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Accumulation of cyanobacterial photosystem II containing the "rogue" D1 subunit is controlled by FtsH protease and the synthesis of the standard D1 protein

Running head: Expression and function of the rogue D1 protein.

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Abstract

Unicellular diazotrophic cyanobacteria contribute significantly to the photosynthetic productivity of the ocean and the fixation of molecular nitrogen, with photosynthesis occurring during the day and nitrogen fixation during the night. In species like *Crocosphaera watsonii* WH8501 the decline in photosynthetic activity in the night is accompanied by the disassembly of oxygen-evolving photosystem II (PSII) complexes. Moreover, in the second half of the night phase a small amount of rogue D1 (rD1), which is related to the standard form of D1 subunit found in oxygen-evolving PSII, but of unknown function, accumulates but is quickly degraded at the start of the light phase. We show here that removal of rD1 is independent of rD1 transcript level, thylakoid redox state and trans-thylakoidal pH but requires light and active protein synthesis. We also found that the maximal level of rD1 positively correlates with the maximal level of chlorophyll biosynthesis precursors and enzymes, which suggests a possible role for rPSII in the activation of chlorophyll biosynthesis just before or upon the onset of light, when new photosystems are synthesized. By studying strains of *Synechocystis* PCC 6803 expressing *Crocosphaera* rD1 we found that accumulation of rD1 is controlled by the light-dependent synthesis of the standard D1 protein which triggers the fast FtsH2-dependent degradation of rD1. Affinity purification of FLAG-tagged rD1 unequivocally demonstrated the incorporation of rD1 into a non-oxygen-evolving PSII complex which we term rogue PSII (rPSII). The complex lacks the extrinsic proteins stabilizing the oxygen-evolving Mn_4CaO_5 cluster but contains the Psb27 and Psb28-1 assembly factors.

Keywords: rogue D1; *Crocosphaera watsonii*; Photosystem II; *Synechocystis*; chlorophyll biosynthesis

Introduction

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis. About half of known cyanobacterial species also have the genetic capacity to fix nitrogen (Stal and Zehr, 2008). Nitrogen fixation, the conversion of atmospheric N_2 to bioavailable ammonium (NH_4^+), is an anaerobic process since the nitrogenase enzyme complex responsible for nitrogen fixation is extremely vulnerable to inactivation by oxygen (Fay, 1992; Gallon and Stal, 1992). On the other hand, ATP and the reductants (NADPH and reduced ferredoxin) required for nitrogen fixation are mostly produced through the light reactions of oxygenic photosynthesis, which generates molecular oxygen as a by-product of the water oxidation reaction within photosystem II (PSII). Therefore, oxygenic photosynthesis will inhibit nitrogen fixation if performed concurrently in the same cell. One strategy to overcome this problem is to perform nitrogen fixation in specialized cells termed heterocysts (Fay, 1992). Another is to separate temporally photosynthetic O_2 evolution and carbon fixation from N_2 fixation. In unicellular diazotrophic cyanobacteria, photosynthesis occurs during the day while N_2 is fixed during the night (Tuit et al., 2004; Mohr et al., 2010; Dron et al., 2012). However, many aspects of temporal separation have not yet been fully elucidated (Berman-Frank et al., 2003; Zehr, 2011).

According to current knowledge, the temporal separation of nitrogen fixation and photosynthesis is accomplished by robust diel metabolic cycles that lead to preferential transcription of photosynthetic genes during the day and nitrogen fixation-related genes in the night (Mohr et al., 2010; Pennebaker et al., 2010; Shi et al., 2010; Dron et al., 2012; Masuda et al., 2018). The diazotroph *Crocosphaera watsonii* WH 8501 (hereafter *C. watsonii*) has proved to be a useful model to study these cycles (for review see Masuda et al., 2022). When grown under 12h light/12h dark (L12:D12) cycles, *C. watsonii* cells fix nitrogen during the dark phase (Tuit et al., 2004; Mohr et al., 2010; Dron et al., 2012) while PSII activity peaks in the middle of the light period. Interestingly, a large fraction of PSII is disassembled during the dusk and most of the dark phase (Mohr et al., 2010; Pennebaker et al., 2010; Rabouille and Claquin, 2016; Masuda et al., 2018) and appears to be related to a general suppression of membrane protein

synthesis (Masuda et al., 2018). Indeed, this especially affects PSII since its key membrane protein D1 exhibits a fast turn-over (for review see Komenda et al., 2012), which can occur even in the dark (Krynická et al., 2015). Intriguingly, a small amount of a paralogue of the D1 protein called rogue D1 (rD1, Murray, 2012) appears to be incorporated into the PSII monomer during the later stage of the night phase and this paralogue again quickly disappears upon the onset of the light phase (Masuda et al., 2018). The appearance of rD1 in the dark has also been proposed (Wegener et al., 2014) and recently confirmed for *Cyanothece* (Liberton et al. 2022; Masuda et al. 2022).

The D1 proteins in cyanobacteria have been recently assigned to several groups and subgroups based on their phylogenetic analysis (Sheridan et al. 2020). rD1 is found in many but not all cyanobacteria and its function remains enigmatic as it lacks the key amino-acid residues involved in binding the oxygen-evolving Mn_4CaO_5 cluster and so is unlikely to catalyze water oxidation (Murray, 2012; Sheridan et al., 2020). Wegener and colleagues (2014) have suggested that the rD1 protein in *Cyanothece* is incorporated into PSII complexes to shut down oxygen evolution. However, given the low abundance of rD1 and its occurrence exclusively in the dark, when no oxygen is evolved, such a role seems improbable. Recent data strongly suggested rD1 incorporation into PSII (Masuda et al., 2018) but definitive experimental evidence is still lacking. Another divergent paralogue of D1 distinct from rD1 belongs to a group of so called super-rogue D1 proteins (srD1; Murray 2012; Sheridan et al. 2020), which has been unequivocally shown to incorporate into a special type of non-oxygen-evolving super-rogue PSII complex (srPSII) essential for the synthesis of chlorophyll (Chl) *f* (Ho et al., 2016; Trinugroho et al., 2020). *C. watsonii* like many other rD1-containing cyanobacteria contains neither the gene for srD1 nor Chl *f*.

In the present study we investigated the environmental factors regulating the accumulation of rD1 during the diel cycle and its role during the dark phase. We also tested whether rD1 was able to assemble into a PSII-like complex. In addition to circadian transcriptional regulation, we found that the standard D1 protein, which is incorporated into oxygen-evolving PSII, plays a crucial role in inducing the fast light-dependent rD1 removal by the FtsH2 protease. The positive correlation between the rise in the level of

Chl biosynthesis precursors and Chl biosynthesis enzymes and the appearance of rD1 leads us to speculate that rPSII might be important for the activation of maximal Chl biosynthesis just before the onset of light when the cell starts to synthesize the photosystems. Importantly, we show that rD1 can assemble into non-oxygen-evolving PSII complexes, lacking the oxygen-evolving-enhancer proteins, which we term rogue PSII (rPSII) complexes.

Results

Diel changes in the expression and accumulation of D1 and rD1 proteins

The genome of *C. watsonii* contains two annotated genes encoding the standard D1 protein (sD1) of PSII and its paralogue rD1. To correlate the expression of sD1 with rD1 we monitored transcript levels of the *psbA1* (*CwatDRAFT_1423*) gene encoding sD1 and the *psbA4* (*CwatDRAFT_4668*) gene encoding rD1 using the RT-PCR method during cultivation of *C. watsonii* cells under 12h light/12h dark cycles (12L:12D) with irradiance having a sinusoidal time course peaking at 400 µmol photons m^{-2} s^{-1}. The *psbA1* transcript level normalized to the transcript level of the constitutively expressed *rotA* gene (CwatDRAFT_6490) increased sharply from the onset of the light period, peaking at around 6 hours of the light period (6L), then decreased from the second half of the light period to the middle of the dark period (6D), before increasing again during the second half of the dark period (Fig. 1A, upper panel). In contrast, the transcript level of the *psbA4* gene encoding rD1 showed an opposite temporal pattern as it decreased at the beginning of the light period, reached a minimum at 6L, and started to rise again towards the end of the light period and the beginning of the dark period, and then was maintained at this level during the entire dark period (Fig. 1A, lower panel).

Using the same light regime, we also monitored the accumulation of sD1 and rD1 proteins by immunoblotting using a purified rD1-specific antibody raised against its unique N-terminus (Masuda et al., 2018). In agreement with the transcriptional data, the level of sD1 increased from the beginning of the

light phase, reached a maximum at 6L-9L and then decreased during the dark period (Fig. 1B). There was also a noticeable increase in D1 levels at 11D just before the onset of the light phase. On the other hand, the signal of rD1 appeared at 6D, peaked at 11D and disappeared during early hours of the light phase (Fig. 1B). The temporal change of Fv/Fm, an index of photosynthetic activity, followed the temporal change of sD1 at both the gene expression and protein level. Thus, the protein level of sD1, and especially rD1, do not necessarily follow their transcript levels during the diel cycle. This is especially apparent at 11L and 12L time points when there is still a high level of sD1 while its transcript level is reduced and there is no detectable rD1 despite accumulation of the *psbA4* transcript.

Expression of the *psbA* genes and accumulation of sD1 and rD1 during extended light and dark phases of the diel cycle

The observed changes in sD1 and rD1 accumulation during the light and dark phases of the diel cycle (Fig. 1) were mostly a consequence of circadian-regulated gene transcription but the observed disconnection of *psbA4* transcription and rD1 accumulation at 11L and 12L time points could be a direct light-driven effect on rD1 synthesis/degradation. To clarify this point, we modified the light regime during the diel cycle. After reaching the maximal irradiance at 6L either the same light intensity was prolonged for another 24 hours, or the dark period was extended after 12D for another 24 hours. In the first case the maximal level of the *psbA1* mRNA reached at 6L was strongly suppressed after an additional 12 (18L) or 17 (23L) hours of high light while the *psbA4* transcript levels observed at 18L and 23L were similar to the transcript levels observed at the same time points in the 12h L/D cycles (Fig. 2A). This result confirmed the robust transcriptional regulation of both genes by the circadian clock regardless of the light conditions. In line with this regulation, the accumulation of sD1 protein was maximal at 6L, sharply decreased at 18L and partially increased again at 23L mirroring the level of the *psbA1* transcript (Figs. 2A and B) and PSII activity (Fig. 2C). Interestingly, the accumulation of rD1 protein mimicked the level of the *psbA4* transcript only in the dark while in the light no rD1 accumulated despite significant levels of *psbA4*

mRNA at 18L and 23L (Figs. 2A and B). Apparently, the synthesis and degradation of rD1 is subject to additional post-transcriptional regulation. This was further confirmed in the second case, when we extended the dark phase for another 18 hours. After an additional 6, 12 and 18 hours the level of rD1 gradually increased while the sD1 content remained low (Fig. 3A). Both sD1 and rD1 were rather stable when chloramphenicol (Cm), an inhibitor of protein synthesis, was added to the cultures during prolonged dark incubation of 30 hours. Thus, in the dark the synthesis of sD1 is inhibited while rD1 is still synthesized during the prolonged dark phase, despite the fact that the circadian clock-regulated transcript level of sD1 transcript should increase and rD1 transcription should be suppressed based on the true light phase of the cycle.

