wang et al. 2016). Additionally, both *gcn1* and many loss-of-function mutants of genes involved in chloroplast translational regulation were cold-sensitive and displayed bleaching of newly emerged leaves at low temperature, such as *cp29a*, *cp31a*, *rbd1*, and *ormm1* (Kupsch et al. 2012; Sun et al. 2013; Wang et al. 2017; Wang et al. 2016). The CP29A and CP31A proteins belongs to the chloroplast ribonucleoproteins (cpRNPs) family. The 23S rRNA processing is incomplete, especially at low temperatures (Kupsch et al. 2012). RBD1 is an RNA-binding protein, and *rbd1* mutants are defective in generating mature 23S rRNAs, leading to deficiencies in chloroplast protein synthesis, revealing it is important for chloroplast protein translation (Wang et al. 2016). ORRM1 (Organelle RRM protein 1), carrying an RNA recognition motif (RRM) at its C terminus is an essential plastid editing factor. In *Arabidopsis* and maize *orrm1* mutants, RNA editing is impaired, subsequently impacting the translation of chloroplast proteins (Sun et al. 2013). It can be found that these genes, along with GCN1, are all involved in the regulation of chloroplast protein translation through 23S rRNA processing or RNA splicing. These evidence strongly support the role of GCN1 in modulating chloroplast protein translation via RH39. Moreover, under 24-h light conditions, chlorophyll synthesis in *gcn1* mutants was reduced compared to the wild type, and *gcn1* mutants exhibited increased sensitivity to lincomycin, a translation inhibitor in chloroplast (Figure. 1c,d). These observations collectively indicate compromised chloroplast function in *gcn1* mutant.

Light is both an energy source and essential environmental signal for plant growth and development. It was reported that light activates GCN2 and eIF2α phosphorylation via reactive oxygen species (ROS) emanating from the chloroplast (Lokdarshi et al. 2020). The level of eIF2α phosphorylation is mitigated by photosynthesis inhibitors and ROS quenchers. We confirmed that eIF2α phosphorylation was modulated by light/dark signal (Figure 3a,b), and found that the levels of phosphorylated eIF2α showed a circadian variation, under a 16-h light (from 7:00 to 23:00) and 8-h dark cycle conditions (Figure 3a), which was consistent with a prior study. However, the eIF2α phosphorylation peak was at the 8th hour (14:00) following the start of lighting, rather than at the 12th (Lokdarshi et al. 2020). Therefore, we need to exclude the influence of circadian rhythms in all experiments related to dark treatment. We harvested both the control and seedlings treated under dark treatment at the same time point (17:00) (Figures 3–5). the findings revealed that the level of eIF2α phosphorylation was dramatically decreased under dark treatments (Figure 3b). Accordingly, the translation of RH39 was reduced, 23S rRNA processing was inhibited, and the translation of chloroplast-encoded proteins was also reduced under dark treatment (Figures 3–5). Taking together, we proposed a complete regulatory loop (Figure 6): under light period, light-harvesting complexes absorb excess light and produce ROS (Takahashi and Badger 2011), and ROS is emitted from the chloroplast and activates GCN2 to phosphorylate eIF2α (Lokdarshi et al. 2020). Phosphorylated eIF2α promotes the translation of RH39 in the cytoplast. The translated RH39 is translocated into the chloroplast to splice the 23S rRNA of ribosomes in the chloroplast. The correctly spiced 23S rRNA is sufficient for the translation of chloroplast-encoded proteins to meet plant growth under light conditions. Under dark period, there is no ROS to activate GCN2 to phosphorylate eIF2α. Therefore, eIF2α phosphorylation is almost undetectable. The non-phosphorylated eIF2α inhibits the translation of RH39. The reduced protein level of RH39 impairs the splicing of 23S rRNA and the impairment of 23S rRNA processing negatively affects the translation process of chloroplast-encoded proteins. The decline of chloroplast-encoded protein translation exactly caters to the plant's relaxation under dark conditions.

