Inositol polyphosphates and target of rapamycin kinase signalling govern photosystem II protein phosphorylation and photosynthetic function under light stress in *Chlamydomonas*

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Summary

- Stress and nutrient availability influence cell proliferation through complex intracellular signalling networks. In a previous study it was found that pyro-inositol polyphosphates (InsP7 and InsP8) produced by VIP1 kinase, and target of rapamycin (TOR) kinase signalling interacted synergistically to control cell growth and lipid metabolism in the green alga *Chlamydomonas reinhardtii*. However, the relationship between InsPs and TOR was not completely elucidated.
- We used an *in vivo* assay for TOR activity together with global proteomic and phosphoproteomic analyses to assess differences between wild-type and vip1-1 in the presence and absence of rapamycin.
- We found that TOR signalling is more severely affected by the inhibitor rapamycin in a vip1-1 mutant compared with wild-type, indicating that InsP7 and InsP8 produced by VIP1 act independently but also coordinately with TOR. Additionally, among hundreds of differentially phosphorylated peptides detected, an enrichment for photosynthesis-related proteins was observed, particularly photosystem II proteins. The significance of these results was underscored by the finding that vip1-1 strains show multiple defects in photosynthetic physiology that were exacerbated under high light conditions.
- These results suggest a novel role for inositol pyrophosphates and TOR signalling in coordinating photosystem phosphorylation patterns in *Chlamydomonas* cells in response to light stress and possibly other stresses.

Introduction

Post-translational modifications (PTMs) rapidly regulate major cellular processes such as transcription, translation and metabolism. Understanding these events is essential to map the complex signalling networks mediated by master regulators such as target of rapamycin (TOR) kinase (Soulard *et al.*, 2010; Yu *et al.*, 2011; Robitaille *et al.*, 2013; Roustan & Weckwerth, 2018; Van Leene *et al.*, 2019; Werth *et al.*, 2019; Scarpin *et al.*, 2020). This highly conserved Ser/Thr kinase responds to nutrient availability, energy levels and stress (Loewith & Hall, 2011; Albert & Hall, 2014; González & Hall, 2017), and is connected to growth regulation, cell cycle progression, circadian clock, photosynthesis, autophagy, and nutrient sensing in photosynthetic organisms (Dobrenel *et al.*, 2016; Pérez-Pérez *et al.*, 2017; Schepetilnikov & Ryabova, 2018; Shi *et al.*, 2018; Caldana *et al.*, 2019; Ford *et al.*, 2019; Couso *et al.*, 2020). TOR also participates in the regulation of processes such as carbon assimilation and lipid accumulation through synergistic coordination with inositol polyphosphates (InsPs) (Couso *et al.*, 2016).

Inositol polyphosphates are phosphorylated derivatives of the myo-inositol ring that can be sequentially and reversibly phosphorylated on all six carbons. InsPs have distinct properties that operate across signalling cascades for the regulation of biological processes throughout the cell, including nutritional sensing and hormone signalling (Tan *et al.*, 2007; Laha *et al.*, 2015; Livermore *et al.*, 2016; Wild *et al.*, 2016; Wu *et al.*, 2016). Among different InsPs, inositol pyrophosphates (PP-InsPs) stand out as secondary messengers due to the presence of ‘high energy’ pyrophosphate moieties and their ubiquitous nature in eukaryotic cells (Wilson *et al.*, 2013; Shears, 2015). The most common PP-InsPs arise from conversion of InsP6 to 1-diphosphoinositol 2,3,4,5,6-pentakisphosphate (1-InsP7 or 1PP-InsP6), 5-diphosphoinositol 1,2,3,4,6-pentakisphosphate (5-InsP7 or 5PP-InsP6) and 1,5-bis-diphosphoinositol 2,3,4,6-tetrakisphosphate (InsP8 or 1,5(PP)2-InsP4) (Supporting Information Fig. S1), in...
reactions catalysed by two distinct classes of enzymes: diphosphoinositol pentakisphosphate kinase (PPIP5K, known as VIH in plants and VIP1 in budding yeast and algae) and inositol hexakisphosphate kinase (IP6K, known as KCS1 in budding yeast) (Saiardi et al., 1999; Saiardi, 2004; Mulugu et al., 2007; Laha et al., 2015; Couse et al., 2016; Shears & Wang, 2019). IP6K is not conserved in green organisms; however ITPKs, which are responsible for the conversion of InsP₃ into InsP₆, has recently been suggested to phosphorylate InsP₆ \textit{in vitro} \textit{in Arabidopsis} (Cridland & Gillaspy, 2020). Although, PP-InsPs constitute a minor portion of the InsPs pool, they are suggested to play a fundamental role in controlling metabolism, interacting with SPX domain-containing proteins that are connected to polyphosphate (Poly-P) synthesis (Wild et al., 2016; Gerasimova et al., 2017) and phosphate signalling in yeast, mammals, and plants (Saiardi, 2012a; Secco et al., 2012; Livermore et al., 2016; Wild et al., 2016; Jung et al., 2018; Zhu et al., 2019; Li et al., 2020; Ried et al., 2021). Furthermore, PP-InsPs have been connected to carbon metabolism in algae (Couse et al., 2016) and jasmonate response in plants (Laha et al., 2015).

In \textit{Chlamydomonas}, a mutation in one of two VIP paralogues \textit{Cre03.g185500}, \textit{vip1-1}, was isolated in a screen for increased sensitivity to the TOR-specific inhibitor rapamycin (Rap) (Couse et al., 2016). \textit{vip1-1} was also hypersensitive to other TOR inhibitors, torin1 and AZD8055, indicating a specific interaction between the TOR and InsPs signalling pathways (Couse et al., 2016). Interestingly, \textit{vip1-1} showed misregulation in carbon assimilation and partitioning, displaying irregular levels of tricarboxylic acid (TCA) cycle intermediates and an overaccumulation of storage lipids. This overaccumulation was exacerbated in the presence of Rap and under nitrogen starvation conditions, both of which downregulate TOR, further supporting an interaction between these two signalling pathways (Couse et al., 2016). However, more work is needed to understand this interaction, as PP-InsPs and TOR crosstalk has only been reported in \textit{Arabidopsis} and \textit{Chlamydomonas} (Couse et al., 2016; Van Leene et al., 2019).

