Supplementary Materials for

Phototropin connects blue light perception to starch metabolism in green algae

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Methods

Algal Material

The strains used in this study included *Chlamydomonas phot* (defective in *PHOT*; gene ID: Cre03.g199000) and *phot-C1* (*phot* strain complemented with WT *PHOT* gene), as well as their background strain CC-125, which have been previously described [1]. Additionally, *Chlamydomonas acry* [2] (defective in animal-type cryptochrome, aka *aCRY*; gene ID: Cre06.g278251), *pcry* (defective in plant-type cryptochrome, aka *pCRY*; gene ID: Cre06.g295200), and *acrypcry* (defective in both animal-type and plant-type cryptochrome) were generated through CRISPR-CAS9 provided by following the protocol described in [2]. The *pmsk1* (defective in phototropin-mediated signaling kinase 1, aka *PMSK1;* gene ID: Cre16.g659400) *and photpmsk1* (defective in both *PHOT* and *PMSK1*) mutants were generated using insertional CRISPR-Cas9 RNP method described by Kim et al. [3] with a few modifications. The target sgRNA sequence of *PMSK1* was designed by Cas-Designer (http://www.rgenome.net/cas-designer) and selected considering the recommendation guideline. To induce early termination of translation, the sgRNA targets were selected in exon 2 (Supplementary Data 1). To form an RNP complex *in vitro*, 100 µg of purified Cas9 protein (Cas9 expression plasmid: Plasmid #62934, addgene, US) and 70 µg of sgRNA synthesized by using GeneArt™ Precision gRNA Synthesis Kit (ThermoFisher, US), were mixed gently. For efficient and fast screening, 0.5 µg of paromomycin-resistance gene cassette was co-transformed with RNP complex. The *Chlamydomonas* cell wall was permeabilized by treatment of Max Efficiency buffer (ThermoFisher, US) following the manufacturer's protocol. The *Chlamydomonas* transformation was performed in the 4 mm gap electroporating cuvette by electroporation with the specific parameter (600 V, 50 µF, 200 Ω). One day after transformation, cells were plated on TAP medium containing 1.5% agar and paromomycin (25 µg/ml). Once colonies appear after transformation, genomic DNA PCR and Sanger sequencing were performed to validate knockout events.

To prepare transgenic lines with a knockdown of GAP1, pChlamiRNA3int-GAP1 was transformed into the *phot* strain. Generation of amiRNA plasmids was performed according to [4]. The oligonucleotides designed for targeting GAP1 (Data S1) using the WEB MicroRNA Designer platform (WMD3: http://wmd3.weigelworld.org/cgi-bin/webapp.cgi. Ossowski Stephan, Fitz Joffrey, Schwab Rebecca, Riester Markus and Weigel Detlef, personal communication) were annealed and ligated into pChlamiRNA3int (SpeI digested) to create pChlamiRNA3int-gap1. Transformed cells were selected and further checked by RT-qPCR.

For the preparation of transgenic lines overexpressing GAP1(Glyceraldehyde 3-phosphate dehydrogenase, aka *GAP1;* gene ID: Cre12.g485150) or different versions of PMSK1, the genomic sequences of *GAP1* and *PMSK1* were PCR amplified from genomic DNA of *Chlamydomonas* CC-125 and cloned into pLM005 in-frame with a C-terminal Venus-3Xflag using Gibson Assembly [5], and then transformed into WT or *phot* strains. The primers used for different gene amplification and point mutations are described in Data S1.

Chlamydomonas reinhardtii cultivation

All *Chlamydomonas* strains were maintained on solid Tris-acetate-phosphate (TAP) [6] agar plates with or without appropriate antibiotic at 22℃ and 5 µmol photons m^{-2} s^{-1}. Prior to the start of the experiments, cells were cultured in 50 mL TAP medium in 250 ml Erlenmeyer flasks at 23 ℃, 120 rpm/min and 15 µmol photons m^{-2} s^{-1}. The experiments were conducted in Sueoka's high salt medium (HSM) [7] at an initial cell density of 1 million cells/ml at 50 µmol photons m^{-2} s^{-1} unless otherwise stated.

For continuous light experiments, the cells were transferred to HSM medium and grew at 23 ℃, 120 rpm/min and 50 μmol photons m^{-2} s^{-1}.

For synchronized experiments, cells were grown in HSM for at least 5 days under a 12h light/12h dark cycle under white light or different light qualities (light intensity was set at 50 μmol photons m^{-2} s^{-1}; temperature was 18℃ in the dark and 23℃ in the light). The light spectrum of the LED lighting system used in this study is the same as previously described [8].

Transformation of *Chlamydomonas reinhardtii*

The transformation was performed by electroporation, which follows the protocol of Zhang et al [9] with minor modification. Cells for transformation were collected at 1-2 h before the end of the light phase in a synchronized (12 h light/ 12h dark) culture. For three reactions 11 ng/kb linearized plasmid was mixed with 400 μl of 1.0 x10^7 *Chlamydomonas reinhardtii* cells/ml and electroporated at a volume of 125 ml in a 2-mm-gap electro cuvette using a NEPA21 square-pulse electroporator, using two poring pulses of 250 and 150 V for 8 msec each, and five transfer pulses of 50 msec each starting at 20 V with a "decay rate" of 40% (i.e., successive pulses of 20, 12, 7.2, 4.3, and 2.6 V). Electroporated cells were immediately transferred to a 15ml centrifugation tube containing 9 ml TAP plus 40mM sucrose. After overnight dark incubation, cells were collected by centrifugation and spread on TAP agar plates which contain the appropriate antibiotic (20 μg/ml paromomycin or 7.5 μg/ml zeocin or 20 μg/ml hygromycin B). Transformants typically appear after 5-7 days.

The putative antibiotic-resistant transformants were transferred into individual wells of a 96-well, flat-bottom transparent microplate, with each well containing 250 μl of TAP medium. Cultures were grown for 3 days under 15 μmol photons m^{-2} s^{-1} light without shaking, refreshed by replacing half of the culture with fresh medium, and allowed to grow for an additional day. Transformants were screened for Venus expression using a fluorescent microplate reader (Tecan Group Ltd., Switzerland), with parameters including Venus (excitation 515/12 nm and emission 550/12 nm) and chlorophyll (excitation 440/9 nm and emission 680/20 nm). The fluorescence signal was normalized to the chlorophyll fluorescence signal, and colonies with a high Venus/chlorophyll value were selected as putative complemented strains. These putative positive transformants were further validated by western blotting and RT-qPCR.

RNA extractions and RT-qPCR analysis

Total RNA for RNA-seq and RT-qPCR was extracted using RNeasy Mini Kit (Qiagen, Germany) and treated with the RNase-Free DNase Set (Qiagen, Germany). 1 μg total RNA was reverse transcribed with oligo dT using Sensifast cDNA Synthesis kit (Meridian Bioscience, US). qPCR reactions were performed and quantitated in a Bio-Rad CFX96 system using SsoAdvanced Universal SYBR Green Supermix (BioRad, US). The *CBLP* gene [10] served as the housekeeping control and relative fold differences were calculated on the basis of the ΔC_t method ($2^{-(Ct\ target\ gene\ -\ Ct\ CBPL)}$) [11–13]. All primers used for the RT-qPCR analyses were synthesized by ThermoFisher (US) or IDT (Integrated DNA Technologies, Inc. Coralville, Iowa, US) and were presented in Data S1.

Analyses of Total Starch content

The total starch content of samples collected daily was determined using Total Starch Assay Kit (K-TSTA-100A, Megazyme, Ireland) as described in its instruction with modifications. The results were calculated according to the standard curve made with glucose solution after starch digestion. To prepare the samples for glucose determination, 10 mL of the liquid culture was pelleted by centrifugation and resuspended in 40 μL of 80% (v/v) ethanol. Next, 400 μL of cold

1.7 M sodium hydroxide solution was added, and the samples were incubated on ice for 15 min. Following this, 1.6 mL of sodium acetate buffer containing calcium chloride (5 mM) was added and mixed well. Subsequently, 20 µL of α-amylase and 20 µL of amyloglucosidase were added, and the samples were incubated at 50 ℃ for 30 min. The supernatant was collected by centrifugation at 13,000 rpm for 5 min and will be ready for glucose determination.

Analyses of Total Protein and Lipid content

The total protein content of samples collected daily was determined using BCA Protein Assay Kit (ThermoFisher, US) with the standardized protocol. The total lipid content of samples collected daily was determined using sulfo-phospho-vanillin (SPV) method [14].

Transmission electron microscopy

Cells were harvested by centrifugation at 700g for 5 min, washed two times in 0.1 M PB (phosphate buffer, pH 7.4) and then were fixed in 0.1 M PB containing 2.5% (v/v) glutaraldehyde for 2h at room temperature and stored overnight at 4 ℃. The cells were then washed five times in 0.1 M PB before being fixed by a 1h incubation on ice in 0.1 M PB containing 2% osmium and 1.5% ferricyanide potassium. After being washed five times with 0.1 M PB , the samples were resuspended in 0.1 M PB containing 0.1% (v/v) tannic acid and incubated for 30 min in the dark at room temperature. The cells were washed five times with 0.1 M PB , dehydrated in ascending sequences of ethanol, infiltrated with an ethanol/Epon resin mixture, and finally embedded in Epon. Ultrathin sections (50–70 nm) were prepared with a diamond knife on a PowerTome ultramicrotome (RMC Boeckeler, US) and collected on 200 µm nickel grids. The ultrathin sections were examined on a Philips CM120 transmission electron microscope operating at 80 kV.

Confocal Microscopy

The preparation of samples for confocal microscopy followed the protocol reported by Mackinder et al [15]. The confocal microscope used in the study was from the cell-imaging platform at IBS, Grenoble, France. All confocal microscopy images were analyzed using Fiji.[16]

Immunoblotting

Protein samples of whole cell extracts (5 µg protein) were loaded on 4-20% SDS-PAGE gels (Mini-PROTEAN TGX Precast Protein Gels, Bio-Rad, US) and blotted onto nitrocellulose membranes. Antisera against ATPB (AS05085, 1:15000) was from Agrisera (Sweden); antiserum against FLAG (F3165, 1:2000) was from Sigma-Aldrich (US); antiserum PHOT (LOV1 domain, 1:5000) was previously described[10]. ATPB was used as a loading control. The anti-rabbit or mouse horseradish peroxidase–conjugated antiserum (Jackson Immuno Research, US) was used for detection at 1:10000 dilution. The blots were developed with ECL detection reagent, and images of the blots were obtained using ImageQuant 800 (Cytiva, UK). For the densitometric quantification, data were normalized with ATPB.

Phos-Tag Gel Electrophoresis

Double-layer Phos-tag gels with a concentration of 12% (w/v) acrylamide/bisacrylamide 37.5:1 and 65 mM of Phos-Tag (Wako Pure, US) were prepared as in [17], with the exception that $Zn(NO_3)^2$ was added equimolarly to the samples to compensate for the absence of EDTA in the lysis buffer. The gels were denatured for 30 min at 37°C prior to loading. In vitro dephosphorylation involved resuspending a cell pellet in 5 mM of HEPES at pH 7.5, 10 mM of EDTA, and 1% (v/v) TritonX 100. An aliquot containing 10 mg of protein was then subjected to

lambda protein phosphatase reaction mix following the manufacturer's instructions (New England Biolabs, US) for 1 hour at 30°C, in accordance to [18].

Fluorescence-based measurements

Fluorescence-based photosynthetic parameters were measured with a pulse modulated amplitude fluorimeter (MAXI-IMAGING-PAM, HeinzWaltz GmbH, Germany). Prior to the onset of the measurements, cells were acclimated to darkness for 15 min. Chlorophyll fluorescence was recorded under different intensities of actinic light; starting with measurements in the dark (indicated as D below the x-axis of the graphs), followed by measurements at 21 µmol photons m^{-2} s^{-1} (indicated as L1 below the x-axis of the graphs) and 336 µmol photons m^{-2} s^{-1} (indicated as L2 below the x-axis of the graphs) and finishing with measurements of fluorescence relaxation in the dark. The effective photochemical quantum yield of photosystem II was calculated as Y(II) = (Fm'-F)/Fm'; *F* and *F*m′ are the fluorescence yield in steady state light and after a saturating pulse in the actinic light, respectively;

Phosphoproteomics analysis

Protein extraction. C. reinhardtii pellets were resuspended in 2000 µL of lysis buffer (100 mM Tris-HCl, PhosphoSTOP inhibitors, protease inhibitors) and ultrasonicated in the Covaris for 4 min each. Samples were diluted by adding 2000 µL of dilution buffer (100 mM Tris-HCl, 5 mM TCEP, 30 mM chloroacetamide, 1 mM sodium orthovanadate, phosphoSTOP inhibitors, 1 mM magnesium chloride) and 1 µL Benzonase. Lysates were shaken at 25 °C for 1 h. 8 mL of methanol was added to each sample, followed by 3 mL chloroform and 3 mL water, with vortexing after each subsequent addition. Samples were centrifuged for 10 min at 3,220 x g and the top layer was removed, leaving the interphase intact. An additional 10 mL of methanol was added and the samples were centrifuged for 20 min at 3,220 x g and the supernatant was removed. Protein pellets were allowed to dry at RT and resuspended in 1 mL of digestion buffer (100 mM Tris-HCl, 2 M urea). Proteins were digested with 12 µg of trypsin overnight and cleaned up via C18 SPE cartridges. Samples were resuspended in 250 µL of water and a BCA assay was performed to determine peptide concentration. 10 µg of digested protein was taken for global analysis, and 500 µg was used for phosphopeptide enrichment.