Testing possible factors controlling degradation of rD1 in light

The results above suggested that the degradation of rD1 is stimulated by illumination via an unknown post-transcriptional mechanism. To better understand the mechanism, we subjected cells containing rD1 (at 12D) to different light regimes in the presence of various effectors that affect nitrogen availability, protein synthesis, the redox state of the plastoquinone (PQ) pool, the membrane pH gradient and activity of proteases.

We found that the rate of light-dependent disappearance of rD1 is rather independent of light intensity. The decrease in rD1 level at 1L and 3L was very similar in cells exposed to the standard diel cycle illumination or with light continuously rising from zero at 12D to 200 µmol photons m^{-2} s^{-1} at 3L or in cells exposed to a constant illumination of 50 µmol photons m^{-2} s^{-1} (Fig. 4A). We also tested whether rD1 degradation could be induced just by a short light pulse and then it would continue during subsequent dark incubation. This would indicate a signaling role of light in the triggering of rD1 degradation. This was assessed by pre-illuminating cells for 5 min before their 3-hour dark incubation. Nevertheless, this short light period did not trigger the dark degradation of rD1, which makes light signaling-mediated

proteolysis improbable. In addition, we tested whether rD1 degradation could be triggered by the availability of nitrogen, either in the reduced (NH_4^+) or oxidized (NO_3^-) form. None of these compounds changed the rate of rD1 disappearance (Fig. 4B).

Illumination has wide-ranging effects on cells including changes to the redox state of the PQ pool and trans-thylakoidal pH gradient. To judge whether light-induced changes in these characteristics underlie the mechanism of rD1 degradation, we treated cells with specific compounds that influence these characteristics. Light-induced rD1 degradation remained unaffected in the presence of DBMIB, an inhibitor of the cytochrome b_6f complex that causes over-reduction of the PQ pool in the light. Similarly, blocking the PSII activity by DCMU leading to over-oxidation of the PQ pool affected neither rD1 degradation (Fig. 4C) nor sD1 and rD1 synthesis (Fig. S1). When DCMU was combined with oxygen depletion using nitrogen flushing, the rate of rD1 degradation was slightly slowed down in comparison with the control suggesting some minor inhibitory effect of oxygen removal (Fig. 4C). Finally, no apparent difference in the rD1 degradation rate was exerted by addition of nigericin, an uncoupler of electron transport and trans-thylakoidal pH gradient, to rD1-containing cells (Fig. 4D). In conclusion, light-induced changes to the redox state of PQ or the trans-thylakoidal pH are not crucial for rD1 degradation.

Although FtsH proteases are considered the main proteases involved in degrading the D1 protein in cyanobacteria (for review see Komenda et al., 2012), we cannot exclude the possibility that rD1 might be a target for a different cyanobacterial proteolytic system like the Deg serine proteases or Clp (Sokolenko et al., 2002). Therefore, we tested whether the serine protease inhibitor (Pefablock) can retard rD1 degradation. At 0.5L and 1L we could see a somewhat higher level of rD1 in Pefablock-treated cells in comparison with untreated cells (Fig. 4D). Thus, some involvement of serine proteases in rD1 removal is possible (see Discussion).

Finally, as illumination of cells could also induce synthesis of new proteins, for instance proteases, that may be crucial for rD1 degradation, we inhibited protein synthesis during the dark period by adding

Cm. Interestingly, at 1L a similar amount of rD1 to that observed at 12D was detected in the membranes and this was similar if cells were illuminated by a constant light intensity of 50 μmol photons m^{-2} s^{-1} instead of the standard diel cycle illumination (Fig. 4E). The inhibitory effect of Cm was less pronounced at 3L of diel illumination but rD1 was quite stable even after 3 hours illumination at a constant irradiance of 50 μmol photons m^{-2} s^{-1}. The presence of Cm also stabilized the sD1 protein during the diel illumination. These data suggest the crucial importance of *de novo* protein synthesis for light-induced rD1 degradation.

Temporal change in biosynthesis and accumulation of Chl during the diel cycle

In our previous study (Masuda et al., 2018) we detected a significant decrease in cellular Chl content during the dark phase of the *C. watsonii* diel cycle. This decrease was verified in the present study (Fig. S2, white columns). Moreover, we also identified a smaller diameter of the cell in the light phase in comparison with the dark phase (Fig. S2). In order to see whether the differences in Chl level during the diel cycle is also accompanied by changes in the level of Chl biosynthesis precursors and enzymes, we assessed their content by HPLC and immunodetection, respectively. The time course of the precursor concentration confirmed the downregulation of Chl biosynthesis during the early stage of the light phase while it resumed during the dark phase with maximal levels reached at its end (Fig. 5A). The only exception was mono-vinyl-chlorophyllide, which decreased during the last 6 hours of dark period in an antiparallel fashion to that of di-vinyl-chlorophyllide. While the former seems to be just partially related to de novo Chl biosynthesis as it partly results from Chl degradation, the latter is clearly a product of de novo Chl biosynthesis (Kopečná et al., 2012). Importantly, the late stages of the dark phase were accompanied by a clear induction of the oxygen-dependent magnesium protoporphyrin IX methylester cyclase (AcsF) which was detected as a double band. We expect that the lower weak band, which was present during the whole diel cycle, is the constitutive 'high-oxygen' AcsF1 cyclase (theoretical calculated mass 40,919 Da). The second band, strongly induced in dark with a maximum observed at 6D

and 11D, is most likely the AcsF2 isozyme (theoretical calculated mass 42,493 Da), found in the majority of cyanobacterial species, which is active even under low-oxygen conditions (Chen et al., 2021). Intriguingly, the light-dependent protochlorophyllide oxidoreductase (LPOR) was also strongly induced at 6D and 11D, despite being inactive in dark, while the light-independent version of the enzyme (DPOR) was induced much less. The increased level of AcsF and LPOR at 6D and 11D correlated very well with the appearance of rD1 (Fig. 5B). Thus, the presence of rD1 in the cells is limited to the dark period showing the highest rate of Chl biosynthesis.

Characterization of *Synechocystis* strains expressing rD1 from *C. watsonii*

Firm evidence for the role of rD1 in Chl biosynthesis could be provided by comparison of the wild-type strain with a mutant strain lacking the *psbA4* gene coding for rD1. However, *C. watsonii* is not easily transformable and we were not able to obtain the corresponding rD1-less transformant. Therefore, we constructed strains of the naturally transformable cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) that either ectopically expresses the rD1 protein encoded by *C. watsonii* or its N-terminal FLAG-tagged derivative. Instead of using a *Synechocystis* wild-type strain, we employed a strain lacking all three *psbA* genes and so unable to synthesize D1 (Debus et al., 1988). The resulting rD1/ΔD1 mutant was unable to grow autotrophically consistent with the inability of rD1 to mediate oxygen evolution. Based on the whole-cell absorption spectrum (Fig. 6), the rD1/ΔD1 strain showed a slightly increased cellular level of Chl in comparison with the control D1-less strain and, in the membranes of the strain, we could detect assembled dimeric and monomeric PSII core complexes (termed rPSII, Fig. 7). Interestingly, rPSII showed much lower Chl fluorescence than standard PSII (Figs. 7 and S3, 1D fluor) indicating the operation of an effective energy quenching process. We also radiolabelled cells of both strains with [^{35}S]-methionine/cysteine and after 2D protein gel analysis and autoradiography we found an increased level of radiolabelled PSI proteins in the rD1-expressing strain in comparison with the control strain (Fig. 7). This

result is in line with the preferential channeling of newly synthesized Chl molecules into PSI (Kopečná et al., 2012).

Isolation and characterization of PSII complexes containing FLAG-tagged rD1

Expression of the FLAG-tagged version of rD1 in the D1-less *Synechocystis* strain (FLAG-rD1/ΔD1) allowed us to isolate rD1-associated complexes using a FLAG-specific affinity column. The purified preparation showed an absorption spectrum very similar to that isolated from the D1-less *Synechocystis* strain expressing FLAG-tagged sD1(FLAG-D1/ΔD1; Fig. 8; Trinugroho et. al., 2020). Its pigment content (32 Chls, 9 β–carotenes and 2 pheophytins per one heme) was also similar to that of FLAG-D1-containing preparation (Trinugroho et al., 2020). Both preparations consisted of PSII core complexes containing FLAG-D1 or FLAG-rD1 and other typical PSII subunits. However, while the FLAG-D1/ΔD1 preparation contained almost equal amounts of PSII dimers and monomers (PSII(2) and PSII(1); Fig. 9), the main component of the FLAG-rD1/ΔD1 preparation was a monomer (rPSII(1)) with just a small amount of the dimer (rPSII(2)). Additionally, we also detected a monomeric PSII complex lacking the CP43 module (rRC47) alone or associated with a monomeric PSI complex (PSI(1)/rRC47). The preparation lacked the PsbO, PsbV and PsbU extrinsic proteins but contained the Ycf48, Psb27 and Psb28-1 accessory proteins, as identified by mass-spectrometry analysis (Table S2). This analysis also revealed the presence of the PSII auxiliary proteins CyanoP (Knoppová et al., 2016), Psb32 (Wegener et al. 2011) and Psb34 (Zabret et al., 2021; Rahimzadeh et al. 2022), which were not seen in the gel and presumably were present at highly sub-stoichiometric levels.

sD1 and FtsH-dependent degradation of the ectopic rD1 in *Synechocystis* mutants transferred from dark to light

We also constructed and characterized other *Synechocystis* strains with ectopically expressed rD1 to study the mechanism of rD1 degradation in the light. Strain rD1 contains the *psbA4* gene encoding *C. watsonii* rD1 in the *psbA2* locus and expresses the standard *Synechocystis* D1 subunit from the *psbA3* locus (rD1 strain). To find out whether rD1 accumulates in this strain just in the dark like in *C. watsonii*, we incubated cells grown in normal light (40 µmol photons m^{-2} s^{-1}, NL) in the dark for 4 hours, and then transferred them back to NL for an additional 2 hours. During this treatment we sampled the cells and analyzed their membranes by immunoblotting using specific antibodies against sD1 and rD1 (Fig. 10A). The results of this analysis confirmed that rD1 in *Synechocystis* behaves in a similar way to native rD1 in *C. watsonii*. There is a gradual appearance in the dark but after transfer to NL, rD1 quickly disappears. In contrast, the level of sD1 remains stable during the whole treatment. However, when we expressed a FLAG-tagged version of rD1 in the *psbA2* locus, the resulting strain FrD1 (Fig. 10B) did not show the light-dependent degradation of FLAG-rD1 and instead its level even slightly increased after transfer from dark to light. These results suggested the possible involvement of the N-terminal tail of rD1 in signaling degradation by the FtsH2/3 protease complex (Komenda et al. 2007). To get stronger experimental evidence for this hypothesis, the gene encoding FtsH2, which has been shown to degrade sD1 in *Synechocystis* (Silva et al. 2003; Komenda et al. 2006) was deleted in the rD1 strain and the resulting strain (rD1/ΔFtsH2) was subjected to the same treatment as the single rD1 mutant. The stable level of rD1 regardless the light condition provided strong support for the crucial role of FtsH2 in rD1 degradation (Fig. 10C). Finally, to test whether sD1 is needed for the disappearance of rD1 after transfer from dark to light, we also subjected cells of the rD1/ΔD1 strain to a dark-light treatment. Subsequent immunodetection revealed that the level of rD1 was constant regardless of the light conditions, implicating the involvement of light-driven synthesis of sD1 in rD1 degradation (Fig. 10D). Interestingly, the absence of sD1 in the rD1/ΔD1 strain led to a large increase of rD1 in comparison with the rD1 levels observed in both the rD1 and rD1/ΔFtsH2 strains (Fig. S4). In all three strains, sD1 and rD1 were

incorporated into PSII complexes as judged by 2D CN/SDS-PAGE (Figs. 7 and S6). Overall, the presence of sD1 appears to limit the accumulation of rPSII much more than degradation of rD1 by FtsH.