In this study, we monitored the phosphorylation levels of RPS6 (a downstream target of TOR signalling) and the autophagy marker ATG8 in \textit{vip1-1} compared with wild-type after Rap treatment. These results indicated a positive downstream synergy of PP-InsPs and TOR kinase on the regulation of these two well known TOR targets. To further explore which processes are either shared or specifically regulated by these signalling pathways, we performed global/phosphoproteomic analysis of \textit{vip1-1} and wild-type before and after Rap treatment. Markedly, the proteomic analysis indicated differential abundance of proteins and decrease in phosphorylation of annotation terms related to photosynthesis between wild-type and \textit{vip1-1}. These results led us to evaluate photosynthetic capacity in \textit{vip1-1} by measuring chlorophyll fluorescence and comparing InsPs levels in \textit{vip1-1} and photosynthetic deficient mutants under low and high light conditions. These data uncovered a novel relationship between TOR and PP-InsPs signalling compounds in governing photosystem II (PSII) and photoprotection that provide new insights in the study of photosynthetic control in the model green alga \textit{Chlamydomonas reinhardtii}.

**Materials and Methods**

**Cell culturing and rapamycin treatment**

\textit{Chlamydomonas reinhardtii} strain CC-1690 wild-type MT+ (Sager 21gr) (Sager, 1955) was used as parental strain to be compared with \textit{vip1-1}. This strain was isolated in an inserional mutant screen in CC-1690 using the hygromycin resistance gene \textit{aph7} (Couse et al., 2016). All cultures were maintained on TAP (Tris acetate phosphate) agar plates and grown in 350 ml TAP liquid cultures at 25°C. Experiments were performed using five replicate cultures grown to exponential phase (1–2 × 10⁶ cells ml⁻¹) for control and Rap treatment, and quenched with cold 40% methanol stored at −80°C before harvesting by centrifuging at 4000 g for 5 min and discarding the supernatant as described in Werth et al. (2019). For rapamycin-treated (LC Laboratories, Woburn, MA, USA) cultures, the drug was added to a final concentration of 500 nM from 1 mM stocks in dimethyl sulfoxide (DMSO) for 15 min before harvesting. For control replicates, just drug vehicle (DMSO) without a chemical inhibitor was added to each replicate culture for 15 min before harvesting.

**Proteomic analysis**

Global protein extraction and phosphopeptide enrichment were performed using frozen pellets as described in Werth et al. (2019) (Fig. S2). Phosphopeptide samples were resuspended in 20 µl 5% acetonitrile, 0.1% TFA while global samples were resuspended to a concentration of 0.2 µg µl⁻¹ in dimethyl sulfoxide (DMSO) for 15 min before harvesting. For control replicates, just drug vehicle (DMSO) without a chemical inhibitor was added to each replicate culture for 15 min before harvesting.

**Determination of chlorophyll fluorescence**

Fluorescence of chlorophyll \textit{a} was measured at room temperature using a pulse-amplitude modulation fluorometer (DUAL-PAM-100; Walz, Effeltrich, Germany) using mid-log phase cultures growing at control (50 µmol m⁻² s⁻¹) and high light (800 µmol m⁻² s⁻¹) conditions. Samples were normalised to 15 µg ml⁻¹ Chla after extraction with 80% acetone (Finazzi et al., 1999). The maximum quantum yield of PSII was assayed...
after incubation of the algal suspensions in the dark for 15 min by calculating the ratio of the variable fluorescence, \( F_v \), to maximal fluorescence, \( F_m \) (\( F_v/F_m \)). The parameters Y(II) and nonphotochemical quenching (NPQ) were calculated using DUAL-PAM-100 software according to the equations in Kramer et al. (2004) and Klughammer & Schreiber (2008). Measurements of linear electron transport rates (ETR II) were based on chlorophyll fluorescence of dark-adapted samples applying stepwise increasing actinic light intensities up to 1250 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Error bars indicate standard deviation (SD) of the values obtained from experiments performed in triplicate. Imaging-PAM M-series Maxi (Walz) was used to monitor \( F_v/F_m \) in Chlamydomonas under 50 and 800 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

**ATP levels**

ATP was assessed via LC-MS/MS analysis and 10 mg of fresh weight powder was extracted with trichloroacetic acid (TCA; Thermo Fisher Scientific, Waltham, MA, USA) as described in Weiner et al. (1987). Recovery experiments were carried out by adding analyte standards of ATP (Merck KGaA, Darmstadt, Germany) to the frozen tissue before the extraction and the analysis was performed as described in Lunn et al. (2006).

**InsPs extraction and analysis**

For InsP7 and InsP8 extraction, 300 ml of mid-log phase culture per sample was collected at a cell density of \( 2 \times 10^6 \) cells ml\(^{-1} \). Samples were extracted as reported in Couso et al. (2016) and 1 \( \mu \text{M} \) 3-fluoro-InsP3 (Enzo Life Sciences, Farmingdale, NY, USA) was used as internal standard for normalisation for relative quantification of the same InsPs species.

LC-MS/MS data were acquired using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a 1200 Capillary LC system (Agilent, Santa Clara, CA, USA) and a 0.5 \( \times \) 150-mm 5-\( \mu \text{m} \) BioBasic AX Column (Thermo Fisher Scientific) using the conditions reported in Couso et al. (2016). Mean data and SD were calculated from three biological replicates, each of which had three technical replicates.

**Results**

TOR activity is misregulated in vip1-1 after rapamycin inhibition

The insertional mutant \( \text{vip1-1} \) is PP-InsPs deficient and displays hypersensitivity to TOR inhibition by Rap (Couso et al., 2016). To further investigate the regulation of TOR in the \( \text{vip1-1} \) mutant, RPS6 phosphorylation was monitored over time in WT and \( \text{vip1-1} \) cells treated with Rap. We previously demonstrated that phosphorylation of RPS6 on Ser245 is a readout of TOR activity in Chlamydomonas (Couso et al., 2020). The basal phosphorylation level of RPS6 Ser245 (P-RPS6) was similar in both strains. However, \( \text{vip1-1} \) showed a significant decrease in P-RPS6 compared with WT (Fig. 1a,b) after 30 min of Rap treatment that was more pronounced after 60 min (Fig. 1b,c) indicating a faster de-phosphorylation in \( \text{vip1-1} \) compared with WT. The P-RPS6/RPS6 levels were similar in WT and \( \text{vip1-1} \) cells only after 90 min.