Phosphopeptide enrichment. Phosphopeptides were enriched using a ProPac Fe-IMAC column (ThermoFisher, US) on a Shimadzu Prominence HPLC system. Before enrichment, the column was charged with 25 mM $FeCl_3$ in 100mM acetic acid. Mobile phase A consisted of 30% acetonitrile in water (v/v) with 0.07% trifluoroacetic acid (v/v). Mobile phase B consisted of 0.3% ammonium hydroxide in water (v/v). Tryptic peptides were diluted to 30% acetonitrile and injected on the column at a flow rate of 0.2 ml/min. After 3 min of loading, flow rate was increased to 2 mL/min. Peptides were eluted by rapidly ramping the gradient to 50% B. Fractions containing phosphopeptides were cleaned up with C18 SPE cartridges (Waters, US) and resuspended in 20 µL of LC-MS water prior to mass spectrometry analysis.

LC-MS analysis. Phosphopeptide samples were analyzed using a nanoACQUITY UPLC (Waters, US) coupled to a TripleTOF 5600 mass spectrometer (Sciex, Canada). Mobile phase A consisted of water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Injections were made to a Symmetry C18 trap column (100 Å, 5 µm, 180 µm x 20 mm; Waters, US) with a flow rate of 5 µL/min for 3 min using 99% A and 1% B. Peptides were then separated on an HSS T3 C18 column (100 Å, 1.8 µm, 75 µm x 250 mm; Waters, US) using a linear gradient of increasing mobile phase B at a flow rate of 300 nL/min. Mobile phase B increased

from 5% to 40% in 90 min before ramping to 85% in 5 min, where it was held for 5 min before returning to 5% in 2 min and re-equilibrating for 13 min.

The mass spectrometer was operated in positive polarity mode. MS survey scans were accumulated across an *m/z* range of 350-1600 in 250 ms optimized at ≥ 30,000 resolution. For data-dependent acquisition, the mass spectrometer was set to automatically switch between MS and MS/MS experiments for the first 20 features above 150 counts having +2 to +5 charge state. Precursor ions were fragmented using rolling collision energy and accumulated in high sensitivity mode for 85 ms across an *m/z* range of 100-1800 optimized at ≥ 30,000 resolution. Dynamic exclusion for precursor m/z was set to 8 s.

Bioinformatic analysis. Raw data files were imported into Progenesis for peak alignment and quantification. Spectra were searched in Mascot against the *C. reinhardtii* phytozome database (v6.1) using a precursor / fragment tolerance of 15 ppm / 0.1 Da, trypsin specificity, two possible missed cleavages, fixed modification cysteine carbamidomethylation, and variable modifications of methionine oxidation, protein N-term acetylation, and phosphorylation (STY). Identifications were imported back into Progenesis for peak assignment, and statistical analysis was performed using the QuantifyR workflow, which can be found on the Hicks Lab Github (github.com/hickslab/QuantifyR). Phosphoproteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [19] with the dataset identifier PXD045599.

Lipidomics

Glycerolipids were extracted from freeze-dried cell pellets frozen immediately in liquid nitrogen after harvesting. Once freeze-dried, cell pellets were resuspended in 4 mL of boiling ethanol for 5 minutes to prevent lipid degradation and lipids were extracted according to [20] by addition of 2 mL methanol and 8 mL chloroform at room temperature. The mixture was then saturated with argon and stirred for 1 hour at room temperature. After filtration through glass wool, cell remains were rinsed with 3 mL chloroform/methanol 2:1, v/v and 5 mL of NaCl 1% were then added to the filtrate to initiate biphase formation. The chloroform phase was dried under argon before solubilizing the lipid extract in pure chloroform. Total glycerolipids were quantified from their fatty acids: in an aliquot fraction, a known quantity of 15:0 was added and the fatty acids present were transformed as methyl esters (FAME) by a 1-hour incubation in 3 mL 2.5% H_2SO_4 in pure methanol at 100°C [21]. The reaction was stopped by addition of 3 mL water and 3 mL hexane. The hexane phase was analyzed by gas chromatography-flame ionization detector (GC-FID) (Perkin Elmer, US) on a BPX70 (SGE; Trajan Scientific and Medical location, Australia) column. FAME were identified by comparison of their retention times with those of standards (Sigma, US) and quantified by the surface peak method using 15:0 for calibration.

The lipid extracts corresponding to 25 nmol of total fatty acids were dissolved in 100 µL of chloroform/methanol [2/1, (v/v)] containing 125 pmol of each internal standard. Internal standards used were PE 18:0-18:0 and DAG 18:0-22:6 from Avanti Polar Lipid and SQDG 16:0-18:0 extracted from spinach thylakoid [22] and hydrogenated as described in [23]. Lipids were then separated by HPLC and quantified by MS/MS.

The HPLC separation method was adapted from [24]. Lipid classes were separated using an Agilent 1200 HPLC system using a 150 mm×3 mm (length × internal diameter) 5 µm diol column (Macherey-Nagel, Germany), at 40°C. The mobile phases consisted of hexane/isopropanol/water/ammonium acetate 1M, pH5.3 [625/350/24/1, (v/v/v/v)] (A) and isopropanol/water/ammonium acetate 1M, pH5.3 [850/149/1, (v/v/v)] (B). The injection volume was 20 µL. After 5 min, the percentage of B was increased linearly from 0% to 100% in 30 min

and stayed at 100% for 15 min. This elution sequence was followed by a return to 100% A in 5 min and an equilibration for 20 min with 100% A before the next injection, leading to a total runtime of 70 min. The flow rate of the mobile phase was 200 µL/min. The distinct glycerophospholipid classes were eluted successively as a function of the polar head group.

Mass spectrometric analysis was done on a 6470 triple quadrupole mass spectrometer (Agilent, US) equipped with a Jet stream electrospray ion source under following settings: Drying gas heater: 230°C, Drying gas flow 10 L/min, Sheath gas heater: 200°C, Sheath gas flow: 10L/min, Nebulizer pressure: 25 psi, Capillary voltage: ± 4000 V, Nozzle voltage ± 2000. Nitrogen was used as collision gas. The quadrupoles Q1 and Q3 were operated at widest and unit resolution respectively. DGTS analysis was carried out in positive ion mode by scanning for precursors of m/z 236 at a collision energy (CE) of 55 eV. SQDG analysis was carried out in negative ion mode by scanning for precursors of m/z -225 at a CE of -55eV. PE, PI, PG, MGDG and DGDG measurements were performed in positive ion mode by scanning for neutral losses of 141 Da, 277 Da, 189 Da, 179 Da and 341 Da at CEs of 29 eV, 21 eV, 25 eV, 8 eV and 11 eV, respectively. Quantification was done by multiple reaction monitoring (MRM) with 30 ms dwell time. DAG and TAG species were identified and quantified by MRM as singly charged ions $[M+NH_4]^+$ at a CE of 19 and 26 eV respectively with 30 ms dwell time. Mass spectra were processed by MassHunter Workstation software (Agilent, US) for identification and quantification of lipids. Lipid amounts (pmol) were corrected for response differences between internal standards and endogenous lipids and by comparison with a quality control (QC). QC extract correspond to a known lipid extract from *Chlamydomonas* cell culture qualified and quantified by TLC and GC-FID as described in [25].

Proteomics analysis

Proteins from total extracts of three biological replicates of WT and *phot Chlamydomonas* reinhardtii were solubilized in Laemmli buffer and heated for 10 min at 95°C. They were then stacked in the top of a 4-12% NuPAGE gel (ThermoFisher, US), stained with Coomassie blue R-250 (Bio-Rad, US) before in-gel digestion using modified trypsin (Promega, US) as previously described [26]. The resulting peptides were analyzed by online nanoliquid chromatography coupled to MS/MS (Ultimate 3000 RSLCnano and Q-Exactive HF, ThermoFisher, US) using a 180-min gradient. For this purpose, the peptides were sampled on a precolumn (300 µm x 5 mm PepMap C18, ThermoFisher, US) and separated in a 75 µm x 250 mm C18 column (Reprosil-Pur 120 C18-AQ, 1.9 µm, Dr. Maisch, Germany). The MS and MS/MS data were acquired using Xcalibur (V2.8, ThermoFisher, US).

Peptides and proteins were identified by Mascot (V2.8.0, Matrix Science) through concomitant searches against the *C. reinhardtii* phytozome database (V5.6) (19526 sequences), the mitochondrion and chloroplast protein sequences (downloaded from NCBI, respectively 69 and 8 proteins), and a homemade database containing the sequences of classical contaminant proteins found in proteomic analyses (human keratins, trypsin..., 126 sequences). Trypsin/P was chosen as the enzyme and two missed cleavages were allowed. Precursor and fragment mass error tolerances were set at respectively at 10 and 20 ppm. Peptide modifications allowed during the search were: Carbamidomethyl (C, fixed), Acetyl (Protein N-term, variable) and Oxidation (M, variable). The Proline software [27] (V2.2.0) was used for the compilation, grouping, and filtering of the results (conservation of rank 1 peptides, peptide length ≥ 6 amino acids, false discovery rate of peptide-spectrum-match identifications < 1% [28], and minimum of one specific peptide per identified protein group). MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [29]with the dataset identifier PXD046943. Proline was then used to

perform a MS1 label-free quantification of the identified protein groups based on razor and specific peptides.

Statistical analysis was performed using the ProStaR software [30] based on the quantitative data obtained with the three biological replicates analyzed per condition. Proteins identified in the contaminant database, proteins identified by MS/MS in less than two replicates of one condition, and proteins quantified in less than three replicates of one condition were discarded. After log2 transformation, abundance values were normalized using the variance stabilizing normalization (vsn) method, before missing value imputation (SLSA algorithm for partially observed values in the condition and DetQuantile algorithm for totally absent values in the condition). Statistical testing was conducted with limma, whereby differentially expressed proteins were selected using a log2(Fold Change) cut-off of 1 and a p-value cut-off of 0.00912, allowing to reach a false discovery rate inferior to 1% according to the Benjamini-Hochberg estimator. Proteins found differentially abundant but identified by MS/MS in less than two replicates, and detected in less than four replicates, in the condition in which they were found to be more abundant were invalidated (p-value = 1).

GO enrichment analysis

GO term enrichment was tested for all proteins significantly differential abundant at a false discovery rate below 5% using the Benjamini-Hochberg estimator [31]. GO annotation of proteins was obtained from phytozome database (v.5.6) and all ancestral GO terms were added to a protein using the R package GO.db. P-values were obtained according to the null hypothesis, that the number of differential abundance proteins bearing a GO term is a random variable whose probability distribution is described by the hypergeometric distribution. The false discovery rate was controlled below 5% using the Benjamini-Hochberg-estimator [31]. Only GO terms linked to at least 6 measured proteins were tested.

Statistical analysis

Prism (GraphPad Software) was used for statistical analysis and all error bars represent standard deviation. ANOVA tests and t-tests were performed, with the p-values or degree of significance provided in the figures and the legends.