Discussion

Transcriptional and post-transcriptional regulation of sD1 and rD1 proteins in *C. watsonii*

Analysis of *C. watsonii* grown under 12L:12D cycles showed that transcription of the genes encoding both rD1 and sD1 is primarily driven by a circadian rhythm (Figs. 1 and 2). This agrees with previous studies showing circadian gene expression under L/D cycles (Chen et al., 1996; Kucho et al.,2005; Toepel et al., 2008; Zinser et al., 2009; Shi et al., 2010). The cellular level of sD1 corresponded well with its *psbA1* transcript level, so that there was an increase in the sD1 content between the second half of the dark period and the middle of the sinusoidal light period peaking at 400 µmol photons m^{-2} s^{-1}. Conversely, the sD1 level decreased during the second half of the light period and first half of the dark period (Fig. 1A, B, C) in line with the observed reductions in photosynthetic activity (monitored by light-saturated rates of O_2 evolution), the rate of carbon fixation and F_v/F_m (Masuda et al., 2018; Figs. 1 and 2). A major role for circadian transcriptional rhythms in regulating sD1 accumulation was supported by analysis of cells, grown under 12L:12D cycle, which had their light period extended from 12 to 24 hours. Immunodetection of sD1 showed the loss of sD1 after 18 hours of light (Fig. 2, 18L), i.e. at midnight in the standard growth regime when the *psbA1* transcript level is at its lowest.

The levels of *psbA4* transcript encoding rD1 went roughly antiparallel with that of sD1, starting to rise at the end of the light phase and increasing further during the dark period. However, the appearance of rD1 did not match the changes in transcript abundance as the protein was detected just in the second half of the night period and quickly disappeared upon the onset of the light period. However, when the light period was extended, rD1 did not appear at the usual time of the diel cycle, despite the rather high level of *psbA4* transcript (Fig. 2, 18L and 23L). Conversely, when the dark period was extended, rD1 content

slowly increased (Fig. 3) despite the circadian clock corresponding to the light phase and low transcript level of rD1 (Fig. 1). Thus, the accumulation of rD1 was primarily determined by post-transcriptional mechanism as its accumulation only occurred in the dark, while in the light the protein was quickly degraded.

What mechanism controls the level of rD1?

As light was apparently the main factor causing the rapid degradation of rD1, we tested various environmental and cellular conditions that could explain the action of light. Our data suggest that light signaling, nitrogen status signaling, the redox state of PQ pool and trans-thylakoidal pH (Fig. 4) do not play major roles. As only a very limited slowdown of rD1 degradation was observed in the presence of the PSII inhibitor DCMU accompanied by nitrogen flushing, oxygen or its reactive species also do not play a major role in the process. We found that Pefablock, a serine protease inhibitor, slightly slowed down the light-induced degradation of rD1 (Fig. 4) suggesting a possible role for serine proteases in this process although this inhibitor might have some side effects due to its non-specific binding (Nduaguibe et al. 2010). An even stronger candidate for the rD1 degradation is the FtsH2/FtsH3 protease complex involved in the light-induced degradation of sD1 in cyanobacteria (Silva et al., 2003; Komenda et al., 2006; Nixon et al., 2010). Its involvement was suggested by the inhibition of FLAG-rD1 degradation in a *Synechocystis* strain expressing FLAG-rD1 together with sD1 (Fig. 10B). The final convincing evidence for the crucial role of this protease complex was obtained using a *Synechocystis* strain expressing rD1 together with sD1 and lacking FtsH2. Unlike the rD1 strain containing this protease, the rD1/ΔFtsH2 strain was now unable to degrade rD1 upon transfer from dark to light (Fig. 10C).

The only treatment we tested that caused a dramatic delay in the light-dependent degradation of rD1 in *C. watsonii* was the inhibition of protein synthesis by Cm. Our evidence that FtsH2/FtsH3 is responsible for light-induced rD1 degradation argues against the involvement of an unknown light-induced/activated protease. Instead, our data support a role for sD1 synthesis in rD1 degradation. First, rD1 expressed in the

rD1/ΔD1 strain was not degraded after transfer of cells from dark to light. Second, in the strain rD1, expressing both rD1 and sD1, rD1 accumulates but after transfer to light, when the level of the *psbA3* transcript encoding sD1 increases and sD1 is intensively synthesized (Fig. S5), rapid degradation of rD1 is induced. The degradation of rD1 appears to be immediately followed by co-translational incorporation of newly synthesized sD1 into PSII complexes as we were unable to detect either rD1 or sD1 in an unassembled state, even in the absence of FtsH2 (Fig. S6).

The mechanistic explanation of how newly synthesized D1 triggers the degradation of "assembled" D1 is unclear. The synthesis of D1 and its incorporation into PSII during assembly occurs in biogenesis centres (Nixon et al. 2010), where precursor PSII complexes with newly inserted D1 might be protected from protease action by auxiliary protein factors like CyanoP, prohibitins and others (Knoppová et al. 2016; Knoppová et al. 2022). In our view the most probable explanation for the synchronization of D1 synthesis and degradation is that intensive synthesis of D1 expels previously assembled/repaired complexes into regions of the membrane where PSII is no longer protected from protease action and might be degraded by the FtsH2/FtsH3 complex. A possible location of rPSII in biogenesis centers would agree with the presence of the Psb27 and Psb28-1 accessory factors in the complex. Given the crucial importance of the N-terminus of D1 for its degradation (Komenda et al. 2007), the N-terminus of rD1, which is markedly different from that of sD1, may trigger rD1 degradation preferentially in comparison with sD1. However, we cannot exclude the possible post-translational modification of rD1 in selective replacement as rD1 migrates as a double band in SDS-PAGE gels, with the upper part retained after transfer from dark to light in the FtsH2-less strain (Fig. 10C).

The overall regulation of sD1 and rD1 synthesis and accumulation is akin to the regulation of expression of the low light (D1:1) and high light (D1:2) forms of D1 in *Synechococcus* sp. PCC 7942, which occurs at both the transcriptional and translational level (Tyystjärvi et al., 2001, 2004). After shifting *Synechococcus* cells from high to low light, D1:2 is rapidly degraded and replaced by D1:1. Given that inhibition of protein synthesis practically blocks D1:2 degradation after transferring to low

light (Komenda et al., 1999), it has been speculated that newly synthesized D1:1 induces the degradation of D1:2. Interestingly, even the degradation of radioactively labeled sD1 in *Synechocystis* is accelerated by the presence of newly synthesized sD1 (Komenda and Barber 1995; Komenda et al., 2000) which would indicate that synchronization between the synthesis and degradation of D1 is a universal feature of the D1 degradation process.

The function of rD1 in cyanobacteria

The physiological role of rD1 in various cyanobacteria has not yet been satisfactorily explained. In the present study, we obtained the first unequivocal evidence for incorporation of rD1 into non-oxygen-evolving PSII complexes (rPSII). A possible role for rPSII in suppressing PSII oxygen evolution during periods of low light to allow the nitrogenase to function more rapidly is an attractive idea (Wegener et al., 2014) but does not seem probable given that the level of rPSII is quite low and nitrogenase acts during the dark phase when no oxygen can be evolved and when there is on-going active respiration. Moreover, the time course of nitrogenase activity does not match the kinetics of the appearance of rD1 (Masuda et al., 2018).

Instead, we obtained several circumstantial pieces of experimental evidence to suggest a role for rPSII in Chl biosynthesis in the dark. Firstly, there was a good correlation between the nocturnal appearance of rD1 and the increase in the dark of the Chl level and the steady-state levels of Chl precursors and enzymes of the Chl biosynthesis pathway (especially AcsF and LPOR). The vast majority of Chl in *C. watsonii* is associated with PSI (Masuda et al., 2018) and its increase should therefore be especially reflected in the higher abundance of PSI. In agreement with this, the transcriptional analysis of *C. watsonii* during the diel cycle (Shi et al., 2010) showed increasing transcript abundance of PsaA/PsaB Chl-binding proteins of PSI as well as the PSI assembly factors Ycf3, Ycf4 and Ycf37 during the later stages of the dark phase (see also Table S3). For this period of the diel cycle the enhanced transcription of

several Chl biosynthesis enzymes and PSII assembly factors like Psb27 and Psb28 was also observed. Particularly, the parallel nocturnal upregulation of AcsF and Psb28-1 supports a relationship between both proteins as revealed from the study of a Psb28-1-less strain of *Synechocystis* (Dobáková et al., 2009). In contrast, the main structural proteins of PSII, with the exception of rD1, did not show such an enhanced transcription (Table S3), which would suggest that assembly of rPSII involves the recycling of PSII subunits originating from the disassembly of sD1-containing PSII complexes. Indeed, the requirement of both PSII activity and assembled PSII complexes alone for maximal rates of Chl biosynthesis and for the accumulation of PSI (the main sink for Chl) has also been shown in *Synechocystis* (Kiss et al., 2019). Chl biosynthesis in cyanobacteria occurs mostly in the light using LPOR enzyme (Kopečná et al., 2013). However, this enzymatic process is also functional in the dark thanks to the presence of the light-independent protochlorophyllide reductase enzyme (DPOR; Fujita and Bauer, 2003), a widespread nitrogenase-like enzyme assumed to be sensitive to oxygen (Yamazaki et al., 2006). Interestingly, LPOR appears even more accumulated in the second half of the dark phase than DPOR (Fig. 5). A possible explanation is that cells prepare for the light phase to immediately start light-dependent Chl biosynthesis and/or LPOR plays a structural or regulatory role in the activity of DPOR and low-oxygen cyclase enzyme formed by the AcsF2 catalytic subunit and the Ycf54 factor (Chen et al., 2021). One possibility is that AcsF2 together with protochlorophyllide reductases may be stabilized in the dark by interacting with rPSII or rRC47 with bound Psb27 or Psb28-1, respectively. Nevertheless, we were not able to detect any of these enzymes in the isolated rPSII, which could be caused by their weak or transient interaction with the complex. Previously AcsF was only detected in RC47 isolated using His-tagged Psb28-1, which might stabilize this interaction (Dobáková et al. 2009). The ability of rPSII to efficiently quench Chl fluorescence (Fig. 8 and S3) may also suggest a possible role of rPSII in photoprotection of biogenesis centers upon the sudden appearance of light, such as at sunrise.