The detection of lipidated ATG8 (ATG8-PE) is an effective method to monitor autophagy; ATG8-PE accumulates under autophagy-inducing conditions including TOR inhibition (Pérez-Pérez et al., 2010). The levels of ATG8 and ATG8-PE were similar in WT and \( \text{vip1-1} \) cells under control conditions (Fig. 1d). However, both ATG8 and ATG8-PE were more highly accumulated in \( \text{vip1-1} \) after 30 min Rap treatment compared with WT (Fig. 1d), indicating a faster and stronger activation of autophagy in \( \text{vip1-1} \). RPS6 was previously demonstrated to be

![Fig. 1](https://newphytologist.com) Immunoblot analysis of P-RPS6/RPS6 as a readout of target of rapamycin kinase activity in Chlamydomonas WT (a) and \( \text{vip1-1} \) (b) in the presence of rapamycin (Rap) along a time course. Relative quantitation of P-RPS6/RPS6 was made using three biological replicates. Error bars represent standard deviation of the mean values. Asterisks represent significant differences (\( P < 0.05 \)) evaluated using Student’s t-test. (c) Immunoblot analysis of ATG8 in the presence or absence of rapamycin for 30 min. FKBP12 was used as loading control (d).

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rapidly turned over by autophagy in *Chlamydomonas* (Couso *et al*., 2018), therefore explaining the reduced abundance of this protein in *vip1-1* after 60 min (Fig. 1b).

**Gene ontology analysis in *vip1-1* proteomics reveals an especial enrichment for PSII**

Quantitative proteomics were performed in WT and *vip1-1* cells under control conditions and following 15 min of Rap treatment as previously reported in Werth *et al.* (2019), through which 2460 proteins were quantified (Table S1). No proteins significantly changed in abundance between control and Rap-treated conditions (Fig. S3A), confirming minimal protein turnover after 15 min Rap treatment. However, we observed significant differences in basal levels of proteins between the noninhibited samples for each strain (Fig. S3B). Despite having similar growth rates as the WT strain (Couso *et al*., 2016), 545 proteins from *vip1-1* were differentially abundant compared with the parent strain, with 373 increased and 172 decreased (Table S1).

Quantitative phosphoproteomic analysis of *vip1-1* and WT cells identified 3986 unique phosphorylated phosphopeptides, referred to as identifiers, derived from 1935 proteins (Table S2). Given the lack of significant changes in protein abundance within a given strain in the global proteomic analysis following Rap treatment (Fig. S3A; Table S1), changes in phosphopeptide abundance is likely to correspond to changes in phosphorylation state rather than changes in total protein abundance, enabling robust analysis of phosphorylation signalling pathways. Following Rap treatment, 1029 identifiers significantly changed in *vip1-1*, with 228 decreasing and 801 increasing (Fig. 2a), while 217 identifiers significantly changed in the parent strain, with 129 decreasing and 88 increasing (Fig. 2b). Comparison of the two strains yielded 1625 identifiers differentially abundant before Rap treatment (Fig. 2c) and 346 identifiers following treatment (Fig. 2d).

Gene ontology (GO) enrichment analysis (Ashburner *et al*., 2000) of the global proteomic dataset of untreated cultures revealed that *vip1-1* was enriched over WT in biological functions related to stress responses, including protein folding, photosystem II (PSII) repair, cellular response to oxidative stress, and protein refolding (Fig. S4A). By contrast, *vip1-1* was deficient in GO terms related to cellular metabolism, including the tricarboxylic acid cycle and electron transport in PSII, among others (Fig. S4B).

Gene ontology analysis of significantly changed phosphopeptides in *vip1-1* following Rap treatment uncovered an enrichment of identifiers involved in RNA processing as well as chromatin and DNA binding (Fig. 3a). By contrast, identifiers significantly decreasing in *vip1-1* under the same treatment were enriched in photosynthesis-related GO terms, including PSII assembly, PSI stabilisation, and the PSII oxygen evolving complex (Fig. 3b), indicating an important role of PP-InsPs in the regulation of photosynthetic-related processes in *Chlamydomonas* that has not been reported in green organisms therefore far. Notably, enrichment of photosynthesis-related proteins was not detected in WT (Fig. S5).

**Differential phosphorylation of known and putative TOR substrates are found in *vip1-1***

In total, 48 phosphorylated identifiers from 22 proteins with homology to known TOR signalling-related proteins were identified in this study (Table S3). Under Rap treatment, 11 of these identifiers significantly increased and two significantly decreased in *vip1-1* while one identifier significantly increased and four significantly decreased in the parent strain, with no overlap between strains (Table S3). One of these identifiers was an uncharacterised phosphosite, S2598 on TOR (Cre90.g400553.t1.1), that was significantly increased in the *vip1-1* mutant following Rap treatment (log2FC: 2.31) but did not change in the parent strain (Table S3).

An identifier from the autophagy-related protein ATG11 (Cre16.g651350.t1.1-S1176) was also found to significantly decrease in *vip1-1* (log2FC: −1.44) (Table S3) compared with WT under control conditions. ATG11 is indispensable for the initiation of autophagy in different eukaryotes (Sun *et al*., 2013; Li *et al*., 2014).

The La-domain RNA-binding (LARP1) protein is an effector of mTORC1 that regulates TOP mRNAs and is connected to cell cycle progression and embryogenesis in mammals (Fonseca *et al*., 2015; Philippe *et al*., 2020). In this study, six significantly changing identifiers were detected in *Chlamydomonas* LARP1 (Cre10.g441200.t1.2), three significantly decreased in *vip1-1* compared with WT in control conditions (S529, T668, S670), two increased in the *vip1-1* mutant under Rap treatment (S670, S812), and only one changed after Rap treatment in WT (S817) (Table S3).