Supplementary Text

Phylogenetic analysis

PMSK1 is found to belong to a family of Serine/threonine-protein kinases (named the PMSK-like family, see material and methods) which is conserved in green algae and vascular plants (**Supplementary Figs. 21 and 22**). In particular, the PMSK-like family is consistent with the known Chlorophyta evolution [32]. Interestingly, when searching for PMSK1 similar sequences into the *Arabidopsis thaliana* genome in the NCBI database [33], next to the PMSK-like *Arabidopsis* members come the *Arabidopsis* HT1 (AT1G62400, NP_176430.2), CBC1 (AT3G01490, NP_186798.1) and CBC2 (AT5G50000, NP_199811.1). It has been found that (i) the best reciprocal best hit for these three proteins when searching for *Chlamydomonas reinhardtii* was PMSK-like family members, especially PMSK1 (ii) this was true when searching for any Chlorophyta species and that (iii) regarding into the three family CBC, HT and PMSK-like, Chlorophyta was only present in the latest (**Supplementary Fig. 23**). A simple molecular clock analysis phylogenetic analysis revealed that the root is likely to be placed between the CBC and the HT/PMSK1 family (**Supplementary Fig. 24**) suggesting that a representative for these three families could have been present in the common ancestor of the Chlorophyta. One explanation for the observed distribution of the sequences is that both HT and CBC representative have been lost before the radiation of the Chlorophyta. It must be noticed that most of the time, duplication in CBC, HT and PMSK-like family seems to have happened after recent speciation events, suggesting that a few members of this family could have been present in the last common ancestor of the Chlorophyta/Charophyta/Land Plants Phylum.

Identification of the PMSK-like family

The gene product of Cre16.g659400, identified as encoding a Ser/Thr protein kinase (**Fig. 3b**) and named kinase Phototropin-mediated signalling kinase 1 (PMSK1), was translated and the amino acid sequence used as a query for a series of BLAST searches in the publicly available National Center for Biotechnology Information (NCBI) database [33]. Both BLASTp and PSI-BLAST [34,35] were performed together with a human-curated process in order to obtain the widest dataset possible and the most robust one. The final dataset (#1) contained 111 amino acid sequences from at least 10 orders of Chlorophyta, Charophyta, Bryopsida, Pteridophyta, Gymnospermae and Angiosperm.

Identification of a relation between the PMSK-like family and both the HT1 and the CBC family

Because both HT1, CBC1 and CBC2 were identified in the BLAST research as first remotely related *Arabidopsis thaliana* sequences in the previous homologous sequences search, a reciprocal best hit research was done using these three sequences together with a human-curated process and results was append to the previous dataset. This new dataset (#2) contained 278 amino acid sequences from at least 10 orders of Chlorophyta, Charophyta, Bryopsida, Pteridophyta, Gymnospermae and Angiosperm. Interestingly, homologous sequences investigations revealed that while the four family Charophyta, Bryopsida, Pteridophyta, Gymnospermae are present in the both the HT and the CDC family, the most closely related Chlorophyta sequences of the CBC/HT *Arabidopsis* thaliana representative were actually the PMSK-like family sequences. A subset of dataset #2 containing 11 sequences was used to compute both a clock-model and a relaxed-clock model to infer the possible root of the three PMSK-like, HT and CBC family.

Multiple Sequence Alignments

Amino acid multiple sequence alignments for each protein segment were created using MUSCLE [36] and then adjusted using Gblocks (version 0.91.1) [37] or the MEGA ML analysis (see below) or manually using Jalview (version 2.11.1.4) [38] for both the PhyML and the Bayesian analysis (see below).

Dataset #1 and #3. Bayesian Inferences

Two independent Metropolis-coupled Markov Chain Monte Carlo (MCMC) analyses were performed using the final curated datasets in the MrBayes (version 3.2.7.a) software [39]. The two datasets were used for Bayesian inference. The first was include in a nexus file resulting from the MUSCLE alignment. The dataset #3 was include in the 278 sequences multiple alignment nexus file from the dataset #2 from which, following a command line in the MrBayes software, it was extracted before setting the algorithm parameters. The Bayesian posterior probabilities (BPP) were estimated by two independent runs of four Metropolis Coupled chains (MCMC): 2 independent runs, together with one cold chain and three "heated" (temperature parameter 0.1, Dataset 3) chains drove the analyses. The analyses were allowed to switch among all the substitution models implemented in the software to identify the best model without any a priori (aamodelpr = mixed). The prior on the branch length was set to unconstrained for the dataset #1, leading to an unrooted tree whereas it was set on both a uniform and a relaxed clock model to infer the root position of the HT, CBC and PMSK-like families.
80,000 and 160,000 generations with sampling every 100 generations were set for Dataset #1 and Dataset #3, respectively. The standard deviation of the split frequency between the two parallel analyses at the end of each analysis were 0.021973 and 0.005348 for Dataset #1 and Dataset #3, respectively. The first 25% of the trees produced were discarded in order to let the analyses stabilize (burnin = 0.25). The convergence of the runs was estimated and the potential scale reduction factor (PSRF +) were all around 1 for all the parameters indicating that the associated chains converged to one target distribution. Large PSRF (PSRF + >1) indicate convergence failure. Nodes with a posterior probability $\geq$0.90 were considered as well supported. Alignments in fasta and trees in newick format are available upon request.

Dataset #2. Maximum Likelihood inferences

Unrooted maximum likelihood (ML) phylogenetic trees were then inferred using both the MEGA (version 11) software package Whelan And Goldman ML model [40] and PhyML (version 3.3.1) [41] based on the best-fit models of amino acid substitution determined by Find Best Protein Model [38]. Non-uniformity of evolutionary rates among sites was modeled using a discrete Gamma distribution (+ G) and a certain fraction of sites were considered to be evolutionarily invariable (+ I). Initial trees for the heuristic search were obtained automatically by applying Neighbor Joining (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions with less than 95% site coverage were eliminated. Statistical tests for branch support for the MEGA ML algorithm was estimated with a Bootstrap procedure [42], using 100 replicates. Statistical tests for branch support concerning the PhyML algorithm was estimated with the aLRT SH-like branch support

method [43]. Trees were drawn to scale with the branch length measured in the number of substitutions per site.

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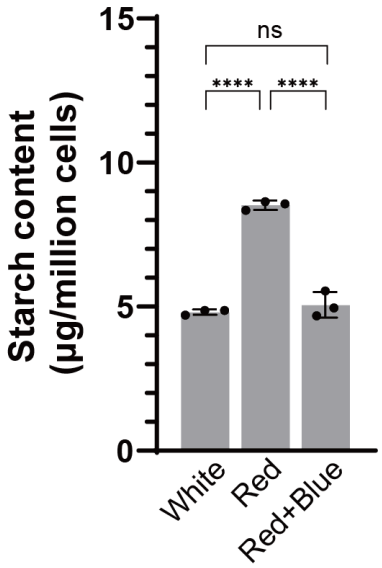
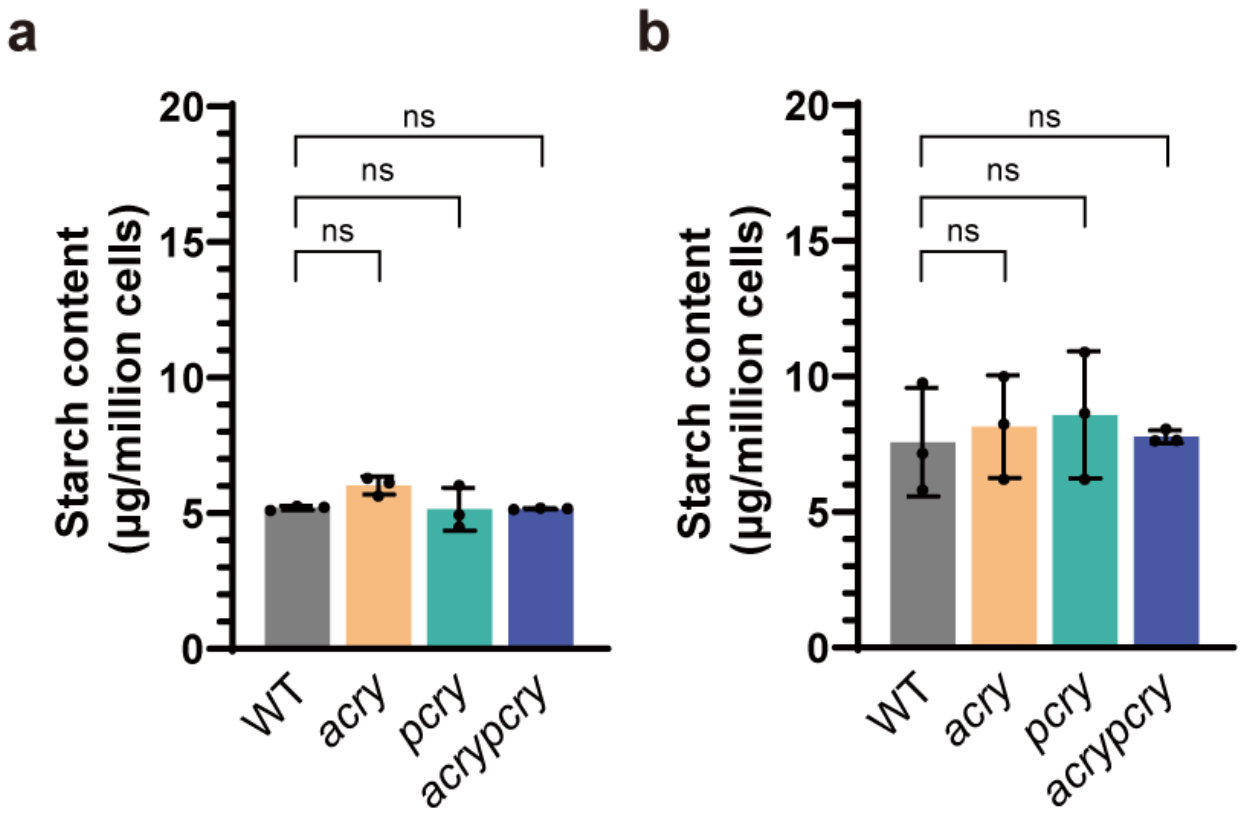
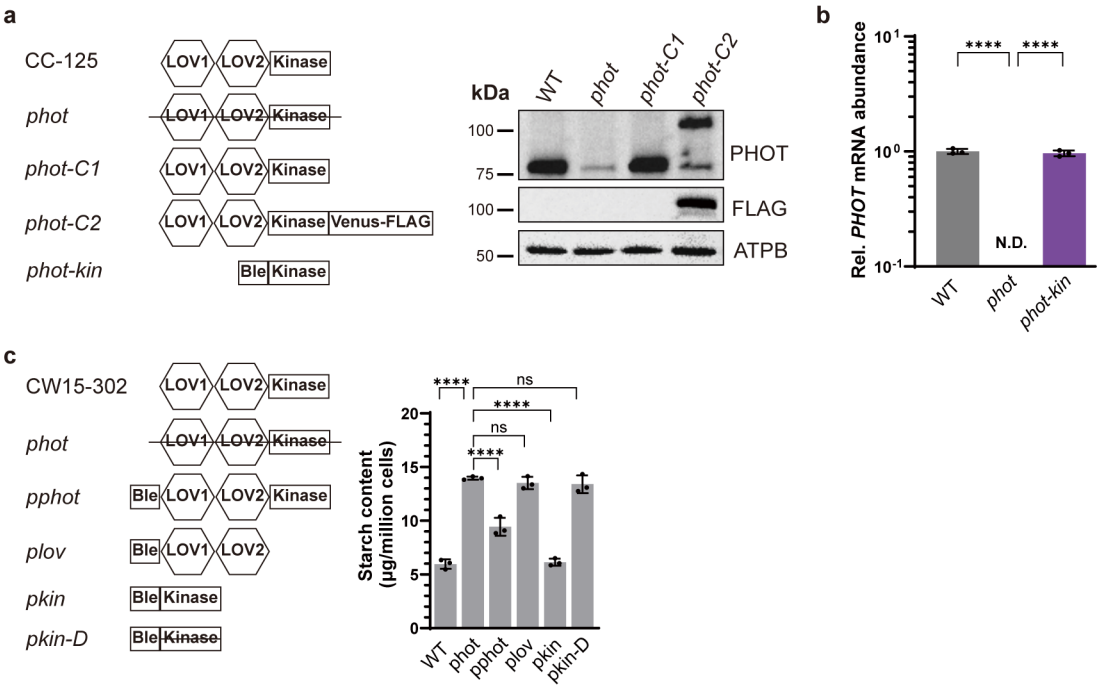