The role of rD1 in Chl biosynthesis is also supported by the analysis of a D1-less *Synechocystis* strain expressing *C. watsonii* FLAG-rD1. It is notable that in the rD1/ΔD1 strain the synthesis and accumulation

of PSI increased in comparison with the control D1-less strain (Figs. 6 and 7) although both strains could not evolve oxygen and grew photoheterotrophically. The FLAG-rD1 pulldown contained mostly monomeric FLAG-rPSII lacking oxygen-evolving-enhancer proteins but was associated with PSII assembly factors Psb27, Psb28 and other auxiliary proteins. Interestingly, among them there was also a hypothetical protein Slr1917, which contains α/β hydrolase fold (Interpro IPR000073) and might represent a *Synechocystis* homologue of plant chlorophyll dephytylases involved in Chl turnover (Lin et al., 2022). This type of enzyme (a homologous gene encoding this enzyme in *C. watsonii* is CwatDRAFT_0930) might be involved in the redistribution of newly synthesized Chl between various Chl-binding proteins as such re-distribution may require phytyl detachment/re-attachment (Vavilin et al., 2005). The FLAG-rD1/ΔD1 eluate also contained a small amount of a complex between a PSI monomer and rRC47. We speculate that this complex might be a PSI synthesis intermediate incorporating new Chl molecules with the assistance of rRC47. Although the involvement of rPSII in Chl biosynthesis is still hypothetical, it is tempting to speculate that rPSII as well as the related srPSII complex (Ho et al., 2016; Trinugroho et al., 2020) might participate in chlorophyll biosynthesis through formation, stabilization and photoprotection of a chlorophyll biosynthesis metabolon.

Materials and methods

Cultivation of *C. watsonii*, experimental conditions and monitoring of growth

Stock cultures of *C. watsonii* WH 8501 were obtained from the Culture Collection Yerseke (the Royal Netherlands Institute for Sea Research, Yerseke, the strain number CCY 0601). Cells were maintained in N-free YBC-II medium (Chen et al., 1996) at 28 ºC in glass flasks under constant white light of 150 µmol photons m^{-2} s^{-1} using a 12:12 hour light:dark (12L:12D) cycle. Diluting them every ~12 days kept cell densities within 200,000 to 6,000,000 cells mL^{-1}. At the beginning of each experiment, cultures were transferred into flat panel photobioreactors (FMT150, Photon System Instruments, Brno, Czech Republic)

(Nedbal et al., 2008) with a sinusoidal 12L:12D growth irradiance peaking at 400 µmol photons m^{-2} s^{-1}. Cultures were acclimated to these conditions and maintained in exponential growth for at least 5 generations (~10 days). The photobioreactors continuously recorded F_o and F_t (every 5 minutes) as well as F_m and F_m' (every 30 minutes), which were then used to calculate *in situ* diurnal changes in F_t and F_m by averaging datasets of 7 consecutive days. Cell abundance was measured by a cell counter (Beckman, Multisizer 4, USA). To determine Chl concentration, we collected 10 ml of culture by centrifugation (8000 rpm × 8 min) and measured Chl absorbance in methanol extract (Porra et al., 1989).

Synechocystis **strains and growth conditions**

All *Synechocystis* strains used in the study were based on the glucose-tolerant wild-type substrain called GT-P (Tichý et al., 2016). The pPD-*N*FLAG vector was used to express the *C. watsonii* rD1 from the *psbA2* locus of *Synechocystis*, either in its native D1 form or with an N-terminal 3xFLAG-tag (Hollingshead et al., 2016). The rD1-encoding *psbA4* gene from *C. watsonii* was amplified using a Phusion High Fidelity PCR Master Mix (New England Biolabs, USA) with genomic DNA as a template and a specific set of primers (Table S1). Plasmids were constructed by In-fusion cloning (Zhu et al., 2007). The resulting plasmids, designated pPD-rD1 and pPD-FLAG-rD1, were used to transform the D1-less triple *psbA* deletion strains (ΔD1; Trinugroho et al., 2020) to yield rD1/ΔD1 and FLAG-rD1/ΔD1 mutants. The strain FLAG-D1/ΔD1 expressing the FLAG-tagged native form of the *Synechocystis* D1 was constructed in a similar way using specific primers (Table S1) as described in Trinugroho et al. (2020).

To generate rD1 and FLAG-rD1 mutant strains in *Synechocystis*, we transformed cells of *Synechocystis* GT-P strain (Tichý et al., 2016) with genomic DNA isolated from rD1/ΔD1 and FLAG-rD1/ΔD1 strains, respectively, where *psbA4* and *FLAG-psbA4* genes are placed under the *psbA2* promoter and carrying kanamycin-resistance cassettes. Cells were then segregated on BG11 plates with kanamycin and segregation of cells was verified with PCR using primers located up and downstream of the *psbA2*

gene (psbA2f 3' TGTCATCTATAAGCTTCGTG 5' and psbA2re 3'ATCCGCCGGCAGACGTTCTTCC 5', Fig. S7A). To generate mutant strains rD1/ΔFtsH2 and FLAG-rD1/ΔFtsH2 in *Synechocystis*, we transformed newly generated rD1 and FLAG-rD1 mutant strains with genomic DNA isolated from ΔFtsH2 strains with erythromycin (Komenda et al., 2006, Mann et al., 2000) and chloramphenicol-resistance cassettes inserted into the *ftsH2 (slr0228)* gene (Komenda et al., 2010), respectively. Full segregation was verified with PCR using primers located in the *ftsH2 (slr0228)* gene (slr0228F 3' AGAACTGCCCTACTTTGG 5' and slr0228re 3' GATAGCGGATTTACGACG 5', Fig. S7B) and with primers psbA2f and psbA2re to verify the background segregation (Fig. S7A).

Synechocystis strains were cultivated on glucose BG-11 agar plates (BG-11 basic mineral medium, supplemented with 0.3 % (w/v) sodium thiosulfate, 10 mM TES-KOH pH = 8.2, 5 mM glucose and 1.5% (w/v) agar). 50 ml liquid BG-11 cultures containing 5 mM glucose (and appropriate antibiotics when necessary) were grown in 250 ml sterile Erlenmeyer flasks on an orbital shaker at approximately 100 rpm. The strains were incubated in a temperature-controlled room set at 27-29 °C and illuminated with fluorescent white light of intensity 35 µmol photons m^{-2} s^{-1}. For the isolation of FLAG-rD1-containing complexes 4 L of the FLAG-rD1/ΔD1 mutant cells were grown in 10 L air-bubbled flask under 35 µmol photons m^{-2} s^{-1}.

Determination of maximum quantum yield of PSII

Chl fluorescence parameter Fv/Fm representing the maximum quantum yield of PSII was measured using a portable AquaPen-C AP-C100 fluorometer (Photon Systems Instruments, Brno, Czech Republic) in 2 mL aliquots withdrawn from the bioreactor as described in Masuda et al. (2018).

RNA isolation, reverse transcription and quantitative PCR

For the RNA extraction, 20 ml of *C. watsonii* culture were collected, centrifuged, pellet was resuspended in PGTX buffer (Pinto et al., 2009) and frozen in liquid nitrogen for RNA isolation. For RNA isolation, cells in the PGTX buffer were incubated at 95°C for 5 minutes and RNA extracted with chloroform and finally RNA was precipitated by isopropanol. After washing with 75% ethanol the precipitate was air dried and dissolved in RNase free water. For the cDNA synthesis, 2 µg of total RNA was DNase-treated by using the TURBO DNA-free kit (Thermo Fisher Scientific) and the Transcriptor first strand cDNA Synthesis kit (Roche Life Science, Mannheim, Germany) was used for cDNA synthesis using gene specific reverse primers (Table 1). Real time-quantitative PCR reactions were performed on the Rotor-Gene 3000 using the iQ SYBR Green Supermix (Bio-Rad). Primer sets for the *psbA4* gene (*CwatDRAFT_4668*) coding for rD1, the *psbA1* gene (*CwatDRAFT_1423*) coding for sD1, and the constitutively expressed *rotA* (*CwatDRAFT_6490*), encoding a peptidyl–prolyl *cis*-trans isomerase (Dyhrman and Haley, 2011) used for normalization, are described in Table S1. The same procedure was applied for quantification of *psbA4* and *psbA3* transcripts in *Synechocystis* strains expressing *psbA4* from the *psbA2* promotor, except for the reverse transcription reaction where random hexamers were used. Primer sets used for quantification of the *psbA4* gene (*CwatDRAFT_4668*) coding for rD1, the *psbA3* gene (*sll1867*) coding for sD1 and *rnpB* (encoding the B subunit of ribonuclease P) as a reference gene (Krynická et al., 2014), are described in Table S1. All analyses were performed in triplicates and the obtained data were analyzed by relative quantification using the 2 (-Delta Delta C[T]) method (Livak and Schmittgen, 2001).

Radioactive labeling of cells

Radioactive pulse labeling of the cells was performed at 500 µmol photons m^{-2} s^{-1} and 30 °C using a mixture of [^{35}S]Met and [^{35}S]Cys (Hartmann Analytic Gmbh, Braunschweig, Germany) as described previously (Masuda et al., 2018).

Isolation of membranes and protein analyses

Cyanobacterial membrane proteins were isolated by breaking cells with glass beads and solubilizing membrane proteins in 1% n-dodecyl-β-D-maltoside to analyze their proteins by clear native (CN) PAGE (Komenda et al., 2012). Samples of equal Chl content (3 μg) were loaded onto the gel. The protein composition of the complexes was analyzed by electrophoresis in a denaturing 16-20% linear gradient polyacrylamide gel containing 7 M urea. Gels with separated proteins were stained with Coomassie Blue. For autoradiography, the membrane with labelled proteins was exposed to a Phosphorimager plate (GE Healthcare, Uppsala, Sweden) overnight. The standard one-dimensional analysis of proteins was performed on the same 16-20% linear gradient polyacrylamide gel containing 7 M urea, which was stained with SYPRO Orange and then transferred onto a polyvinylidene difluoride membrane. Membrane proteins were incubated with specific primary antibodies against functional D1 present in cyanobacteria (Boehm et al., 2012), N-terminus of the *C. watsonii* rD1 (Masuda et al., 2018), LPOR enzyme (Kopečná et al., 2012), L subunit of the DPOR from *Leptolyngbia* (Yamazaki et al., 2006), plant CHL27 homologue of AcsF (Tottey et al., 2003), and then with secondary antibody-horseradish peroxidase conjugate (Sigma, St. Louis, MO, USA).