**Phosphorylation of PSII core components are downstream PP-InsPs and TOR signalling**

In this study, global protein analysis uncovered 155 proteins related to photosynthesis, photorepair, and chlorophyll biosynthesis.
Among them, 50 proteins displayed differential abundance in the vip1-1 strain compared with the parent strain, with 34 increased and 16 decreased under control conditions (Table S4). These data suggest less abundant PSII with a significant decrease in four out of the six catalytic subunits, including D1 (gi|41179021|refNP_958377.1) and D2 (gi|41179063|refNP_958420.1), with log2FC of −2.07 and −2.13, respectively, and the two reaction centre proteins, psbC (CP43; gi|41179065|refNP_958422.1) and psbB (CP47; gi|41179032|refNP_958388.1), with log2FCs of −1.89 and −2.17, respectively (Table S4).

Phosphopeptide analysis of the photosynthesis-related proteins revealed 72 identifiers from 31 proteins, with 27 identifiers upregulated and 16 downregulated in vip1-1 compared with WT (Table S5). Additionally, 15 identifiers were differentially phosphorylated in vip1-1 compared with WT following TOR inhibition (5 upregulated and 10 downregulated) (Table S5). Phosphorylation of psbC-S456 (log2FC −3.46) and psbB-T501 (log2FC −4.98) were highly downregulated in vip1-1 compared with WT (Fig. 4a; Table S5). Following Rap treatment, the phosphorylation of these identifiers increased in vip1-1 but no significant differences were detected between WT and vip1-1 (Table S5). STL1 (Cre12.g483650.t1.2), the Chlamydomonas paralogue of STN8 kinase, had significantly less phosphorylation on T126 in vip1-1 compared with WT before Rap treatment.
(log2FC $-2.06$), and this downregulation was compensated after Rap where it was upregulated by a log2FC of 1.81 (Table S5). This kinase plays a central role in the phosphorylation of the PSII core proteins (Rochaix et al., 2012).

We performed PAM fluorometry under a light induction curve to further investigate PSII defects in vip1-1. $F_v/F_m$ was decreased in both the vip1-1 and WT cells treated with Rap (Table 1) similar to the results reported using the TOR inhibitor AZD 8055 (Ford et al., 2019; Upadhyaya & Rao, 2019). However, we observed that the electron transfer rate of PSII (ETR II) in vip1-1 was significantly decreased compared with WT after 600 $\mu$mol m$^{-2}$ s$^{-1}$, with the ETRII of vip1-1 comprising only 12–19% of the rate of the WT (Fig. 4b). Furthermore, at high fluences Rap treatment decreased ETRII of WT that shows similar levels than vip1-1 cells, which did not change after Rap addition (Fig. 4c). This result suggests that PP-InsPs are involved in the maintenance of electron transfer during high light stress and work constructively with TOR to provide positive regulation of the photosynthetic apparatus under noninhibited conditions, as Rap treatment results in the same decreased ETRII in WT as it is observed in control conditions of vip1-1. In addition, the abundance of several photosynthetic repair-specific proteins were upregulated in vip1-1, including vesicle-inducing protein in plastids 1 (VIPP1, Cre13.g583550.t1.2; log2FC 1.62) (Table S4), a multifunctional protein involved in the maintenance of photosystems (Nordhues et al., 2012; Theis & Schroda, 2016; Gupta et al., 2021), and chloroplast DNAJ-like protein 2 (CDJ2, Cre07.g316050.t1.2; log2FC 3.54) (Table S4), which interacts with VIPP1 to regulate thylakoid biogenesis (Liu et al., 2005).

Oxygen evolving complex proteins PsbO and PsbP were also differentially phosphorylated (Fig. 4a; Table S5). We found 15 identifiers of PsbO (Cre09.g396213.t1.1) (Table S5), 14 of them upregulated in vip1-1 before Rap exposure. Two sites, S103 and T237, were highly upregulated in control conditions (log2FC 4.73 and 5.20, respectively) and highly downregulated (log2FC $-2.93$ and $-2.33$) after Rap treatment. A conserved T212 was also upregulated in the mutant (log2FC 2.63). Additionally, four identifiers of PsbP (Cre12.g550850.t1.2;S73; -S133; -S156; -S174) (Table S5) were highly upregulated in vip1-1 (log2FC $2.66$; $3.49$; 2.21 and 4.13, respectively) while S169 was downregulated (log2FC $-2.68$) in the mutant. S73 and S174 were downregulated in vip1-1 after Rap treatment (log2FC $-1.71$ and $-1.70$, respectively).

**Table 1** $F_v/F_m$ in *Chlamydomonas reinhardtii* WT, vip1-1 and complemented line under different conditions.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rap</th>
<th>High light (800 $\mu$mol m$^{-2}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.71 ± 0.003</td>
<td>0.60 ± 0.003</td>
<td>0.63 ± 0.005</td>
</tr>
<tr>
<td>vip1-1</td>
<td>0.65 ± 0.002</td>
<td>0.58 ± 0.002</td>
<td>0.08 ± 0.004</td>
</tr>
<tr>
<td>vip1-1:VIP1</td>
<td>0.68 ± 0.001</td>
<td>0.61 ± 0.002</td>
<td>0.52 ± 0.003</td>
</tr>
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Values are mean ± SD; $n = 6$. **Fig. 4** (a) Differential phosphorylation of the photosynthetic apparatus in the vip1-1 mutant compared with the *Chlamydomonas* parent strain. Proteins coloured green show proteomic coverage in the dataset while proteins coloured grey do not. Each significantly changing phosphosite was localised on a unique phosphopeptide. Nontransformed fold changes are reported. Pink arrows represent significant upregulated phosphorylation of the indicated identifiers while blue arrows represent significant downregulated phosphorylation of the indicated identifiers (b) Electron transfer rate of PSII (ETR II) measured in a light induction curve using photosynthetically active radiation (PAR) from 0 to 1250 $\mu$mol m$^{-2}$ s$^{-1}$ under control conditions and rapamycin treatment (c) in WT, vip1-1 and the complemented line (vip1-1:VIP1). Errors bars indicate standard deviation of the mean values from three biological and three technical replicates.
PP-InsPs control abundance and phosphorylation of light harvesting complex II proteins in coordination with TOR

State transitions involve the reversible transfer of a fraction of the light harvesting complex II (LHCII) from photosystem PSII to PSI as a result of protein phosphorylation (Goldschmidt-Clermont & Bassi, 2015). This process balances the excitonic flux of the two PSs to meet the cellular demand for ATP following changes in environmental conditions and protects from overexcitation (Cardol et al., 2009). Under stress conditions, the transition from state 1 to state 2 induces the switch from linear to cyclic electron flow (CEF). Our proteomic analysis indicated that LHCBM5 (Cre03.g156990.t1.12) abundance is decreased in vip1-1 compared with WT (Table S4). This protein is a component of the light harvesting antenna that migrates from PSII to PSI under state transitions (Takahashi et al., 2006). The downregulation of LHCBM5 was confirmed by immunoblotting, with an observed protein reduction of c. 20% (Fig. 5a,b) in vip1-1 compared with the WT and the complemented line.