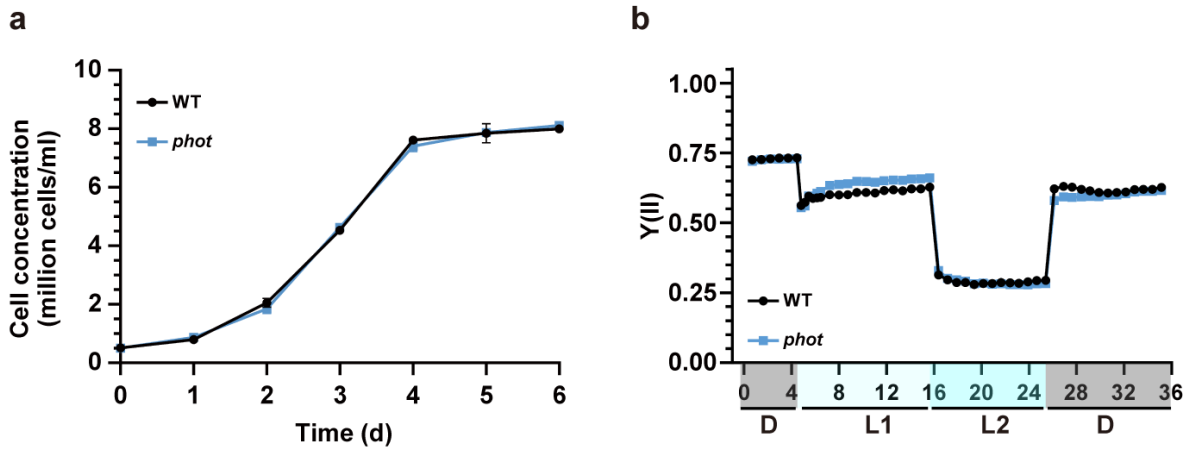
Supplementary Fig. 1. Effect of superimposing low intensity blue light on the red light on starch content. The starch content of WT under different continuous light conditions. White, 50 µE white light; Red, 50µE red light; Red+Blue, 45µE red light plus 5µE blue light. Data are represented as mean ± SD (n = 3 biologically independent samples). Asterisks indicated the p-values (****, $p < 0.0001$; ns, not significant)



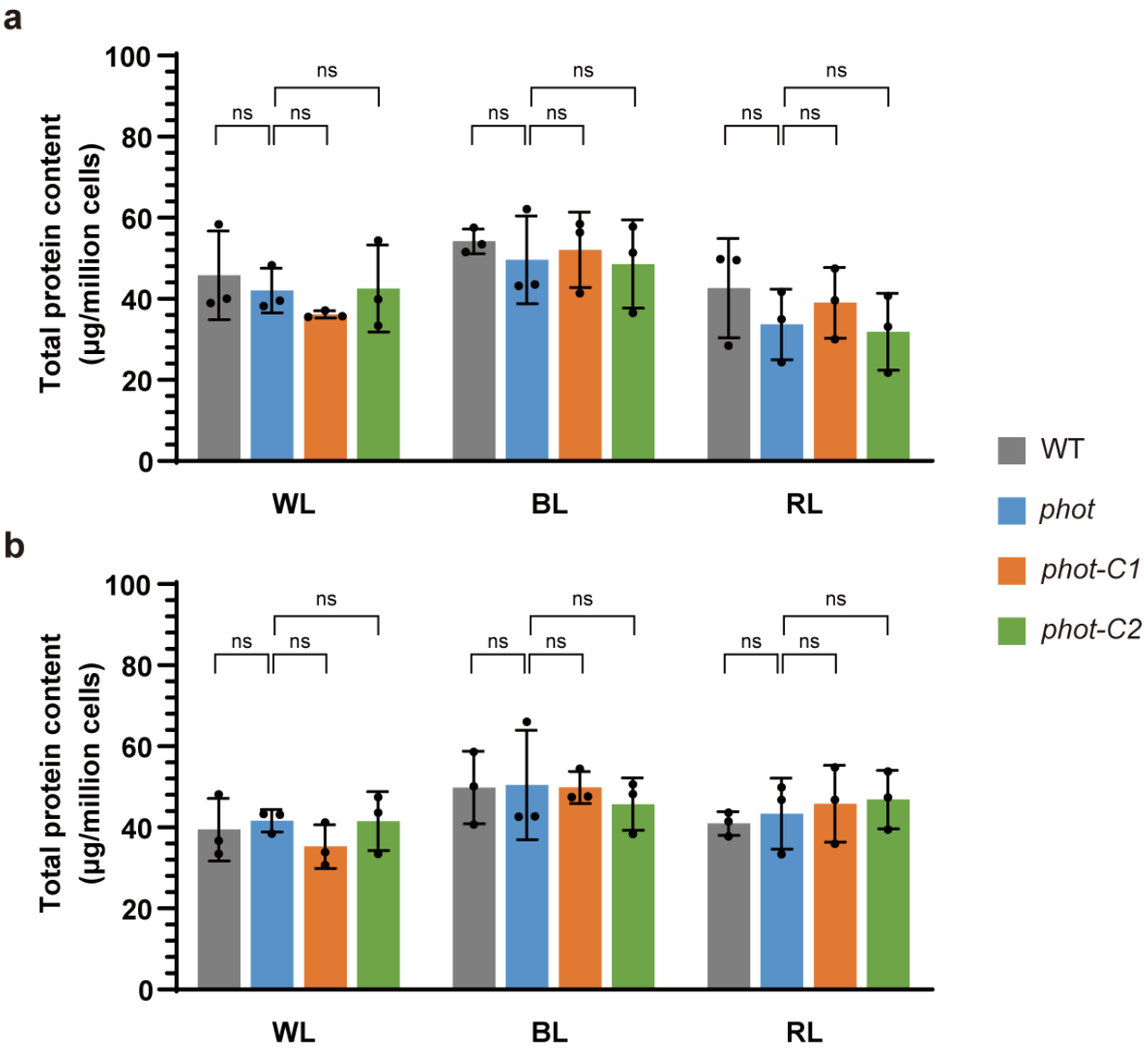
Supplementary Fig. 2. Mutants devoid of animal- or plant-like cryptochromes accumulate WT-levels starch. The starch content of *acry, pcry and acrypcry* synchronized to a 12 h/12 h light dark cycle (white light). Samples were collected at the start (**a**) and at the end of the light phase (**b**). Data are represented as mean ± SD (n = 3 biologically independent samples). ns, not significant.



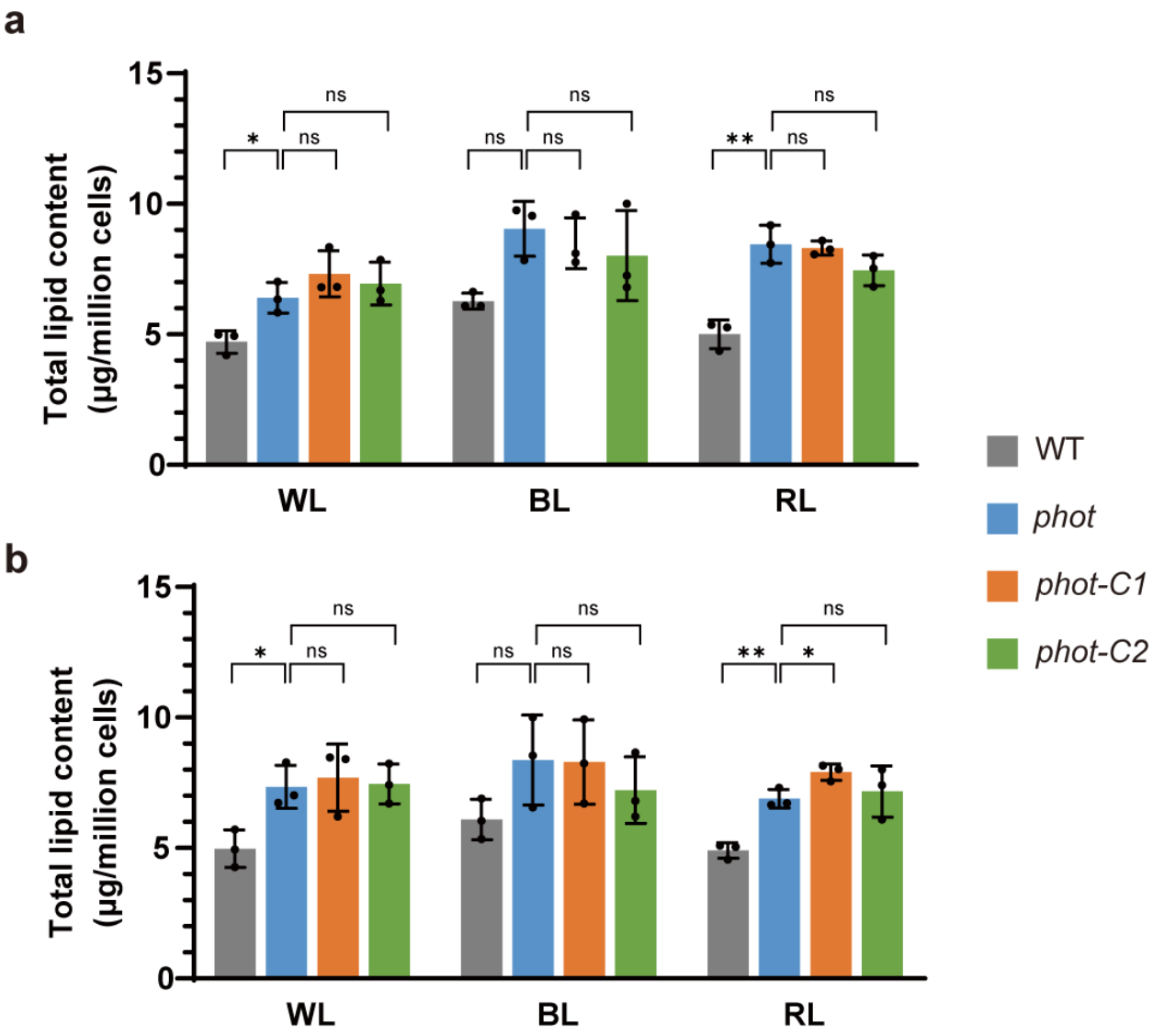
Supplementary Fig. 3. PHOT inhibits starch accumulation in *Chlamydomonas reinhardtii.* (**a**) Left: Domains of the *PHOT* gene in WT and complemented *phot* lines. LOV1 and LOV2, photosensory domains; Kinase, kinase domain; Venus-FLAG, yellow fluorescent protein with FLAG tag; Ble, gene conferring resistance to bleomycin. Right: Immunoblot analyses of PHOT level in WT (CC125), *phot* and complements strains. (**b**), RT-qPCR analysis of *PHOT-Kinase* transcription level in WT and *phot-kin*. N.D., not detected. (**c**), Left: Domains of the *PHOT* gene in WT (*cw15-302*) and complemented *phot* lines. Right: Starch content of WT, *phot* and various *phot*-complemented lines grown under continuous white light. Data are represented as mean ± SD (n = 3 biologically independent samples). Asterisks indicated the p-values (*, p<0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant).



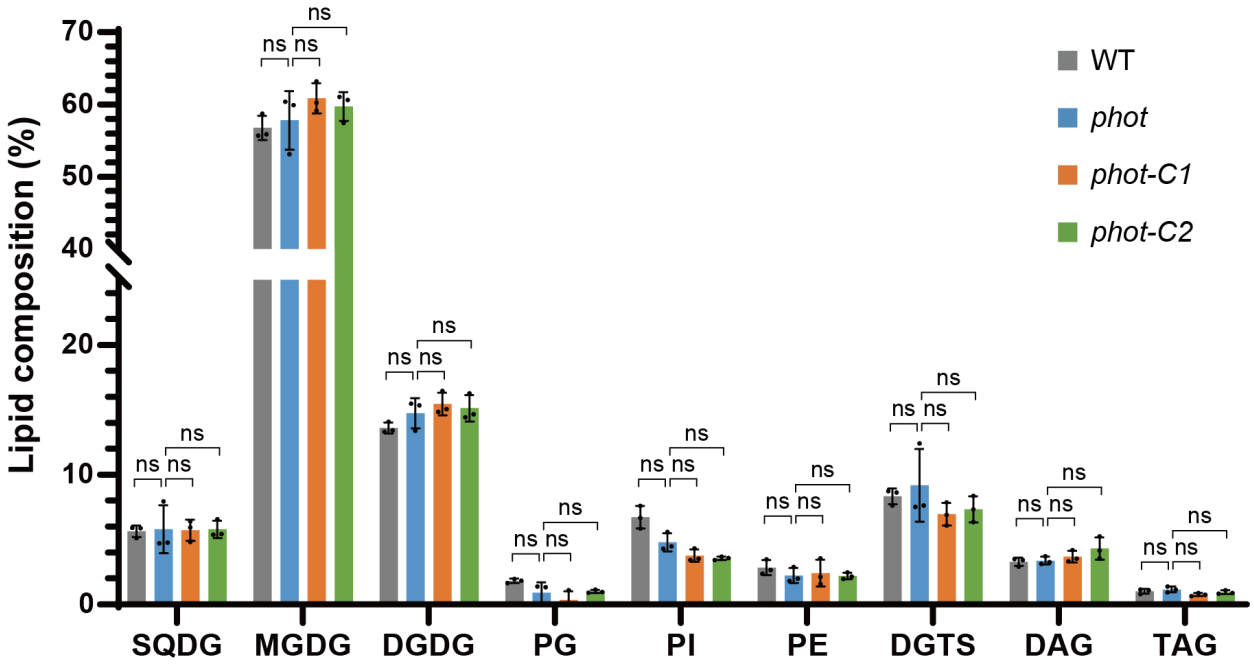
Supplementary Fig. 4. The *phot* mutant shows WT-like growth and photosynthetic efficiency. Growth curves (**a**) and photosystem II efficiency (YII) (**b**) of WT and *phot* grown under continuous white light. *In vivo* chlorophyll fluorescence was recorded in the dark (labelled as "D"), at 21 (labelled as "L1") and 336 (labelled as "L2") µmol photons m^{-2} s^{-1} as indicated in the graphs. Y(II) values calculated as (Fm'-F)/Fm' (n = 3 biological samples, mean ± SD). Please note that in some cases the error bars are smaller than the data point symbols.