Isolation of the rD1-containing complexes

Large-scale membrane preparations for the purification of proteins and their complexes were isolated using Mini-Beadbeater-16, the membranes were solubilized with n-dodecyl-β-D-maltoside and FLAG-tagged proteins were isolated using the anti-FLAG M2 affinity gel (Sigma-Aldrich, USA) as described in detail in Koskela et al. (2020).

Whole cell absorption spectroscopy and pigment determination

Absorption spectra of whole cells were measured at room temperature using a UV-3000 spectrophotometer (Shimadzu, Japan) and were measured in the cultures with the identical OD750 nm. For routine Chl determination, pigments were extracted from cell pellets with 100% methanol and the Chl concentration was determined spectroscopically (Porra et al., 1989). For detailed analysis of Chl biosynthesis precursors they were extracted by an excess of 70 % methanol from 2 mL of cell cultures. Their detection was performed by an Agilent 1200 HPLC instrument (Agilent Technologies). The fluorescence detector FLD1 was set to excitation/emission maxima of 440/660 nm to detect the Chl precursors mono-vinyl-chlorophyllide, di-vinyl-chlorophyllide and di-vinyl-protochlorophyllide, while coproporphyrin III and protoporphyrin IX were detected at 400/620 and 400/630 nm, respectively. Mg-protophorphyrin IX and Mg-protophorphyrin IX monomethyl ester were detected using the fluorescence detector FLD2 set to excitation/emission maxima of 416/595 nm (Pilný et al., 2015).

Data Availability

The data underlying this article are available in the article and in its online supplementary material.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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Legends to figures:

Fig. 1. Temporal changes in *psbA1* and *psbA4* transcript levels (A), accumulation of sD1 and rD1 proteins (B), and changes in PSII activity reflected by Fv/Fm ratio (C) in *C. watsonii* during the regular 12L:12D diel cycle. A: Content of sD1 and rD1 transcripts during the diel cycle related to the value of both at 12D (=1); **B**: Membranes isolated from cells at particular time were analyzed by 1D SDS PAGE, gel was stained by SYPRO Orange for general proteins to document equal loading, and electroblotted to a polyvinylidene difluoride membrane, which was probed with antibodies specific for sD1 and rD1; each loaded sample contained 2 μg of Chl, 1L* indicates 50% loading of 1L; **C**: Diurnal pattern of the growth irradiance (bell shaped dotted line) and values of PSII photochemical yield Fv/Fm (filled circles).

Fig. 2. Temporal changes in *psbA1* and *psbA4* transcript levels (A), accumulation of sD1 and rD1 proteins (B), and changes in PSII activity reflected by Fv/Fm ratio (C) in *C. watsonii* during the diel cycle with prolonged light exposure. A: Content of sD1 and rD1 transcripts during the diel cycle related to the value of both at 6D (=1); **B**: Membranes isolated from cells at particular time were analyzed by 1D SDS PAGE, gel was stained by SYPRO Orange for general proteins to document equal loading, and electroblotted to a polyvinylidene difluoride membrane, which was probed with antibodies specific for sD1 and rD1. Each loaded sample contained 2 μg of Chl. **C:** Diurnal pattern of the growth irradiance (dotted line) and values of PSII photochemical yield Fv/Fm (filled circles).

Fig. 3. Temporal changes in accumulation of sD1 and rD1 proteins (A) and changes in PSII activity reflected by Fv/Fm ratio (B) in *C. watsonii* during the diel cycle with prolonged dark incubation. A: Membranes isolated from cells at particular time were analyzed by 1D SDS PAGE, gel was stained by SYPRO Orange for general proteins to document equal loading, and electroblotted to a polyvinylidene difluoride membrane, which was finally probed with antibodies specific for sD1 and rD1. Each loaded sample contained 2 μg of Chl. **B**: Diurnal pattern of the growth irradiance (dotted line) and values of PSII photochemical yield Fv/Fm in cultures non-treated (filled circles) and treated (empty circles) with protein synthesis inhibitor chloramphenicol.

Fig. 4. Temporal changes in accumulation of sD1 and rD1 protein levels after various treatments affecting light intensity (A), nitrogen presence (B), redox state of plastoquinone pool (C), trans-thylakoidal pH, serine protease activity (D), and protein synthesis (E). A: The content of rD1 was followed after the dark phase of the regular diel cycle and after subsequent 1 h and 3 h of constantly increasing diel light intensity (reaching about 200 μmol photons m^{-2} s^{-1} after 3 h; left panel), 3 h of constant illumination of 50 μmol photons m^{-2} s^{-1} (middle panel), or 5 min pre-illumination at 50 μmol photons m^{-2} s^{-1} followed by 1 h and 3 h dark incubation (right panel); **B**: The content of rD1 was followed after the dark phase of the regular diel cycle and after subsequent following 0.5 h and 1 h of constantly increasing diel light intensity in the presence of 0.8 mM and 5 mM NH_4Cl, or 0.8 M $NaNO_3$; **C**: The content of rD1 was followed after the dark phase of the regular diel cycle and after subsequent 0.5 h and 1

h of constantly increasing diel light intensity in the presence of 5 μM DBMIB, 5 μM DCMU, and 10 μM DCMU under constant flushing (400 mL/min) with nitrogen; **D**: The content of rD1 was followed after the dark phase of the regular diel cycle and after subsequent 0.5 h and 1 h of constantly increasing diel light intensity in the absence and presence of nigericin (50 μM) and serine protease inhibitor Pefablock SC (500 μM); **E:** The content of sD1 and rD1 was followed after the dark phase of the regular diel cycle and after subsequent 1 h and 3 h of constantly increasing diel light intensity or constant illumination of 50 μmol photons m^{-2} s^{-1} in the absence and presence of chloramphenicol (final conc. 15 mM). Each loaded sample contained 2 μg of Chl.

Fig. 5. Cellular content of Chl biosynthesis precursors (A) and Chl biosynthesis enzymes in correlation with rD1 content (B) in *C. watsonii* during the regular 12L:12D diel cycle. A: Culture of *C. watsonii* was tested during the regular diel cycle for the content of Chl biosynthesis precursors using HPLC analysis. Precursors were quantified as described in Material and Methods. Abbreviations: CoPP, coproporphyrin III; PPIX, protoporphyrin IX; MgP, magnesium protoporphyrin IX; MgPME, magnesium protoporphyrin IX methyl ester; DV Pchlide, di-vinyl-protochlorophyllide; MV Chlide, mono-vinyl-chlorophyllide. **B**: Content of Chl biosynthesis enzymes in *C. watsonii* during the regular diel cycle was monitored by 1D SDS PAGE in combination with immunodetection using the specific antibodies raised against the Chl biosynthesis enzymes and rD1. Each loaded sample contained 0.5 μg of Chl.

Fig. 6. Whole cell absorption spectra of *Synechocystis* strains lacking D1 (ΔD1) and lacking D1 but expressing rD1 from *C. watsonii* (rD1/ΔD1). The spectra of cultures adjusted to OD_{750nm} = 0.1 were measured using the Shimadzu UV3000.

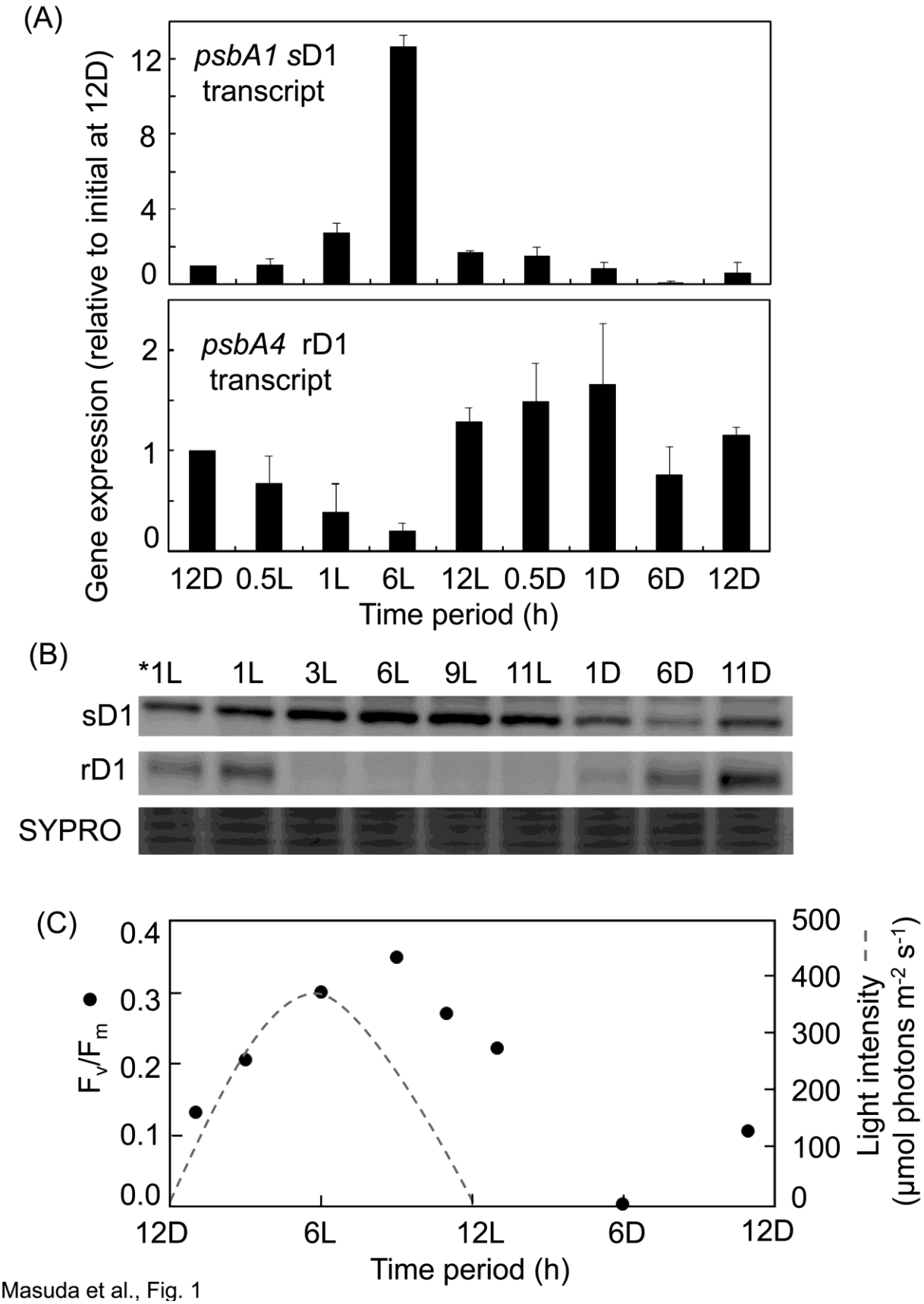
Fig. 7. Analysis of protein accumulation and synthesis in *Synechocystis* strains lacking D1 (ΔD1) and lacking D1 but expressing rD1 from *C. watsonii* (rD1/ΔD1). Membranes from ΔD1 and rD1/ΔD1 cells were analyzed by clear-native polyacrylamide gel electrophoresis (CN-PAGE) in the first dimension. The gel was photographed (1D color) and scanned for Chl fluorescence (1D fluor). After the separation in the second dimension the 2D gel was stained using Coomassie blue (2D CBB stain), dried and exposed to Phosphoimager plate for 24 h (2D autorad). Designations of complexes: RCCS, supercomplex of PSI and PSII; PSI(3), trimeric PSI; rPSII(2), dimeric PSII complex containing rD1; rPSII(1), monomeric PSII complex containing rD1; PSI(1), monomeric PSI complex; CP47m and CP43m, unassembled modules of CP47 and CP47 antennae; FP, free pigments. Red arrows designate the large labeled PsaA/B subunits of PSI. The loading of samples was performed on the same OD_{750nm} basis corresponding to 3 μg of Chl of the ΔD1 membranes.