LHCB4 (CP29) is a heavily phosphorylated protein in the thylakoid membrane of Chlamydomonas (Lemeille et al., 2010; Bergnet et al., 2015), with six different identifiers found in this study (Table S5). Among them T11, T17 and S39 were significantly decreased in the mutant under control conditions (log2FC/C0 2.68, -2.97 and -1.41, respectively) and T11 remained downregulated after Rap treatment. Four identifiers belonging to LHCB5 (Cre16.g673650.t1.1-T10, -S63, -T187, -S202) were also identified (Table S5), although only S63 was upregulated in vip1-1 compared with WT (log2FC 4.11). This upregulation was prevented in the presence of Rap, further indicating the interaction of PP-InsPs and TOR in controlling the phosphorylation state of LHCB5.

CEF and ATP levels are aberrantly regulated in vip1-1

In Chlamydomonas, PGRL1 (Cre07.g340200.t1.12) forms a supercomplex with PSI and cytochrome b6/f to initiate CEF (Iwai et al., 2010). In our study, a nonconserved identifier in PGRL1 (Cre07.g340200.t1.12; S50) was downregulated in vip1-1 (log2FC: -2.11) under control conditions and in WT after Rap treatment (log2FC: -2.01) (Fig. 4a; Table S5). In vip1-1, this downregulation is alleviated after Rap and showed no significant difference compared with WT. These data suggest a compensation mechanism for PGRL1 phosphorylation that is regulated by PP-InsPs and TOR.

Cyclic electron flow is activated after high light stress and rewires energy to increase ATP yield. We identified significant increases in two ATP synthase subunits of vip1-1 (Table S1): subunit E (gi|4117902|ref|NP_958379.1; log2FC 1.67), which forms the connection between the luminal and stromal hemispheres, and subunit II (Cre11.g481450.t1.2; log2FC 1.37), which regulates ATP synthesis based on the proton gradient.

Fig. 5 (a) Immunoblot analysis of LHCBM5 under control conditions and rapamycin treatment (30 min) in Chlamydomonas wild-type (WT), vip1-1 and the complemented line (vip1-1:VIP1). (b) Quantitation of LHCBM5 was made using three biological replicates. Error bars represent standard deviation (SD) of the mean values. Asterisks represent significant differences (P < 0.05) evaluated using Student’s t-test. (c) Quantitation of ATP in WT and vip1-1 mutant under control and rapamycin conditions. Error bars represent SD of the mean values from three biological replicates. Asterisks represent significant differences (P < 0.05) evaluated using Student’s t-test. (d) Nonphotochemical quenching (NPQ) was measured in WT, vip1-1 and the complemented line (vip1-1:VIP1) on an induction curve using photosynthetically active radiation (PAR) from 0 to 1250 µmol m⁻² s⁻¹ in the presence or absence of 500 nM rapamycin (Rap). Error bars indicate SD of the mean values from three biological and three technical replicates.
Nonphotochemical quenching and PP-InsPs biosynthesis are likely to be connected by a feedback loop

In *Chlamydomonas*, LHCSR3 (Cre08.g367400.t1.1) mediates the induction of NPQ (Peers et al., 2009) and can work together or independently with PGRL1 during high light (HL) acclimation (Chaux et al., 2017). By contrast with the observed downregulation of GrPGRL1-S50, three identifiers of LHCSR3 (Cre08.g367400.t1.1; -S165, -T161, -Y170) (Table S5) were upregulated in *vip1-1* (log2FC 1.48, 1.70 and 2.67, respectively). The phosphorylation of two of these phosphosites was decreased following Rap exposure, while Y170 remained upregulated in the mutant under the same conditions (Table S5). To investigate this further, we compared NPQ in WT, *vip1-1* and a complemented line using light response curves and PAM fluorometry in the presence or absence of Rap (Fig. 5d). NPQ was highly downregulated in the *vip1-1* mutant curve compared with the other two strains, reaching a difference of 72% under very HL (1250 µmol m⁻² s⁻¹) (Fig. 5d). Although, WT and the complemented line keep NPQ levels above 0.4 after reaching HL (750 µmol m⁻² s⁻¹), *vip1-1* further downregulates NPQ reaching 0.16, indicating that NPQ is not supported by *vip1-1* photosynthetic machinery under HL. In the presence of Rap, NPQ did not change significantly in any of the strains compared with control conditions.

Additionally, F₂/F₅ and Y(II) were analysed in WT and *vip1-1* cells under control light (50 µmol m⁻² s⁻¹), HL (800 µmol m⁻² s⁻¹) and Rap (control light conditions) conditions (Fig. S6; Table 1). Despite few differences in F₂/F₅ under control conditions, *vip1-1* showed significantly reduced F₂/F₅ and Y(II) values when subjected to HL, further indicating a misregulation of the light stress compensation mechanisms (Fig. S6A,B; Table 1).