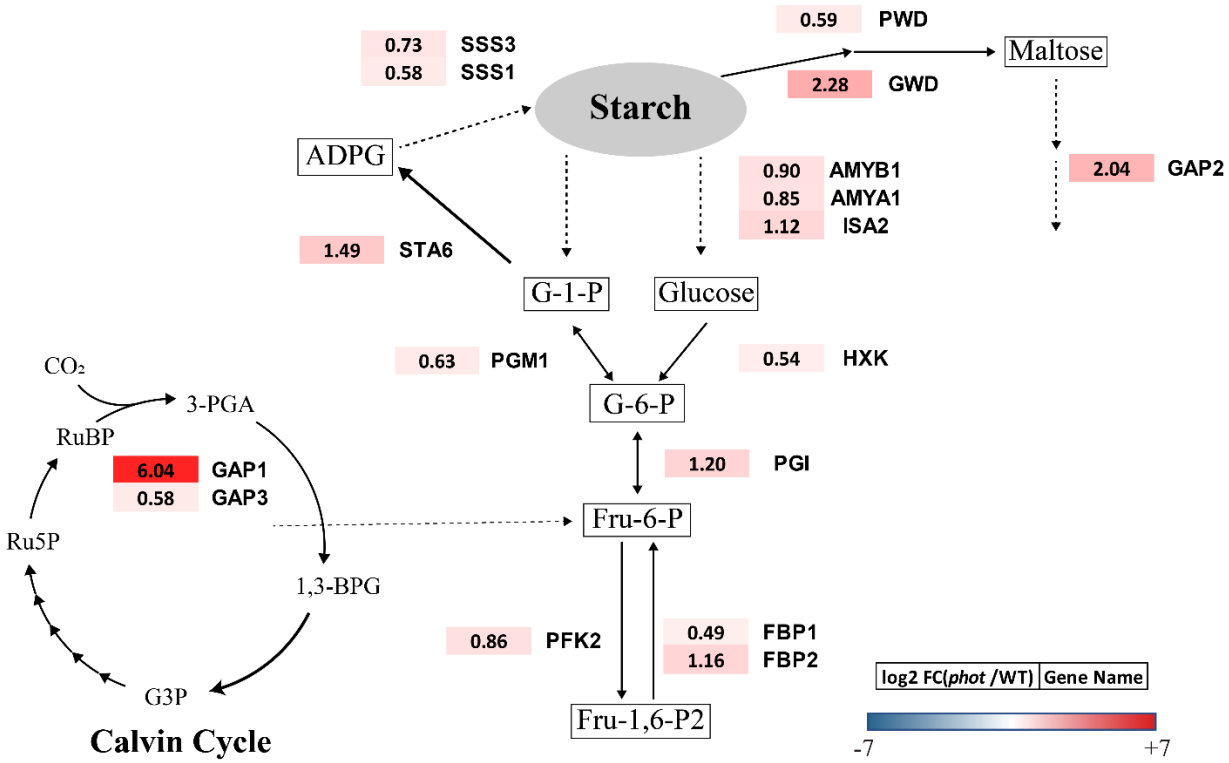
Supplementary Fig. 5. The *phot* mutant has WT-like total protein content. The total protein content of WT, *phot* and complemented *phot* lines synchronized to a 12 h/12 h light dark cycle under different light qualities (WL, white light; BL, blue light; RL, red light). Samples were collected at the start of the start (**a**) and at the end (**b**) of the light phase. Data are represented as mean ± SD (n = 3 biologically independent samples). ns, not significant.



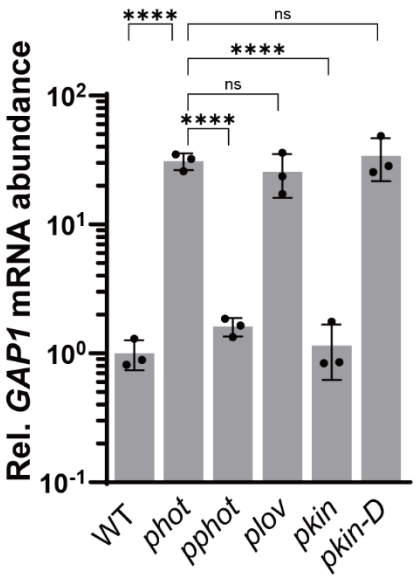
Supplementary Fig. 6. The *phot* mutant has WT-like total lipid content. The total lipid content of WT, *phot* and complemented *phot* lines synchronized to a 12 h/12 h light dark cycle under different light qualities (WL, white light; BL, blue light; RL, red light). Samples were collected at the start of the start (**a**) and at the end (**b**) of the light phase. Data are represented as mean ± SD (n = 3 biologically independent samples). Asterisks indicated the p-values (**, $p < 0.01$; ns, not significant).



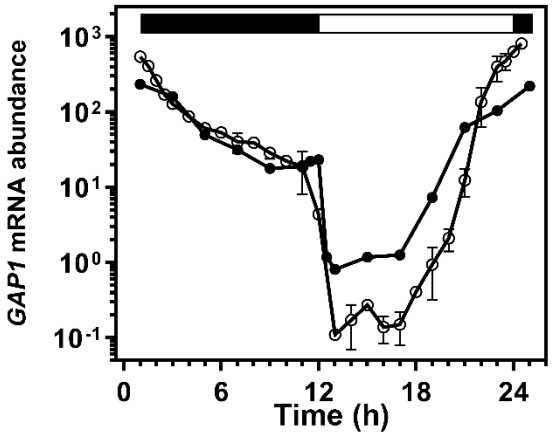
Supplementary Fig. 7. The *phot* mutant has WT-like lipid composition. The lipid composition of WT, *phot and* complemented *phot lines* under 12h/12h light dark conditions. Samples are collected at the end of the light phase. Data are represented as mean ± SD (n = 3 biologically independent samples). SQDG, sulfoquinovosyl diacylglycerol; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; PG, Phosphatidylgylcerols; PI, Phosphatidylinositols; PE, Phosphatidylethanolamines; DGTS, diacylglycerol-trimethyl homoserine; DAG, diacylglycerols; TAG, triacylglycerols. In some cases, the error bars are smaller than the data point symbols. ns, not significant.



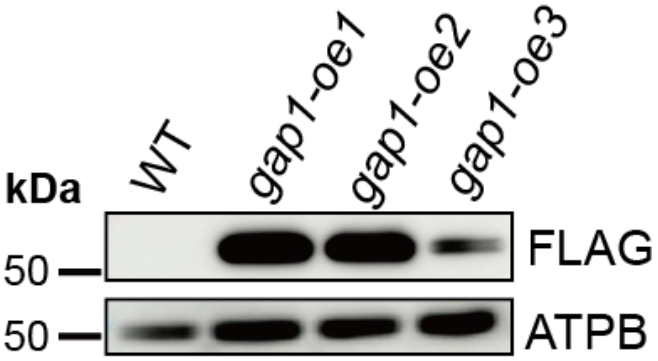
Supplementary Fig. 8. The mRNA abundance of genes involved in starch metabolism is increased in the *phot* mutant. WT and *phot* were synchronized to a 12h/12h light dark cycle under white light. Samples were taken in the middle of the light phase (6h) and the extracted RNA was analyzed by RT-qPCR to quantify mRNA levels of the following genes: GAP, glyceraldehyde-3-phosphate dehydrogenase; PFK, phosphofructokinase; FBP, Fructose-1,6-bisphosphatase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; HXK, Hexokinase; STA6, ADP-glucose pyrophosphorylase small subunit; SSS, starch synthase; AMY, α-amylase; ISA, isoamylase; PWD, phosphoglucan water dikinase; GWD, α-glucan water dikinase. Fold change between *phot* and WT was calculated as log2 FC (*phot*/WT).



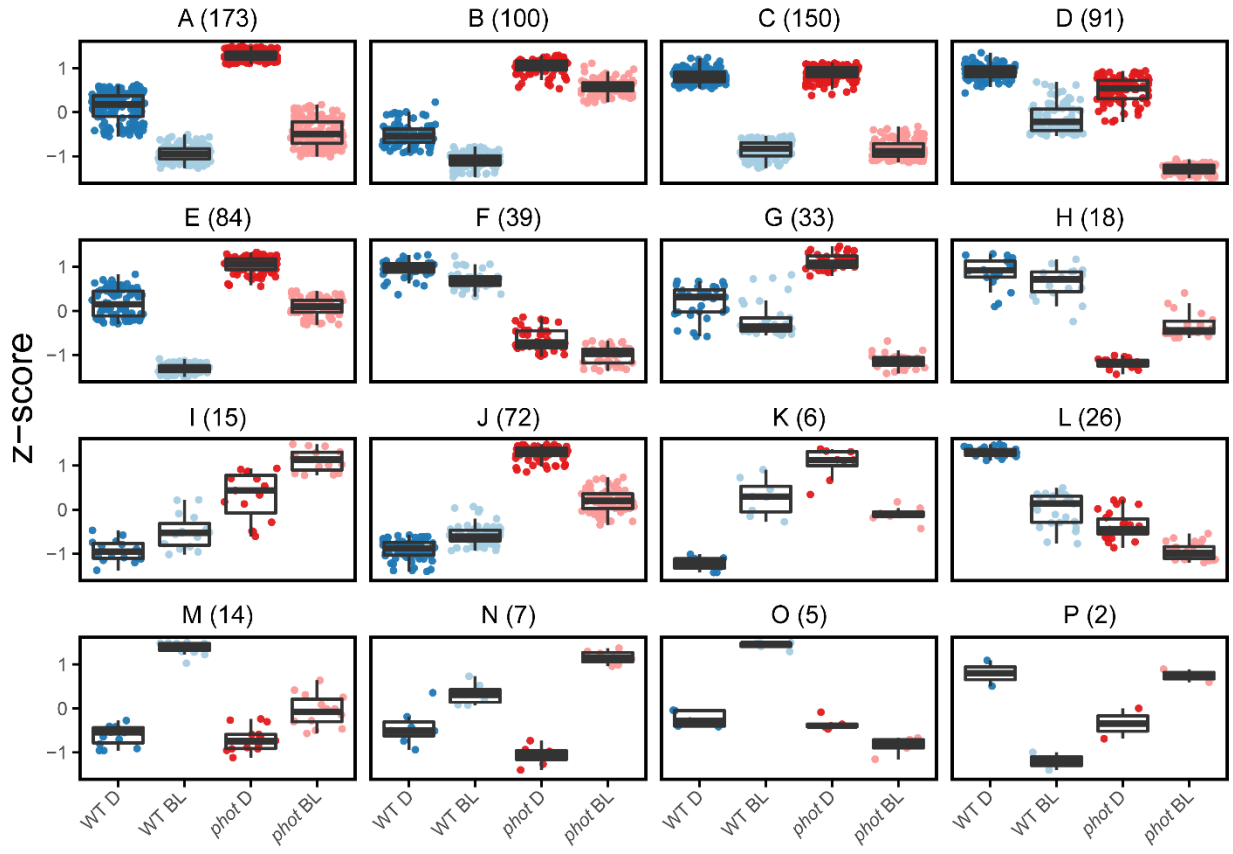
Supplementary Fig. 9. PHOT suppresses *GAP1* mRNA accumulation. *GAP1* relative mRNA abundance in WT (*cw15-302*) and in different *phot*-complemented lines, grown under continuous white light. Data are represented as mean ± SD (n = 3 biologically independent samples). Asterisks indicated the p-values (*, $p<0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant).