Fig. 8. Absorption spectra of preparations isolated from *Synechocystis* strains lacking D1 but expressing *Synechocystis* FLAG-tagged D1 (FLAG-D1/ΔD1) or *C. watsonii* FLAG-tagged rD1 (FLAG-rD1/ΔD1). The spectra of preparations were measured using the Shimadzu UV3000 and normalized to the red absorption Chl peak and the spectrum of the FLAG-rD1/ΔD1 was shifted by 0.25.

Fig. 9. Analysis of preparations isolated from *Synechocystis* strains lacking D1 but expressing *Synechocystis* FLAG-tagged D1 (FLAG-D1/ΔD1) or *C. watsonii* FLAG-tagged rD1 (FLAG-rD1/ΔD1). Preparations isolated from FLAG-D1/ΔD1 and FLAG-rD1/ΔD1 cells were analyzed by clear-native polyacrylamide gel electrophoresis (CN-PAGE) in the first dimension (1D color). The gel was photographed (1D color) and scanned for Chl fluorescence (1D fluor). After the separation in the second dimension the 2D gel was stained using SYPRO Orange (2D SYPRO stain). Designations of complexes as in Fig. 7: PSI(1)/rRC47, the complex of PSI monomer and PSII core lacking CP43 and containing rD1; PSII(2), dimeric PSII complex; PSII(1), monomeric PSII complex; RC47, PSII monomer complex lacking CP43; rRC47, PSII monomer complex lacking CP43 and containing rD1. Each loaded sample contained 0.5 μg of Chl.

Fig. 10. Temporal changes in accumulation of sD1 and rD1 proteins in *Synechocystis* strains (A) containing the original D1 and expressing rD1 from *Crocosphaera* (rD1), (B) containing the original D1 and expressing FLAG-tagged rD1 (FrD1), (C) containing the original D1, expressing rD1 from *Crocosphaera* and lacking FtsH2 (rD1/ΔFtsH2), and (D) lacking the original D1 and expressing rD1 from *Crocosphaera* (rD1/ΔD1). The content of sD1 and rD1 in cells of all four strains grown in the presence of 5 mM Glc was followed in control cells (NL), cells incubated in the dark for 1, 2 and 4 hours (1D, 2D and 4D) and after subsequent transfer from dark to light for 1 and 2 hours (4D1L and 4D2L). Cells were used for isolation of membranes which were analyzed by SDS-PAGE. Gel was stained with SYPRO Orange to document equal loading, transblotted to PVDF membrane and sD1 and rD1 were detected by specific antibodies. Each loaded sample contained 1 μg of Chl, samples 50% NL just 0.5 μg of Chl.