Similarly to downregulated NPQ mutants (*npq1* and *npq2*), *vip1-1* showed decreased NPQ under HL stress (Niyogi et al., 1997, 1998; Kress & Jahns, 2017) (Fig. 6a). To evaluate the link between NPQ, PP-InsPs and TOR, InsP₇ and InsP₈ abundances were determined in WT, *vip1-1* and *npq2* under control and HL conditions. In WT cells, InsP₇ and InsP₈ levels increased significantly after HL treatment (Fig. 6b,c). In fact, InsP₈ levels reached a 50% increase under HL (Fig. 6c). As previously reported (Couso et al., 2016), InsP₇ and InsP₈ were diminished in *vip1-1* compared with WT, which was constant under HL (Fig. 6b,c). For *npq2*, InsP₇ decreased compared with WT in control conditions and further decreased under HL treatment (Fig. 6b). InsP₈ was also highly downregulated in *npq2* under HL, reaching 75% and 60% decrease, respectively (Fig. 6c). NPQ and InsP₇/
InsP₈ were also monitored following Rap treatment (30 min) in all strains. There were no significant differences in NPQ after Rap treatment in any of these strains compared with control conditions (Fig. 6a). After Rap treatment, InsP₈-an InsP₈ levels in npq2 were comparable to the level detected under control light conditions (Fig. 6b,c). These data indicate a novel connection between PP-InsPs levels and NPQ that may control HL stress compensation in *Chlamydomonas* that acted independently of TOR.

**Discussion**

**Pyro-inositol polyphosphates modulate TOR activity in *Chlamydomonas***

The macrolide rapamycin partially arrests cell growth in *Chlamydomonas* (Crespo *et al.*, 2005; Couso *et al.*, 2016), suggesting that it does not completely inhibit TOR activity. This is conserved in mammals (Thoreen *et al.*, 2009), in which differential sensitivity of mTORC1 phosphorylation sites to Rap has been reported as well (Kang *et al.*, 2013). We isolated *vip1-1* in a genetic screen due to its hypersensitivity to Rap compared with WT and have demonstrated a genetic link between pathways by restoring this phenotype in the *vip1Δkbp12* double mutant (Couso *et al.*, 2016). In this study, we found that *vip1-1* accelerates TOR inactivation under Rap conditions (Fig. 1a–c), indicating that these signalling pathways are likely to be controlling similar downstream targets either coordinately and/or independently of each other.

InsP₇ is a phosphate donor for different nucleolar proteins in yeast (NSR1 and SRP40) and mammals (Nopp140 and TCOF1) (Saiardi, 2004, 2012b, 2016; Bhandari *et al.*, 2007). The InsP₇-derived phosphate is thought to be attached to a pre-existing phosphorylation (β-pyrophosphorylation), which may provide a unique mode of signalling to proteins. However, the impact of PP-InsPs on phosphorus-signalling networks in photosynthetic organisms has not yet been reported. We used differential analysis of global proteomics and phosphoproteomics in WT and *vip1-1* in the presence or absence of Rap to reveal both the overlap with the TOR signalling network as well as the mechanistic connection between PP-InsPs and PTMs that has not been reported in green organisms therefore far.

While the number of identifiers shown to significantly change in phosphorylation in WT cells following Rap treatment mirrored previous studies (Fig. 2b) (Werth *et al.*, 2019), *vip1-1* yielded a larger change in phosphopeptide abundance following TOR inhibition than the WT (Fig. 2a). However, the number of identifiers found to be differentially phosphorylated was significantly lower when comparing WT and *vip1-1* before (1625 identifiers) and after (346 identifiers) TOR inhibition (Fig. 2c,d). These results strongly suggested that TOR kinase and PP-InsPs operated in the same signalling cascade in agreement with their previously observed genetic interaction (Couso *et al.*, 2016). We cannot disregard that they could directly interact, as we found an upregulation of S2598 on TOR in *vip1-1* after Rap treatment (Table S3) when TOR activity is faster inhibited in the mutant (Fig. 1a–c). Previously, this phosphosite was identified in *Chlamydomonas* WT, but no change was detected in response to different TOR inhibitors (AZD8055, Torin 1 and Rap) (Werth *et al.*, 2019). Our results suggest TOR phosphorylation at S2598 may be regulated by PP-InsPs, although the impact on TOR activity is still unknown.

We also identified a novel connection between PP-InsPs and established processes under TOR control (Table S3). Autophagy is inhibited by active TOR signalling in diverse eukaryotes including algae and plants (Díaz-Troya *et al.*, 2008; Yu *et al.*, 2018). Recently, VIP1 has also been linked to the regulation of autophagy by modulating the level of ATG proteins in *Candida albicans* (Ma *et al.*, 2020). We found an overaccumulation of lipidated ATG8, therefore indicating an overactivation of the recycling process of autophagy in *vip1-1* under Rap conditions (Pérez-Pérez *et al.*, 2010) (Fig. 1d). We also found an ATG11 phosphosite (ATG11-S1176) (Table S3) that was significantly downregulated in *vip1-1* under control conditions. ATG11 is a well-known scaffold protein that interacts with phosphorylated ATG29 or ATG32 in yeasts to induce mitophagy and the organisation of the phagophore assembly site (Aoki *et al.*, 2011; Mao *et al.*, 2013) and encourages starvation-induced phosphorylation of ATG1 in Arabidopsis, a downstream TOR target (Li *et al.*, 2014). Taking these results together, we conclude that autophagy is regulated coordinately with TOR and PP-InsPs signalling pathways in *Chlamydomonas*, but that PP-InsPs may also act independently of TOR to potentiate this recycling process.

LARP1 proteins are direct effectors of mTORC1 in mediating mRNA translation (Thoreen *et al.*, 2012) and are found in many eukaryotes (Dergon & Bouquet-Antonelli, 2015). However, none of the significantly changing phosphorylated identifiers found in *Chlamydomonas* LARP1 (Table S3) was conserved in yeasts or humans. AtLARP1 showed significantly decreased phosphorylation on S644 and S649 following TOR inhibition (Van Leene *et al.*, 2019); the former is conserved in *Chlamydomonas* (LARP1-S810) and identified as a phosphosite in Werth *et al.* (2017), but did not significantly change following TOR inhibition (Werth *et al.*, 2019). However, LARP1-S817 phosphorylation decreased following TOR inhibition in the same study (Werth *et al.*, 2019), which was also observed here in WT after Rap inhibition however no significant differences were seen in *vip1-1* (Table S3). Instead, three different phosphosites were significantly decreased in *vip1-1* compared with WT (LARP1-S529, -T668 and -S670) and only -S670 was upregulated after Rap treatment (Table S3). These results suggest that PP-InsPs act partly through the TOR signalling cascade and partly through TORC1-independent mechanisms to effect LARP1 phosphorylation.