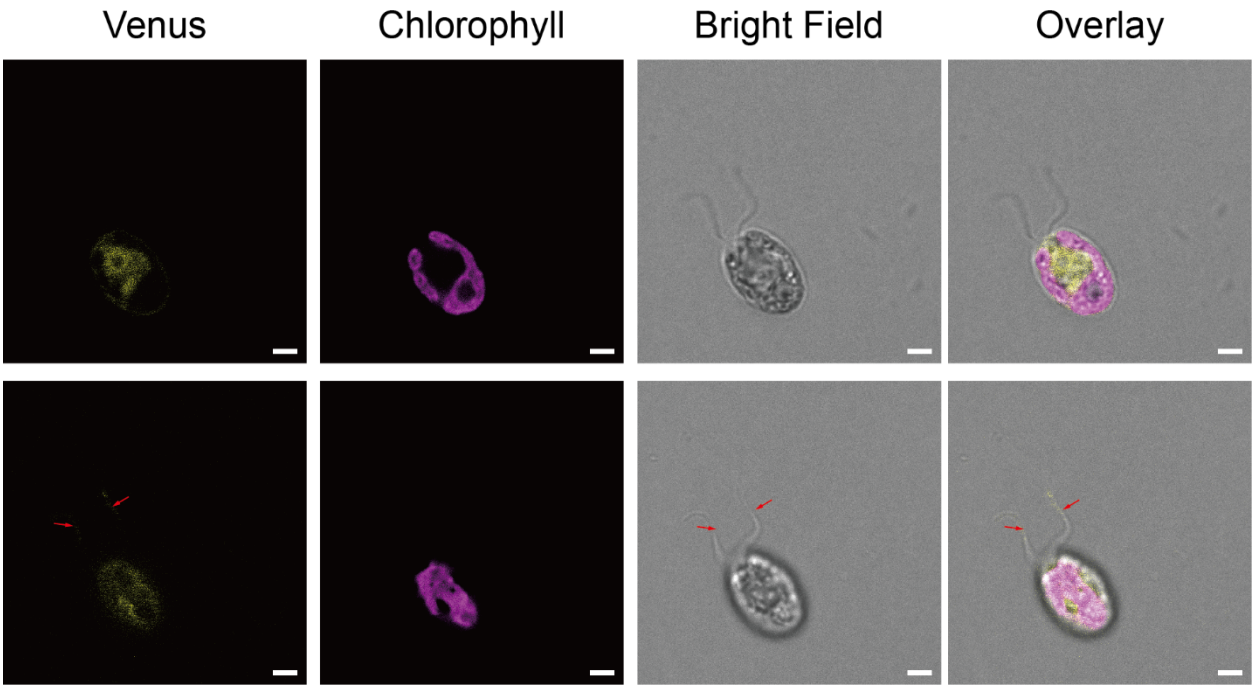
Supplementary Fig. 10. ***GAP1* transcription level reaches its peak at dusk.** *GAP1* expression profile in cells synchronized to a 12h/12h light dark cycle under white illumination, from *Strenkert et al.* (ref. 28, closed circles) and *Zones et al.* (ref. 27, open circles). The dark phase is indicated by black bars above the graphs; the light phase by white.



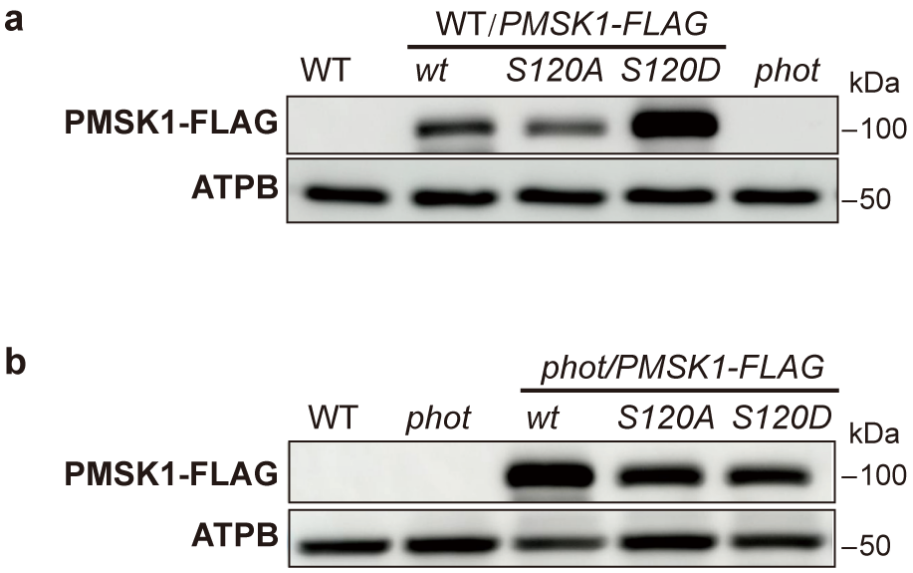
Supplementary Fig. 11. **Immunoblot analyses of *gap1-oe* lines.** Samples were collected from cultures grown in mixotrophic medium containing acetate (TAP medium) under continuous white light. Total protein extracts were probed with anti-FLAG antibody to quantify GAP1-FLAG levels. ATPB served as a loading control.



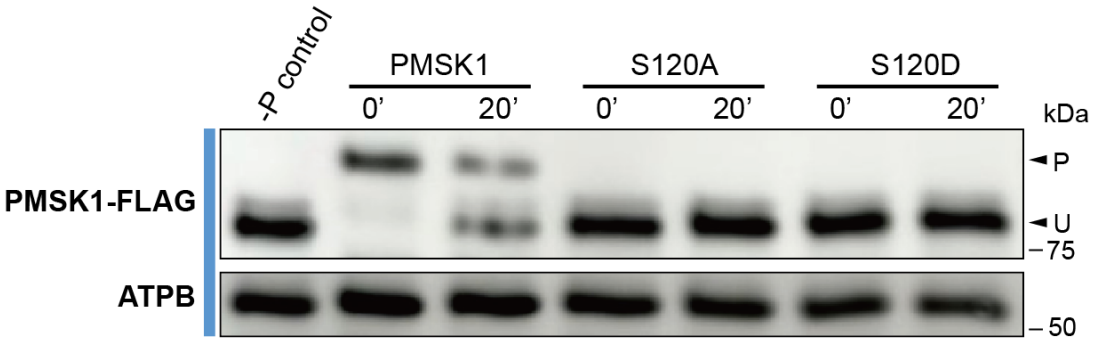
Supplementary Fig. 12. Unsupervised hierarchical clustering analyses of phosphoproteome. Significantly changing phosphopeptides across treatments based on a one-way ANOVA (FDR-adjusted *p*-value < 0.05). Each cluster is labelled A-P, and the number of phosphopeptides contained in each cluster are in parenthesis. Z-scores represent the relative abundance of each peptide across condition.



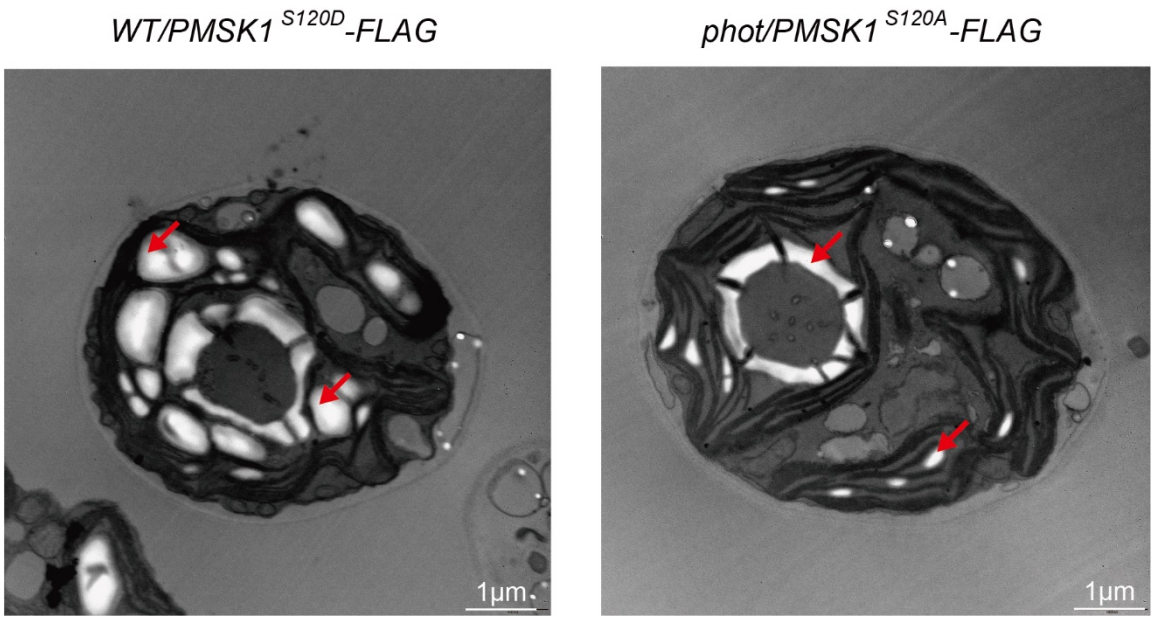
Supplementary Fig. 13. PMSK1 subcellular localization. Representative confocal fluorescent microscopy images PMSK1-Venus (Yellow) constitutively expressed in WT. Scale bar: 2 μm, red arrows indicate flagella. Two focal planes representative of the volume at 2.1 μm in distance were shown.



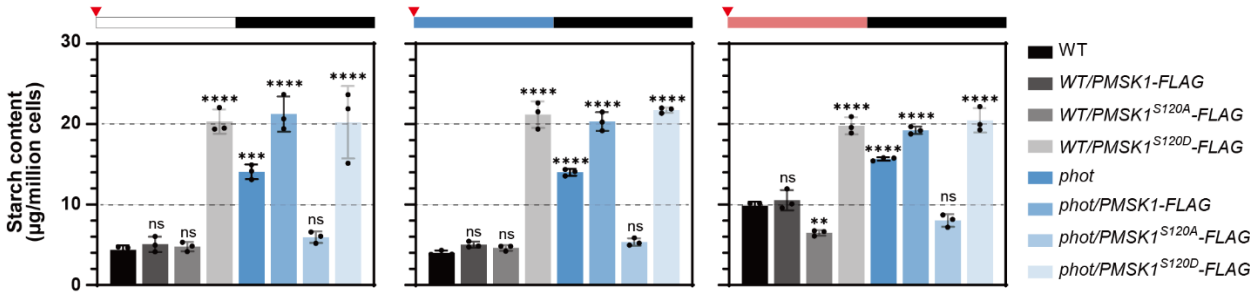
Supplementary Fig. 14. PMSK1-FLAG protein levels in WT/*PMSK1-FLAG* and *phot*/*PMSK1-FLAG* lines. Samples were collected from cultures grown in mixotrophic medium containing acetate (TAP medium) under continuous white light. Total protein extracts were probed with anti-FLAG antibody to quantify PMSK1-FLAG levels in the WT **(a)** or *phot* **(b)** background. ATPB served as a loading control.