Polysomal profiling is often used to analyze the translation state of mRNAs. The polysome fractions are isolated by sucrose

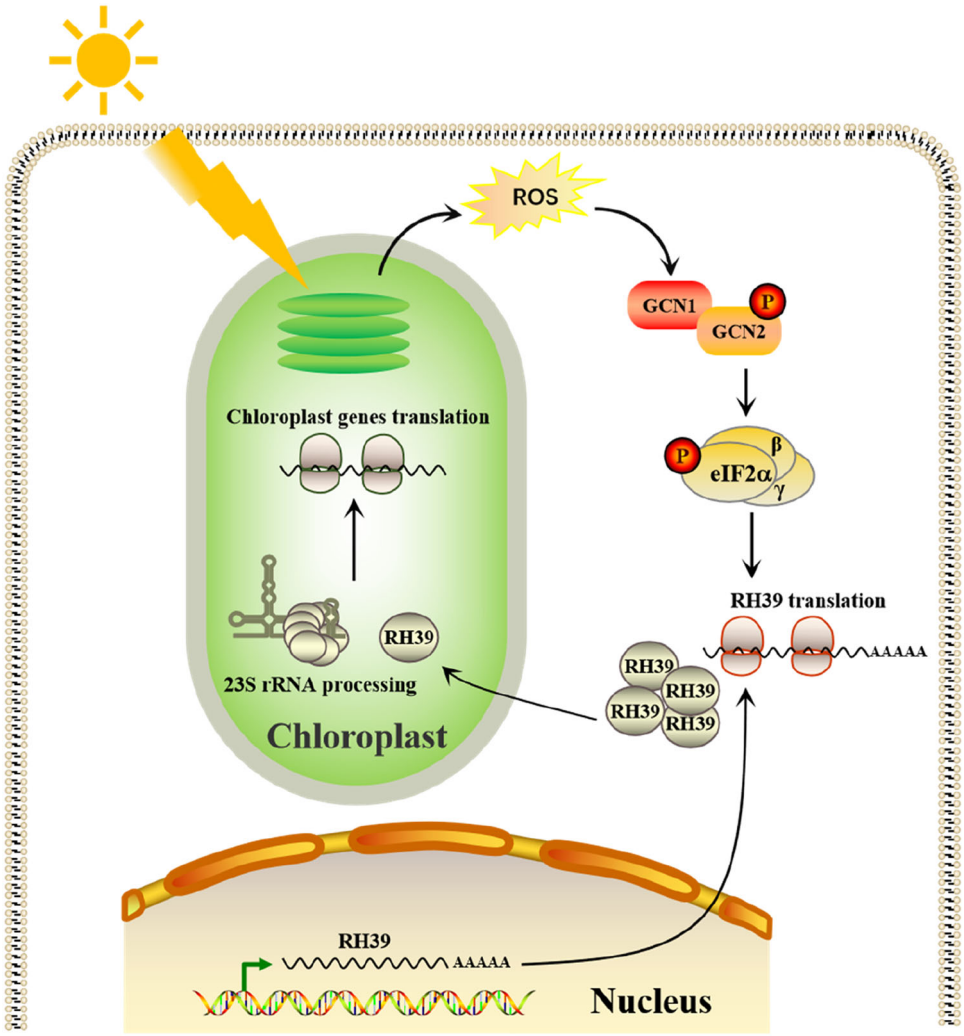


**FIGURE 6** | Work model: Translation regulation of chloroplast-encoded proteins through eIF2α phosphorylation and RH39 during light/dark cycles. During the light period, light-harvesting complexes absorb excess light and generate reactive oxygen species (ROS) (Takahashi and Badger 2011). The emission of ROS from the chloroplast activates GCN2, leading to the phosphorylation of eIF2α (Lokdarshi et al. 2020). Phosphorylated eIF2α promotes the translation of RH39 in the cytoplast. The RH39 proteins are then translocated into the chloroplast to spice the 23S rRNA of chloroplast ribosomes. Properly spliced 23S rRNA is essential for the translation of chloroplast-encoded proteins, thereby supporting plant growth under light conditions. In contrast, during the dark period, the absence of ROS results in a lack of GCN2 activation, leading to minimal phosphorylation of eIF2α. Consequently, the translation of RH39 declines through an unclear mechanism. This reduction in RH39 translation compromises the splicing of 23S rRNA, which subsequently negatively impacts the translation of chloroplast-encoded proteins. The decrease in chloroplast-encoded protein translation aligns with the relaxation of plant growth under dark conditions. [Color figure can be viewed at wileyonlinelibrary.com]