**PP-InsPs and TOR control phosphorylation of photosynthetic apparatus**

Gene ontology analysis revealed an unexpected enrichment of photosynthetic targets (Fig. 3) that were not described in previous studies reporting protein phosphorylation patterns under TOR inhibition in either *Chlamydomonas* or Arabidopsis (Roustan & Weckwerth, 2018; Van Leene *et al.*, 2019; Werth *et al.*, 2019).
However, proteomic analysis of reversible cysteine oxidation, a PTM known to crosstalk with phosphorylation, indicated that photosynthesis is regulated by TOR in *Chlamydomonas* (Ford *et al.*, 2019). Similarly, our data here showed significant differences in photosynthesis-related proteins in global and phospho-proteomic data between WT and *vip1-1* under control conditions (Tables S4, S5). These results indicated reduced levels of the catalytic subunits D1 and D2 and reaction centres psbC (CP43) and psbB (CP47). Also, psbC-S456 and psbB-T501 were highly downregulated in the mutant under control conditions but not after Rap treatment (Table S5). D1, D2 and psbC are primarily phosphorylated in response to light stress and endogenous circadian rhythm at their N-terminal threonine residues (Elich *et al.*, 1992; Booij-James *et al.*, 2002) by the Ser/Thr kinase STATE TRANSITION8 (STN8) (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005; Rochaix *et al.*, 2012). The *Chlamydomonas* parologue of STN8, STL1 (Cre12.g483650.r1.2) showed a significant downregulation on T126 in *vip1-1* that was compensated in the presence of Rap (Table S5). Although this kinase is regulated by the redox state of the PQ pool (Bennett, 1991; Fristedt *et al.*, 2009), it has also been reported to be subjected to phosphorylation (Reiland *et al.*, 2011). Additionally, STL1-T126 was previously reported in *Chlamydomonas* (Bergner *et al.*, 2015). The phosphorylation of PSII core proteins is part of the PSII repair cycle that proceeds before the proteolytic degradation of damaged D1 protein, preventing its degradation by proteases (Koivuniemi *et al.*, 1995). Additionally, STN8 is thought to control the transitions from linear to CEF by controlling the phosphorylation of PGRL1 in *Arabidopsis* (Reiland *et al.*, 2011). Our data could indicate that the downregulation of D1 found in *vip1-1* is a consequence of the differential phosphorylated identifiers in STL1 and core components found in this mutant and that PP-InsPs and TOR act coordinately upstream of this process controlling PSII photochemistry, possibly facilitating the transition between linear and CEF under high irradiances.

We also determined the light harvesting antenna protein LHCBM5 (Cre03.g156900.r1.2) has decreased abundance in *vip1-1*. Although LHCBM5 has no homologue in plants, it has been suggested that this protein plays a similar role to the CP24 protein in *Arabidopsis* (Takahashi *et al.*, 2006). CP24-deficient plants displayed altered kinetics of state transitions (Kovács *et al.*, 2006). The downregulation of LHCBM5 detected in the *vip1-1* mutant could link PP-InsPs to state transitions in *Chlamydomonas* as LHCBM5 is the more abundant LHCI1 (type II) polypeptide found in PSI-LHCI under state 2 (Takahashi *et al.*, 2006).

Phosphorylation of LHC4 and LHC5 (CP26) is considered fundamental for the detachment of LHCBM polypeptides from PSII during the transition from state 1 to state 2 (Iwai *et al.*, 2008). Three identifiers belonging to LHCBM polypeptides in *Chlamydomonas* (Schoz *et al.*, 2019). Another identifier found in this study, LHCBM-S103 (Table S5), has been previously linked to kinase STT7 activity in this alga (Bergner *et al.*, 2015), but we could not find any significant difference in the conditions tested. We also found four identifiers in LHCBS1 (T10, -S63, -T187, -S202) (Table S5), although S63 was the only one significantly changing in *vip1-1* under control conditions. The upregulation of S63 was then prevented after Rap treatment indicating that TOR and PP-InsPs share this target. Also, LHCBS1-T10 has previously been identified after mapping in vivo phosphorylation sites in integral and peripheral membrane proteins (Vener, 2007). These data suggest that PP-InsPs and TOR control state transitions at different levels, with LHCBM5 protein abundance and the phosphorylation state of LHC4 and LHC5 influencing the transition to state 2 when light compensation mechanisms are required. Additionally, repair-specific proteins (VIPP1 and CDJ2) were upregulated in *vip1-1* mutant. In *Chlamydomonas*, *vipp1* amiRNA knockdown strains are sensitive to HL, which is likely to be due to structural defects in PSII (Nordhues *et al.*, 2012). The upregulation of this protein in *vip1-1* suggests that PSII repair mechanisms are more active in this mutant, and are likely to be as a consequence of PSII malfunction. This is supported by decreased Fv/Fm (Table 1) and ETR II (Fig. 4b) values detected in the mutant.

PP-InsPs and TOR are involved in the maintenance of cell energy levels

In *Arabidopsis*, PGRL1-T62-T63 has been reported to be a possible target of STN8 kinase (Reiland *et al.*, 2011). We found one identifier in PGRL1 (S50) to be downregulated in *vip1-1* under control conditions and in WT after Rap treatment. The levels of PGRL1-S50 were alleviated in the presence of Rap, but only in the mutant, therefore indicating a fine modulation mediated by PP-InsPs and TOR that correlates to the tight control of the initiation of CEF mediated by PGRL1 (Johnson *et al.*, 2014).

Under stress, CEF provides ATP for CO2 fixation (Lucker & Kramer, 2013), balances overproduction of PSI, and readjusts the ATP poise, leading to increased lumen acidification that is important for photoprotection (Alric, 2010; Peltier *et al.*, 2010; Leister & Shikanai, 2013; Shikanai, 2014). ATP synthase sub-units E and II are increased in *vip1-1* (Table S1) that corresponds with the high ATP levels found in this mutant (Fig. 5c). Although PP-InsPs have been previously linked to the control of intracellular ATP in yeast kes14 mutants (Szigiaryto *et al.*, 2011), *vip1* mutants have not previously been directly connected with this phenotype. While no IP6K homologue is found in algae or plants, the detection of InsP8 (1,5(PP)2-InsP4) suggests the presence of a functional IP6K enzyme (Desai *et al.*, 2014; Laha *et al.*, 2015; Couso *et al.*, 2016), or a noncanonical ITPK function (Cridland & Gillaspy, 2020) that could be regulating ATP levels in coordination with VIP1. Recently, VIP1 was also reported to have a bifunctional kinase/pyrophosphatase activity that produces and destroys 1-PP-InsPs at the expense of consuming ATP in yeast (Dollins *et al.*, 2020). This could contribute to the increase in ATP observed in the *Chlamydomonas* *vip1-1* mutant. Additionally, mTOR is a homoeostatic ATP sensor that adjusts ribosome biogenesis to ATP intracellular levels (Dennis, 2001).
Although \( vip1-1 \) displays higher ATP levels than WT, we did not detect any difference in TOR activity before Rap treatment, suggesting that this activation pathway may not be conserved in this alga.