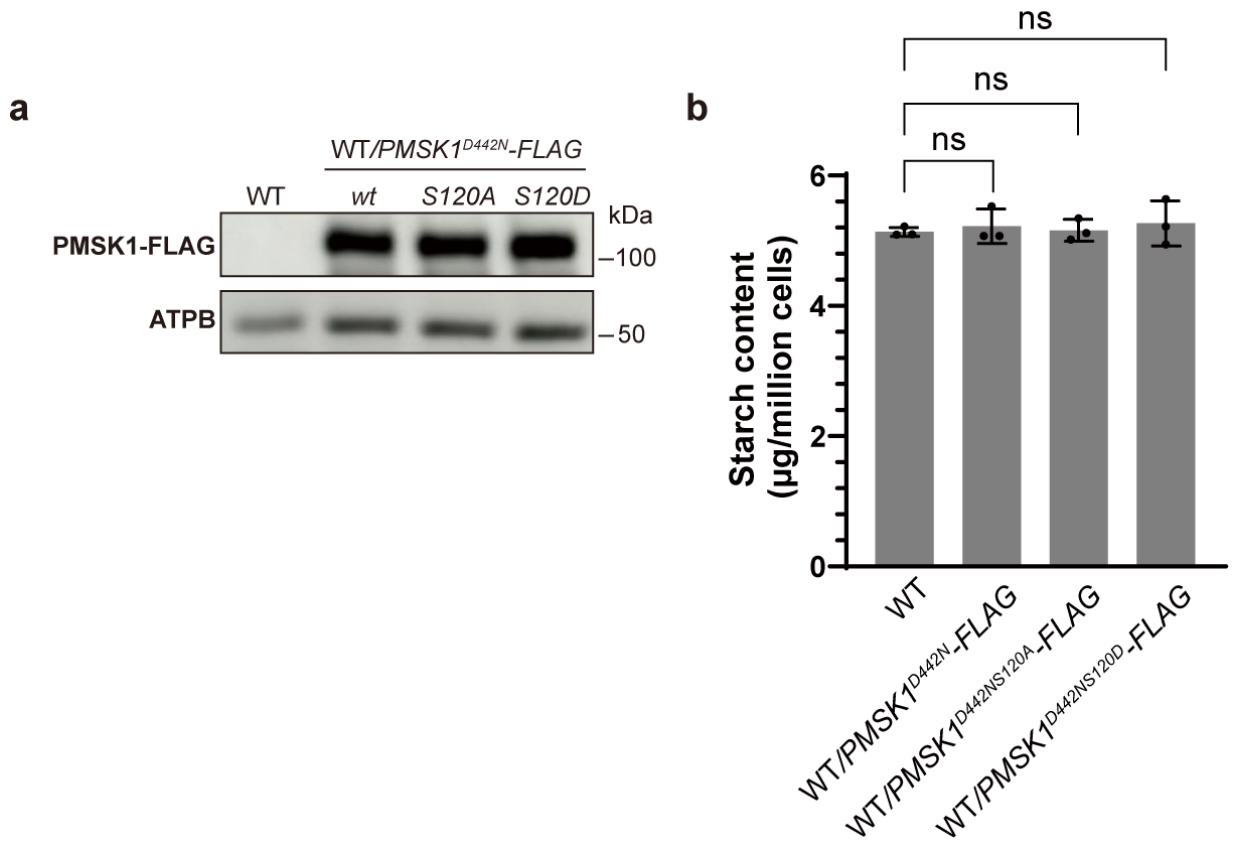
Supplementary Fig. 15. The blue-light triggered mobility shift of the PMSK1-FLAG protein is mediated by serine 120. Changes in the phosphorylation level of PMSK1-FLAG in WT/*PMSK1-FLAG, WT/PMSK1*^{S120A}*-FLAG* and *WT/PMSK1*^{S120D}*-FLAG* lines. Samples were collected after 24h of acclimation to darkness (t=0’) and 20’ after exposure to blue light (100 µmol photons m^{-2} s^{-1}). Detection was performed by Phos-tag SDS-PAGE; ATPB was used as a loading control. Phosphatase-treated WT/*PMSK1-FLAG* sample was also loaded on the gels. “U” and “P” indicate the unphosphorylated and phosphorylated PMSK1-FLAG respectively.



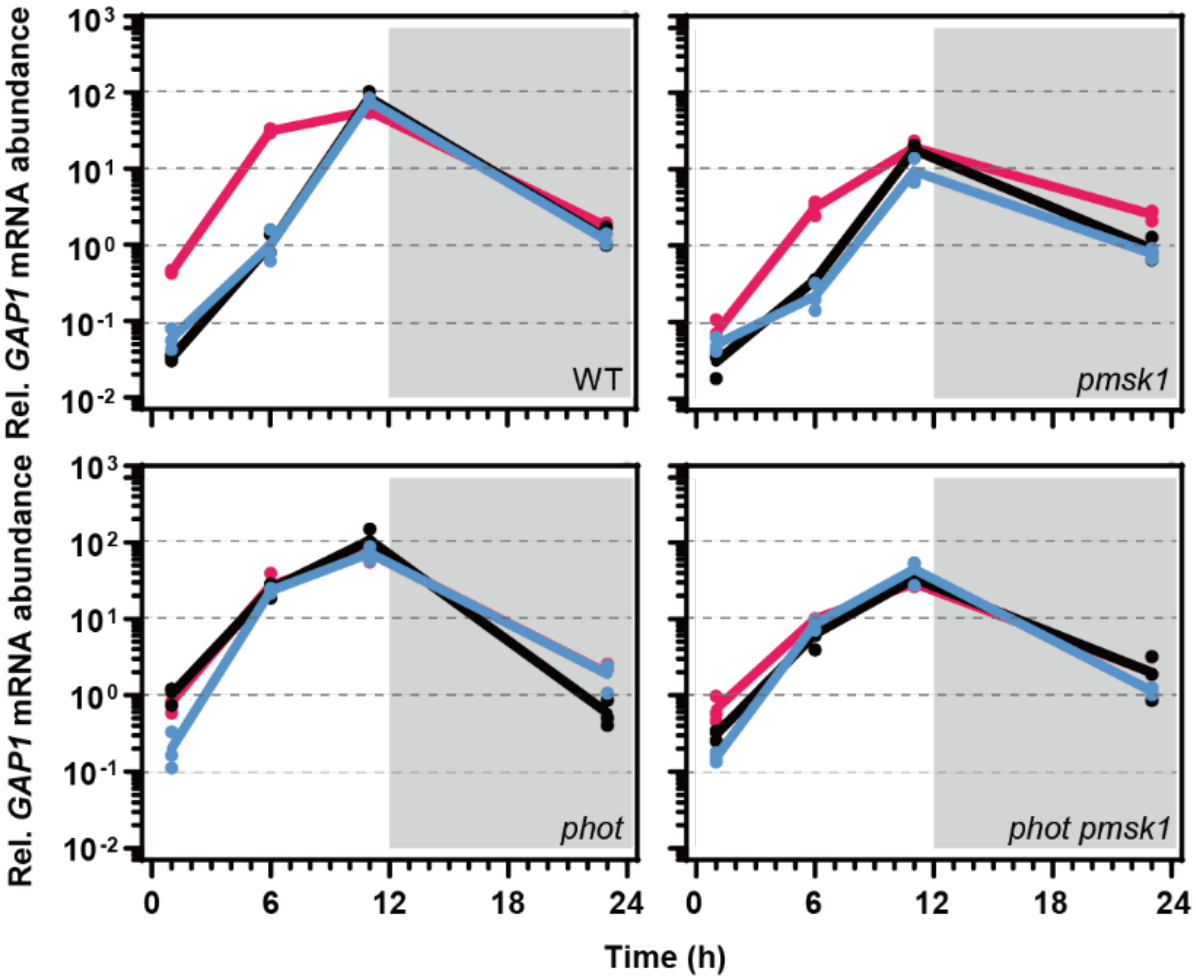
Supplementary Fig. 16. Transmission electron microscopy pictures of WT/*pmsk1*^{S120D} and *phot/pmsk1*^{S120A}. The two strains were synchronized to a 12/12 light dark cycle under white light and samples were collected at the end of the light phase. Bar scale: 1 µm. Red arrows indicate starch granules.



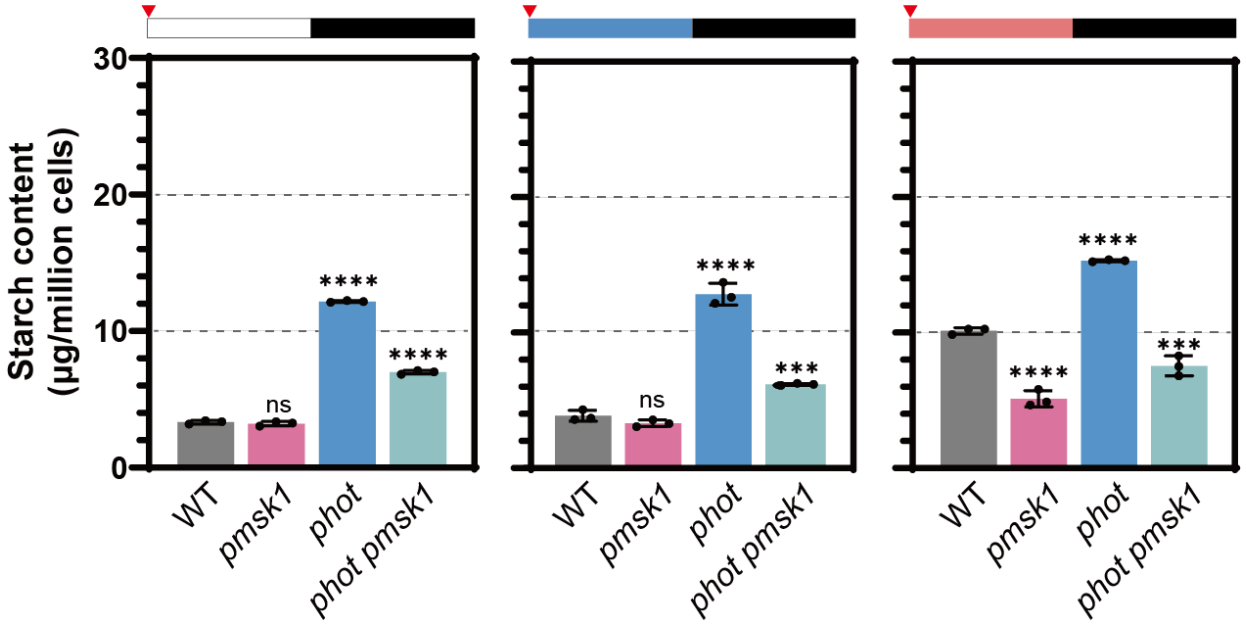
Supplementary Fig. 17. Impact of PMSK1-FLAG overexpression in WT and *phot* on starch metabolism. Starch content in WT or *phot* overexpressing PMSK1-FLAG. Cells were synchronized to a 12h/12h light dark cycle under different light qualities. The dark phase is indicated by black bars above the graphs; the light phase by white, blue or red bars, depending on the light quality used. Red triangles indicate sample collection time. Data are represented as mean ± SD (n = 3 biologically independent samples). Asterisks indicate the *P* values compared to WT. (*, p<0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant). Please note that in some cases the error bars are smaller than the data point symbols.



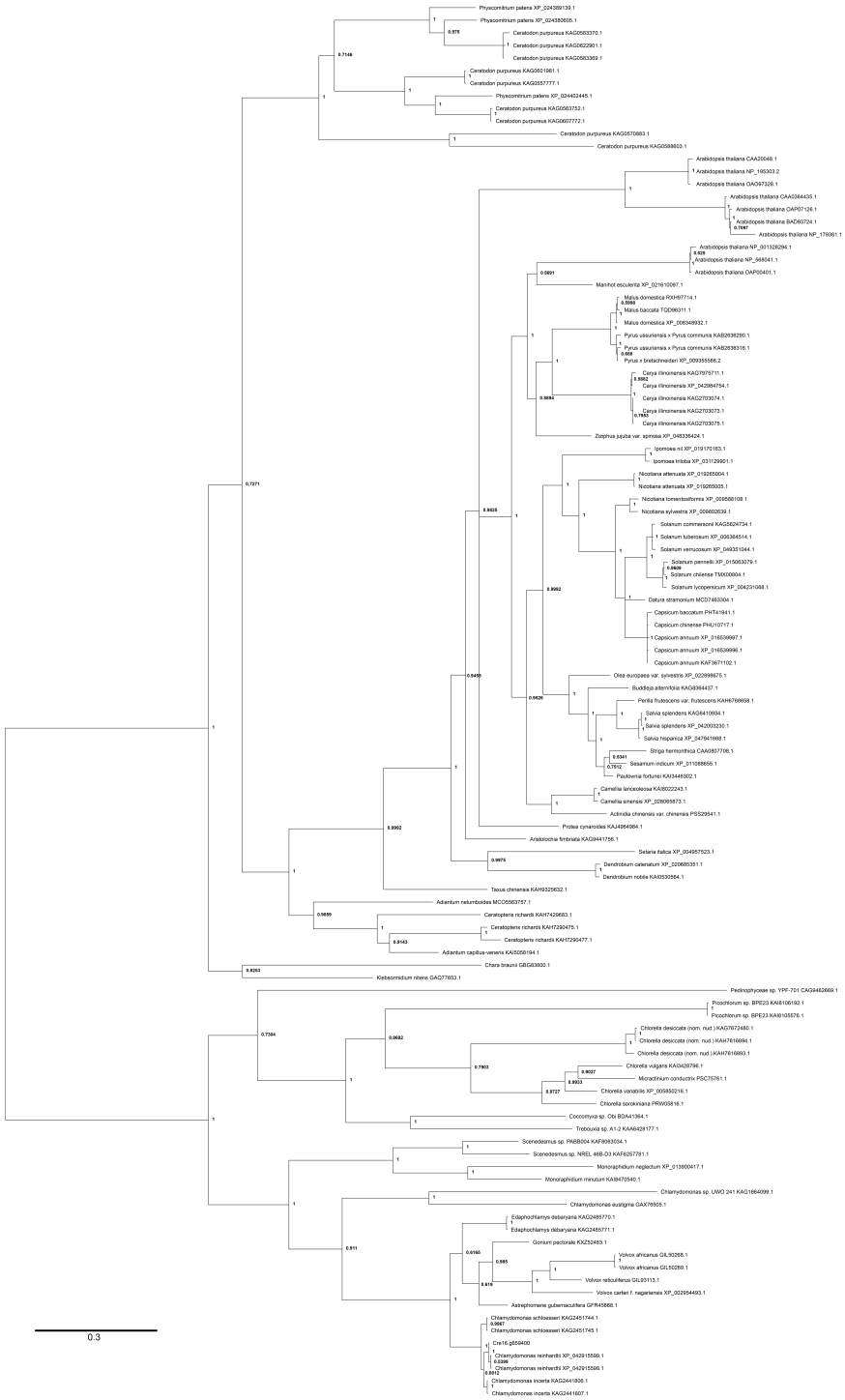
Supplementary Fig. 18. PMSK1 kinase activity is necessary to mediate light quality dependent starch metabolism in *Chlamydomonas reinhardtii* (**a**) Immunoblot analyses of PMSK1-FLAG in various PMSK1-FLAG overexpressing lines. ATPB served as a loading control. (**b**) Starch content of various dead kinase PMSK1-FLAG overexpression lines grown under continuous white light. Data are represented as mean ± SD (n = 3 biologically independent samples). ns, not significant.