density gradients, and then be analyzed using RNA sequencing, RT-qPCR, or Northern blot to assess translational efficiency and identify specific mRNA interactions with ribosomes (Missra and von Arnim 2014). It is known that disruptions in translation initiation or termination can impact polysome formation, while defects in translation elongation do not significantly affect ribosome binding to mRNA (Barkan 1993; Motohashi et al. 2007; Pesaresi et al. 2001). There were no significant differences in the sedimentation profiles of rbcL and psbA mRNAs between WT and *nara12-1* mutant via polysomal profiling, indicating that the *nara12-1* mutant has defect in the elongation step of chloroplast translation, but the initiation and termination steps are not affected (Nishimura et al. 2010). Therefore, the chloroplast translation regulated by GCN1 through RH39 also primarily impacts the elongation phase. Additionally, the accumulation of chloroplast protein triggered by the dark signal also due to the disruption of translation elongation step, because the levels of ribosomes binding to chloroplast mRNA was unchanged in the dark conditions, while the synthesis of chloroplast proteins decreased (Burch-Smith et al. 2018; Klein et al. 1988). Consequently, we detected the accumulation of chloroplast proteins in *gcn1* or under dark treatment to indirectly reflect protein translation level (Figures 1f and 5). However, protein accumulation is also regulated by degradation mechanisms involving proteases, the ubiquitin-proteasome system (UPS) or uautophagy, in addition to the biosynthesis (Li et al. 2022a; Ling et al. 2019; Sun et al. 2023; Sun et al. 2022). Moreover, the decrease in chloroplast translation levels induced by light/dark signals may also be attributed to the modulation of the redox state of photosynthetic electron chain proteins and

ATP production. Therefore, we do not exclude the possibility that the reduction in chloroplast proteins under darkness may also be modulated by other mechanisms, besides the regulation of RH39 by the GCN1-GCN2-eIF2α pathway.

Although we have shown that cells modulate the translation of chloroplast-encoded proteins via eIF2α phosphorylation and mediated RH39 translation during light and dark conditions, it is still unclear how eIF2α phosphorylation mediates the translation of RH39. In mammals and yeast, it is reported that eIF2α phosphorylation selectively promotes the translation of ATF4 or GCN4 with upstream open reading frames (uORFs). uORFs are potent regulatory elements located in 5′ mRNA transcript leaders. The translation of uORFs usually inhibits the translation of downstream main open reading frames (mORF). (Dever et al. 1992; Vattem and Wek 2004). Similar translational regulation by uORF was also found in plants. For instance, pathogen induced eIF2α phosphorylation, thus releasing the inhibitory effects of uORFs on TBF1 transcription factor translation to turn on growth-to-defense transition (Pajerowska-Mukhtar et al. 2012). There are also some uORFs known to enhance mORF translation (Guo et al. 2022; Lin et al. 2019; Yang et al. 2020). Although half of the *Arabidopsis* genes contain uORF (Cui et al. 2021; Niu et al. 2020), there are no uORFs in the 5′-untranslated region of *RH39*. We speculated that *Arabidopsis* eIF2α phosphorylation promotes the translation of RH39 in a manner different from the above-mentioned genes. Therefore, the underlying mechanism awaits future investigation.

Based on all the results, we propose a complete regulatory loop: chloroplasts absorb lights to accumulate ROS (Lokdarshi et al. 2020); ROS activates eIF2α phosphorylation through GCN1-GCN2 pathway (Lokdarshi et al. 2020); phosphorylated eIF2α promotes the translation of nuclear-encoded RH39; RH39 in turn regulates the translation of chloroplast-encoded proteins (Nishimura et al. 2010). In this way, plant cells can adjust the biosynthesis of chloroplast-encoded proteins to match the environment change of light/dark alterations.


### Acknowledgements

We acknowledge the funding supported by the National Natural Science Foundation of China to H.Z. (31270309, 30900357). We thank Dr. Xiaohong Zhu (State Key Laboratory of Crop Stress Adaptation and Improvement, School of Life Sciences, Henan University) for her kind gift of CP-HA transformed *Arabidopsis* seeds and constructive revision of the manuscript.


### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.
**Figure S1:** Transcriptional levels of chloroplast-encoded and nuclear-encoded genes. **Figure S2:** The translational regulation of *eEF1α* under dark treatment. **Figure S3:** Analysis of chloroplast-encoded proteins accumulation under chlorsulfuron treatment. **Figure S4:** Transcription analysis of *RH39*. **Table S1:** List of primer sequences used in this study.