NPQ, PP-InsPs and TOR coordinate to protect cells to excessive irradiance

The photoprotective process NPQ is activated in almost all photosynthetic organisms in PSII antenna to dissipate excess light as heat (Ruban, 2016). NPQ is catalysed by Light Harvesting Complex Stress Related (LHCSR) subunits, with a major role observed for LHCSR3 (Girolomoni et al., 2019). We found three identifiers of LHCSR3 (-S165, -T161, -Y170) (Table S5) increased in \( vip1-1 \) and only Y170 remained upregulated in the presence of Rap. LHCSR3 phosphorylation has also been reported as nonessential for NPQ activation in \( Chlamydomonas \) (Bonente et al., 2011). However, that work compared phosphorylated with dephosphorylated LHCSR3 that contrasts with the over-phosphorylated identifiers found in \( vip1-1 \). Protein levels of LHCSR3 have previously been connected to the regulation of NPQ (Peers et al., 2009) but we could not detect any significant difference in \( vip1-1 \) compared with WT (Table S4), suggesting that PP-InsPs and possibly TOR are regulating NPQ at post-translational level. LHCSR3 phosphorylation at the N-terminus (-S26, -S28-T32 and -T33) has been reported to operate as a molecular switch modulating LHCB4 phosphorylation, which in turn is important for PSII-LHCII disassembly before state transitions (Bergner et al., 2015; Scholz et al., 2019). Also, Arabidopsis \( koLhcb4 \) mutants present lower activation of NPQ (de Bianchi et al., 2011). These results indicated a tight control of photoprotective mechanisms mediated by PP-InsPs that act independently (-Y170) and coordinately with TOR (-S165, -T161) over the phosphorylation of LHCSR3. We also need to consider that the deregulation of LHCB4 (CP29) phosphorylation in \( vip1-1 \) is suggesting that NPQ is finely controlled by PP-InsPs at different levels in the photosynthetic machinery. In vivo measurements of NPQ levels in the WT, \( vip1-1 \), and complemented line revealed highly deceased NPQ levels after HL in \( vip1-1 \) that were not observed following Rap treatment (Figs 5d, 6a). Although NPQ does not seem to be affected by TOR inhibition in any of the strains, we cannot disregard that LHCSR3-S165 and -T161 levels were compensated after Rap treatment (Table S5). This is another example of the multiplex regulation mediated by PP-InsPs and TOR kinase over the same targets that are mechanistically difficult to delineate.

Nonphotochemical quenching, InsP, and InsP levels were also compared among WT, \( vip1-1 \) and the NPQ defective mutant \( npq2 \). We found an important decrease of PP-InsPs in \( npq2 \) that does not respond to Rap treatment (Fig. 6b,c). These data indicated a possible feedback loop in the regulation of NPQ and PP-InsPs biosynthesis that is independent of TOR signalling (Fig. 6b,c). We also found that PP-InsPs levels and NPQ were highly different from WT levels under HL conditions, indicating that this feedback regulation may be especially relevant under stress conditions where NPQ is activated.

Conclusion

Overall, our data indicated a strong relationship between TOR kinase and VIP1/PP-InsPs that impacted autophagy and TOR activity in \( Chlamydomonas \). We have uncovered that PP-InsPs share common targets with TOR in controlling photosynthetic machinery and compensation mechanisms including state transitions and CEF (Fig. 7). We have also identified PP-InsPs as key components of the signal transduction machinery that can act independently of TOR controlling NPQ under high irradiance or energy levels (Fig. 7). We have begun unravelling the influence of these essential signalling compounds in \( Chlamydomonas \) PTMs; however, several questions still remain. Future work should address the role of PP-InsP signalling in specific cell compartments as well as the conditions in which PP-InsPs signal transduction act independently and/or coordinately with TOR signalling over different targets to maintain cell homeostasis.

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Data availability
The data that support the findings of this study are openly available in the ProteomeXchange Consortium via the PRIDE partner repository and can be accessed with the Identifier PXD023085.

References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Schematic model of InsP₆ phosphorylation steps in *Chlamydomonas reinhardtii*.

**Fig. S2** Proteomic workflow for analysis of *Chlamydomonas vip1-1* and wild-type cells treated with rapamycin.

**Fig. S3** Global proteomic results.

**Fig. S4** Global proteomic gene ontology (GO) analysis for proteins more abundant in *Chlamydomonas vip1-1* and wild-type.

**Fig. S5** *Chlamydomonas* wild-type phosphoproteomic gene ontology (GO) analysis.

**Fig. S6** Imaging-PAM analysis in *Chlamydomonas* WT and *vip1-1* comparing low (LL) and high light (HL) conditions.

**Methods S1** Proteomic analysis additional information and references.

**Table S1** Global proteomic analysis of *Chlamydomonas* WT and *vip1-1* strains under control and rapamycin conditions.

**Table S2** Phosphoproteomic analysis of *Chlamydomonas* WT and *vip1-1* strains under control and rapamycin conditions.

**Table S3** Target of rapamycin (TOR) related targets found in phosphoproteomic analysis of *Chlamydomonas* WT and *vip1-1* strains under control and rapamycin conditions.

**Table S4** Photosynthesis-related targets found in global proteomic analysis of *Chlamydomonas* WT and *vip1-1* strains under control and rapamycin conditions.

**Table S5** Photosynthesis-related targets found in phosphoproteomic analysis of *Chlamydomonas* WT and *vip1-1* strains under control and rapamycin conditions.

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