Fig. 1



Masuda et al., Fig. 1

Fig. 2

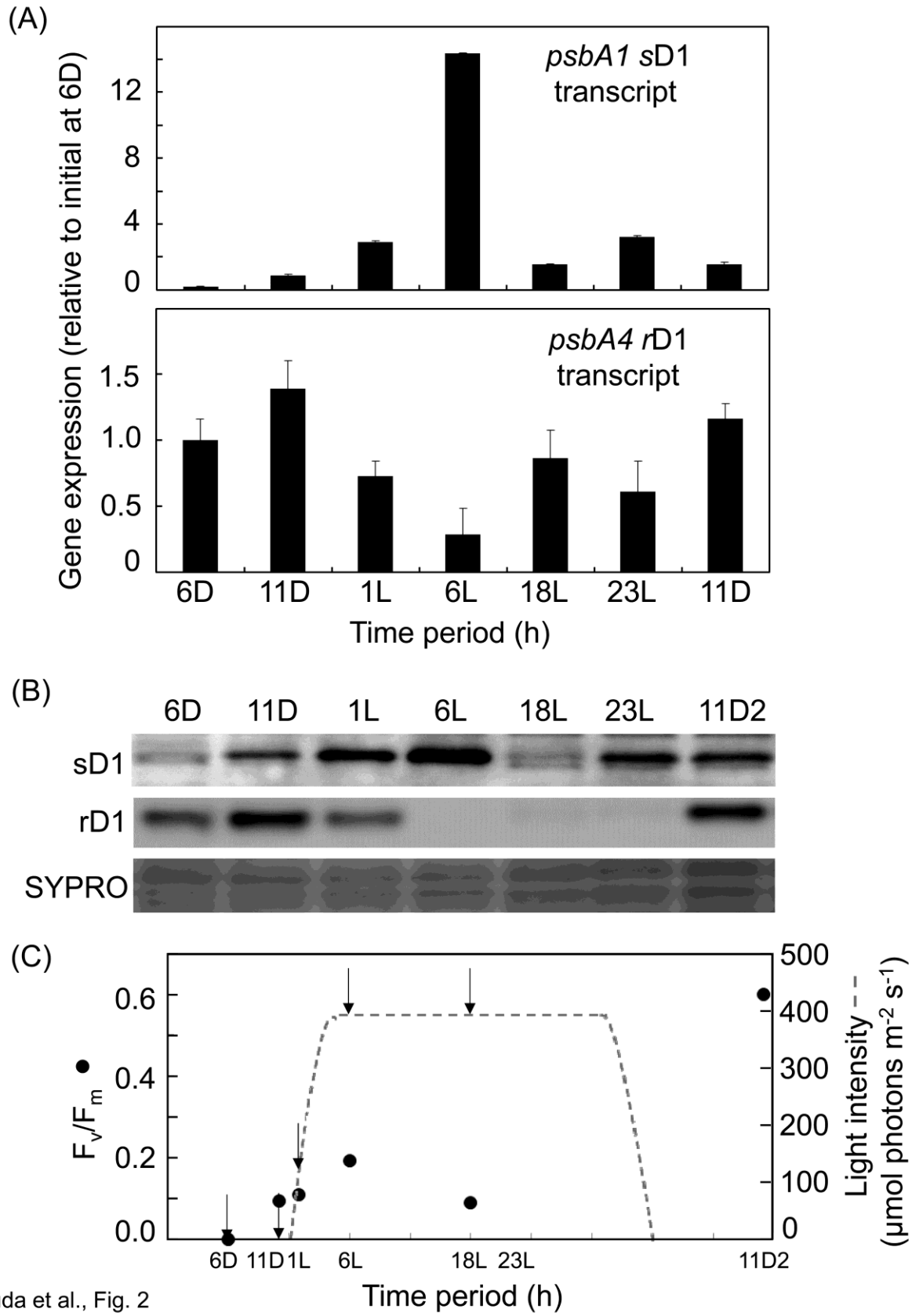


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Fig. 3

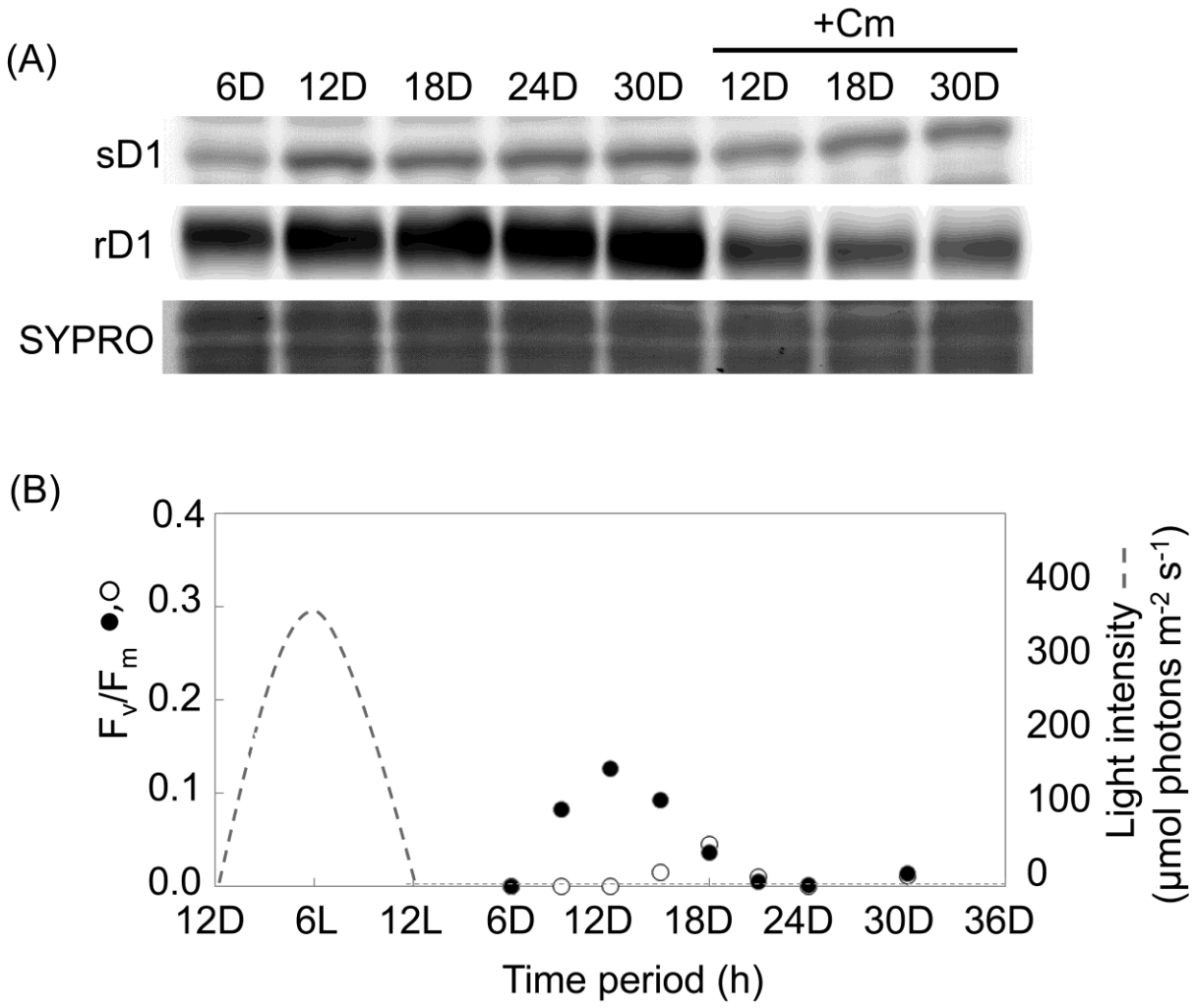

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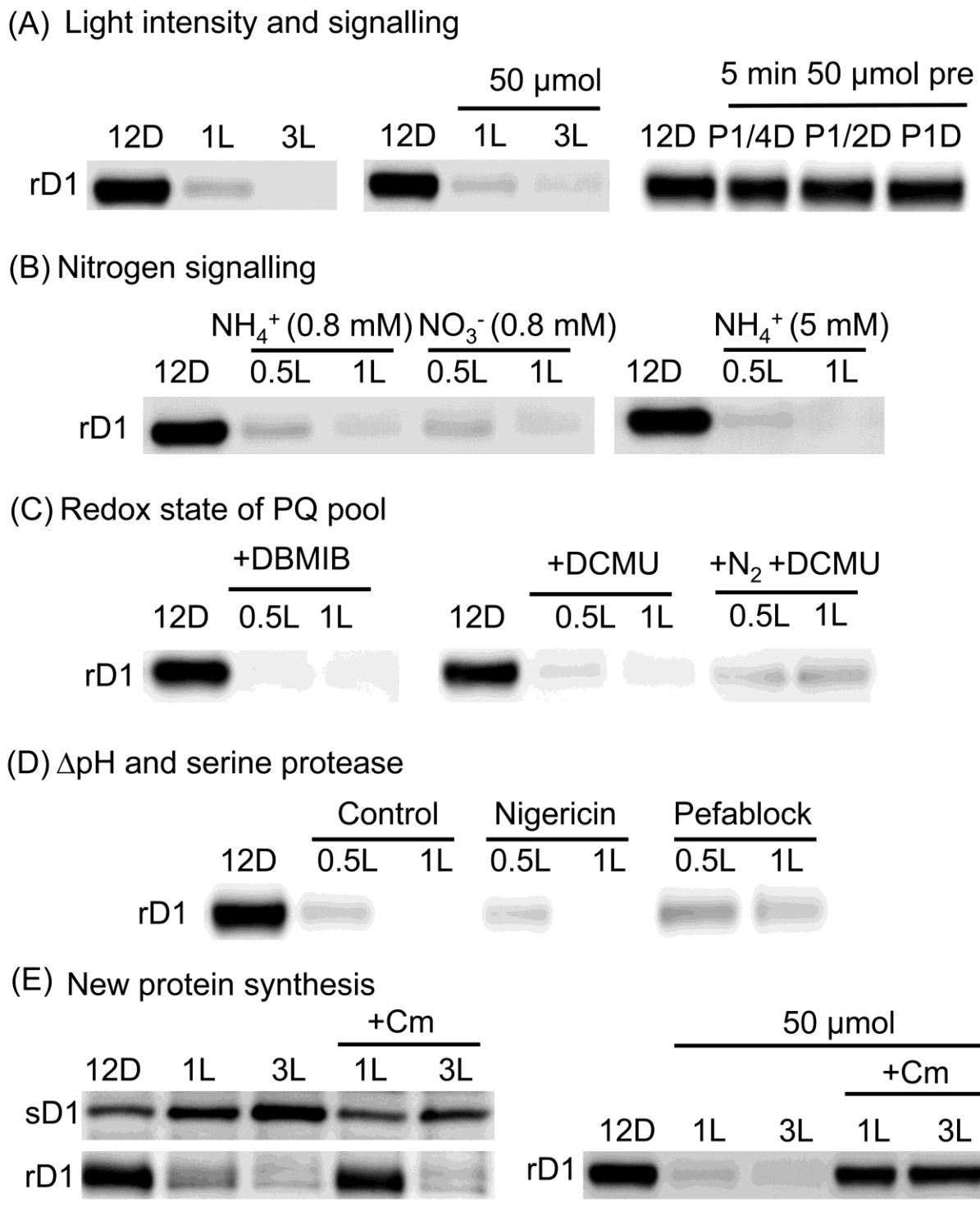
Fig. 4



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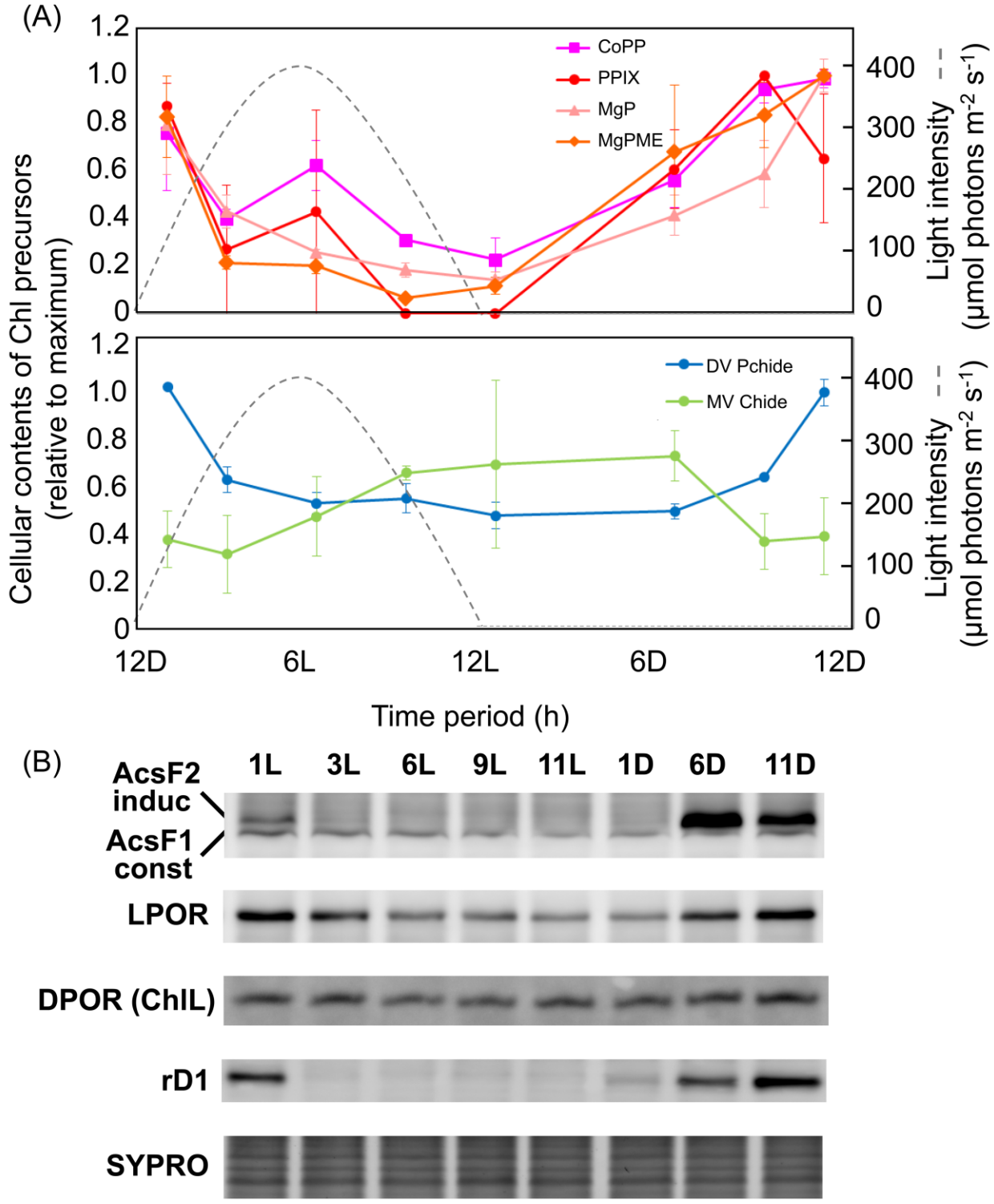

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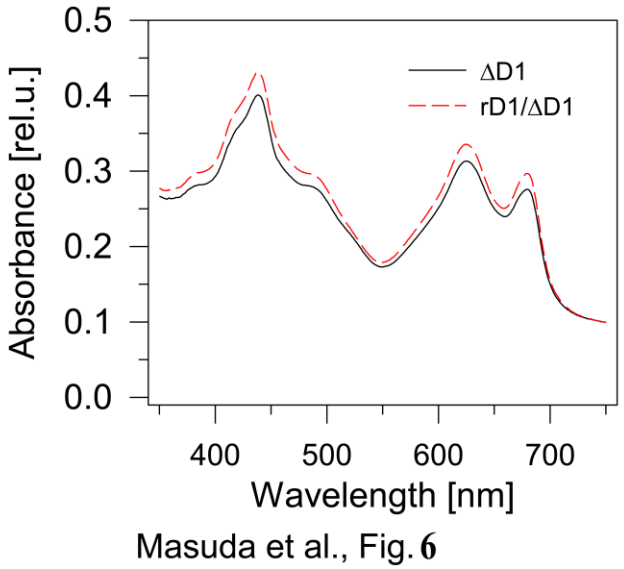
Fig. 6.