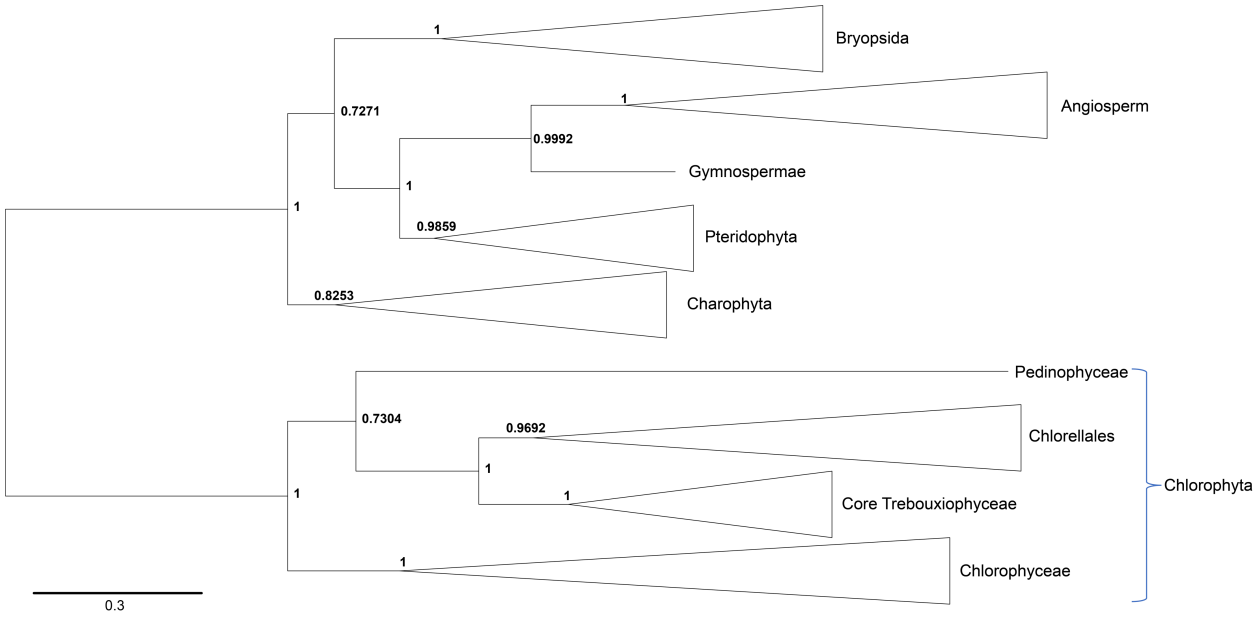
Supplementary Fig. 19. *GAP1* relative mRNA abundance in WT and in single and double *phot pmsk1* mutants synchronized to a 12h/12h light dark cycle under different light qualities. Phases are indicated by white and gray shading. Line colors indicated light qualities. Black, white light; Red, red light; Blue, blue light. Data are represented as mean ± SD (n = 3 biologically independent samples). Please note that in some cases the error bars are smaller than the data point symbols.



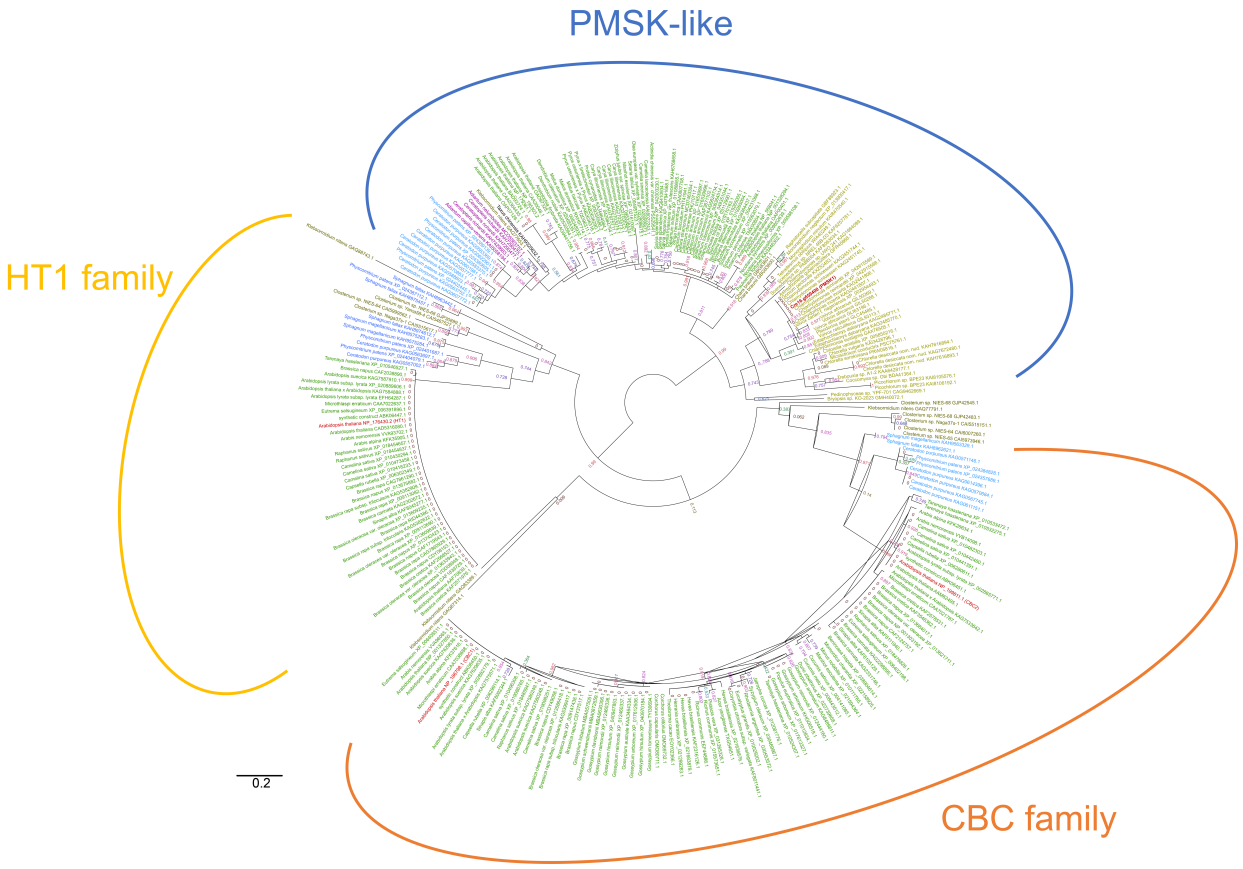
Supplementary Fig. 20. Starch content in WT and in single and double *phot pmsk1* mutants sampled at dawn. Cells were synchronized to a 12h/12h light dark cycle under different light qualities. The dark phase is indicated by black bars above the graphs; the light phase by white, blue or red bars, depending on the light quality used. Red triangles indicate sample collection time. Asterisks indicated the p-values compared to WT. (*, $p<0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant). Data are represented as mean ± SD (n = 3 biologically independent samples). Please note that in some cases the error bars are smaller than the data point symbols.



Supplementary Fig. 21. Phylogenetic tree of the PMSK-like family. Complete Unrooted phylogenetic tree of the PMSK-like family (dataset #1) identified as described in the "Identification of the PMSK-like family" from Chlorophyta representatives (Chlorophyceae, core Trebouxiophyceae, Chlorellales, Pedinophyceae), Charophyta, Briopsida, Pteridophyta, Gymnosperma and Angiosperma. The tree presented was inferred by Bayesian analysis as described in the "Methods" section. Bayesian Posterior Probability (BPP) values are reported at each node.

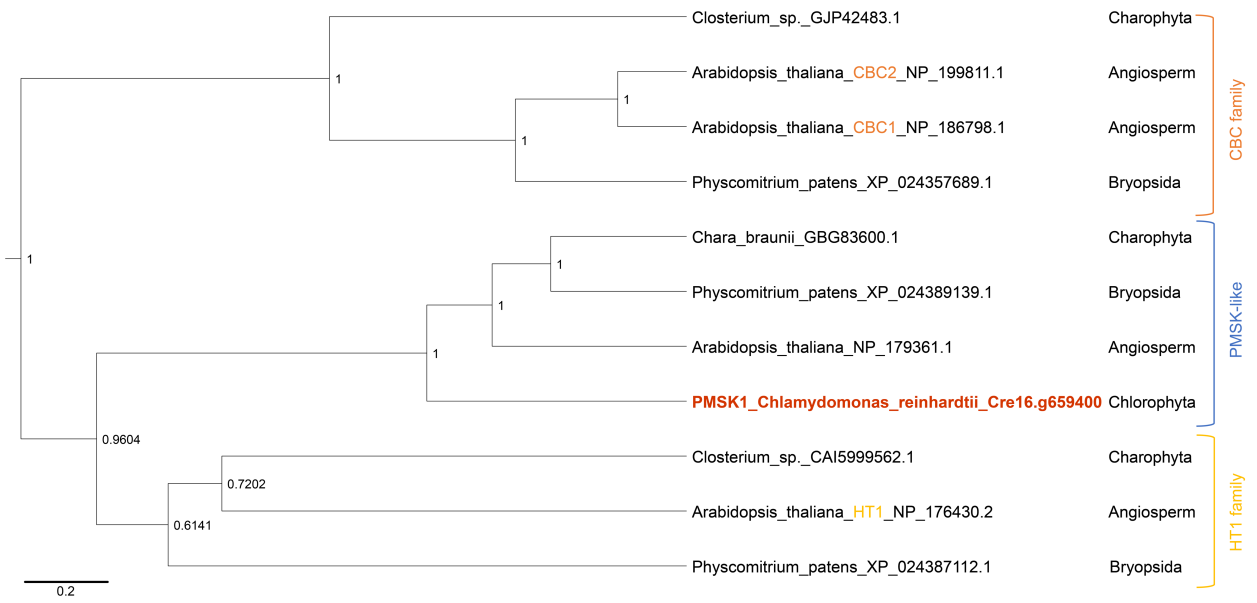


Supplementary Fig. 22. Evolutionary relationship between major green lineage clades inside the PMSK-like family. Simplified version of the **Supplementary Fig.21** phylogenetic tree of the PMSK-like family (dataset #1). The tree presented was inferred by Bayesian analysis as described in the "Methods" section. Bayesian Posterior Probability (BPP) values are reported at each node. This tree highlights the monophyletic origin of the PMSK family. The root represented on this tree is here to help the identification of major clades with the **Supplementary Fig.21** is not indicative of an ancestral state.



Supplementary Fig. 23. Evolutionary analysis by Maximum Likelihood method of the PMSK-like family with the HT1 and the CBC family

The evolutionary history was inferred from the dataset #2 by using the PhyML maximum likehood method from Chlorophyta representatives (Chlorophyceae, core Trebouxiophyceae, Chlorellales, Pedinophyceae), Charophyta, Briopsida, Pteridophyta, Gymnosperma and Angiosperma. The tree with the highest log likelihood (-4797.84912) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the WAG model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 1.283)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 15.3% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 278 amino acid sequences. Evolutionary analyses were conducted in MEGA11.



Supplementary Fig. 24. Phylogenetic clock tree of representatives of the PMSK-like, the HT1 and the CBC families. Rooted phylogenetic tree of representatives of the PMSK-like, the HT1 and the CBC families. The tree presented was inferred by Bayesian analysis with a clock-uniform method as described in the "Methods" section. Bayesian Posterior Probability (BPP) values are reported at each node. The root position was evaluated during the phylogenetic computation and is indicative of the evolutionary relationship between the three represented proteins families.