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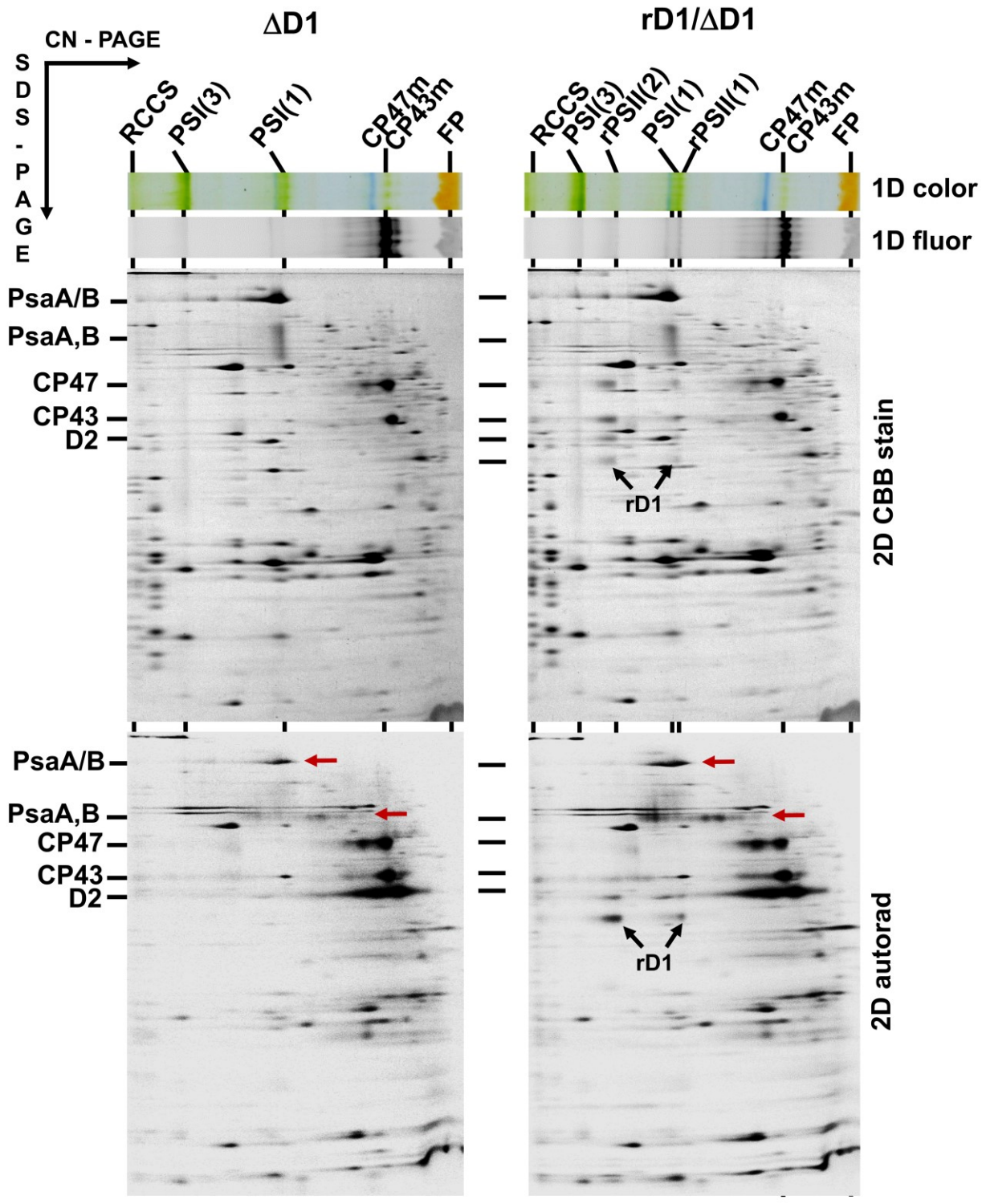

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Fig. 8

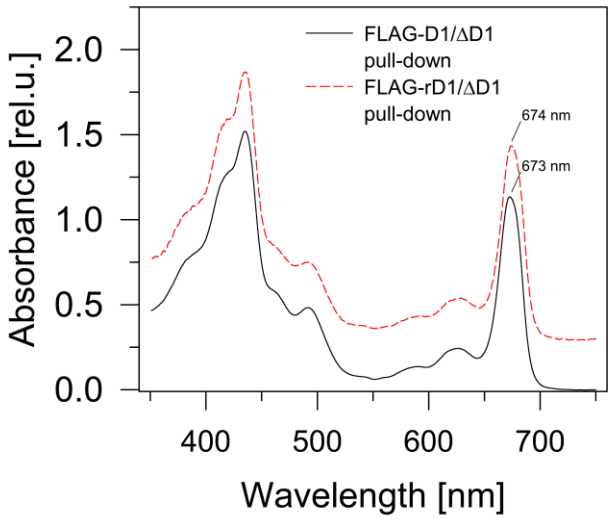

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Fig. 9

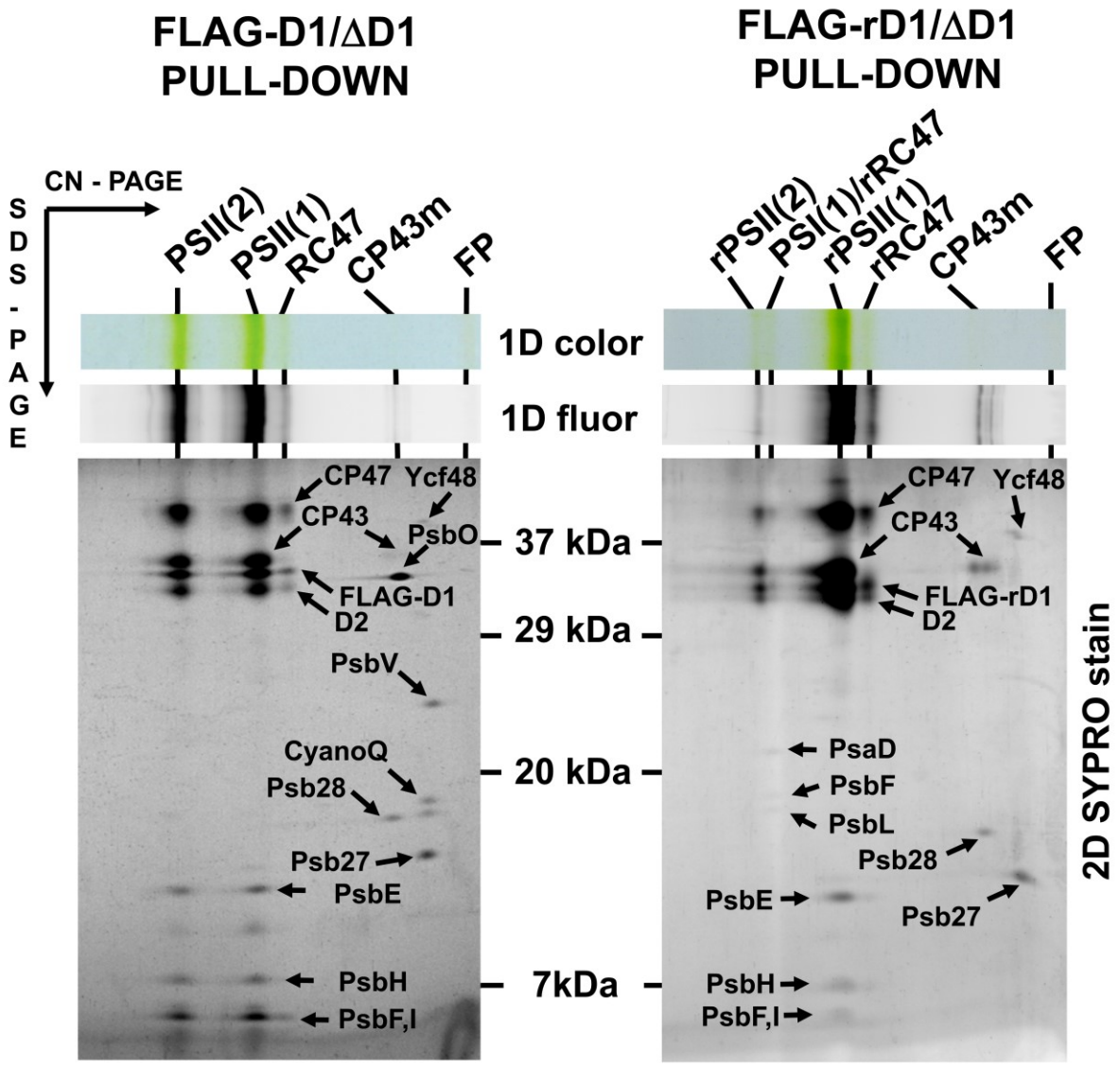

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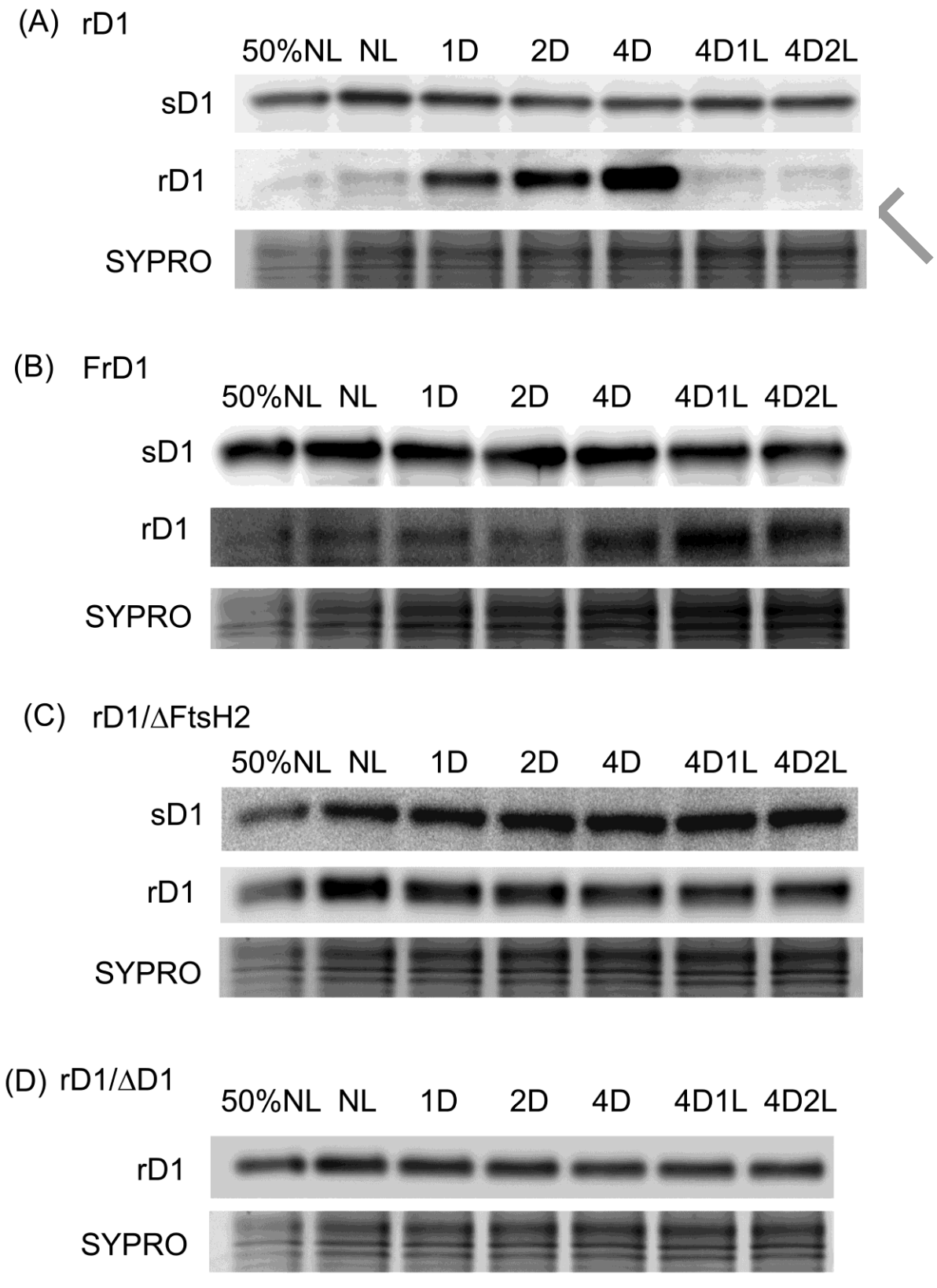
Fig. 10



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