

RESEARCH PAPER

Identification and characterization of chloroplast casein kinase II from *Oryza sativa* (rice)

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Abstract

Plastid casein kinase II is an important regulator of transcription, posttranscriptional processes, and, most likely, different metabolic functions in dicotyledonous species. Here we report the identification and characterization of pCKII from the monocotyledonous species *Oryza sativa*. OspCKII activity was enriched from isolated rice chloroplasts using heparin-Sepharose chromatography, in which it co-elutes with the transcriptionally active chromosome (TAC) and several ribosomal proteins. Inclusion mass scanning of the kinase-active fraction identified the gene model for OspCKII. Transient expression of GFP fused to the 184 N-terminal amino acids of the OspCKII sequence in rice confirmed the chloroplastic localization of the kinase. OspCKII activity shows the characteristic features of casein kinase II, such as the utilization of GTP as phosphate donor, inhibition by low concentrations of heparin and poly-lysine, and utilization of the canonical pCKII motif E-S-E-G-E in the model substrate RNP29. Phosphoproteome analysis of a protein extract from rice leaves combined with a meta-analysis with published phosphoproteomics data revealed differences in the target protein spectrum between rice and *Arabidopsis*. Consistently, several pCKII phosphorylation sites in dicotyledonous plants are not conserved in monocots and algae, suggesting that details of pCKII regulation in plastids have changed during evolution.

Key words: Casein kinase II, chloroplast, mass spectrometry, phosphorylation, substrates, transcriptionally active chromosome.

Introduction

Relatively few chloroplast protein kinases have been identified and functionally characterized to date (Baginsky and Gruissem, 2009). Among these are STN7 and STN8, which are associated with the thylakoid membrane system and phosphorylate photosystem core, as well as light-harvesting complex subunits. They are involved in the regulation of state transitions and cyclic electron flow, and probably also in the regulation of other plastid functions such as gene expression (Rochaix, 2013; Schonberg and Baginsky, 2012). Another

thylakoid-associated kinase, TAK1, interacts with a major light-harvesting protein in yeast two-hybrid experiments (Snyders and Kohorn, 1999; Snyders and Kohorn, 2001). The exact function of TAK1 is not known, but the phosphorylation state of several thylakoid membrane proteins is altered in the *tak1* mutant. Msk4, a homologue of glycogen synthase kinase 3 (GSK-3), was identified and initially characterized in the plastids of *Medicago sativa* where it was found to regulate carbohydrate metabolism under environmental stress

conditions (Kempa *et al.*, 2007). In addition, two kinases with a function in the regulation of gene expression were identified; i.e. the chloroplast sensor kinase (CSK) and a plastid casein kinase II homologue (pCKII). CSK is a homologue of bacterial histidine sensor kinases and has a role in the regulation of chloroplast transcription, most likely via interaction with pCKII and sigma factor 1 (SIG1) (Puthiyaveetil *et al.*, 2008).

Plastid CKII co-purifies with the plastid RNA polymerase complex in heparin-Sepharose chromatography and subsequent glycerol density-gradient centrifugation. With this purification scheme, the plastid RNA polymerase can be isolated as a multisubunit enzyme complex with an apparent molecular mass >700 kDa (Pfannschmidt and Link, 1994; Baginsky *et al.*, 1997; Schröter *et al.*, 2010). This protein complex is a stable subcomplex of a larger complex that comprises the plastid-encoded RNA polymerase subunits and numerous additional associated factors that were identified from detergent-solubilized chloroplast extracts after gel filtration on Sepharose 4B columns (Reiss and Link, 1985; Pfalz *et al.*, 2006). This higher-order organization form of the plastid RNA polymerase was named 'transcriptionally active chromosome (TAC)'. The TAC subunits include proteins involved in RNA and DNA metabolism, the core subunits of the plastid encoded RNA polymerase, and regulatory factors that integrate the transcription machinery into different chloroplast processes (Pfalz *et al.*, 2006). *In vitro* transcription analyses showed that pCKII—as one of these associated factors—mediates transcriptional regulation by phosphorylation of RNA polymerase/TAC subunits (Baginsky *et al.*, 1999). Because of its association with higher order assemblies of the plastid RNA polymerase, pCKII has been named 'plastid transcription kinase (PTK)' (Baginsky *et al.*, 1997, 1999).

PTK was later identified as a CKII α -subunit homologue in several dicotyledonous species (Ogrzewalla *et al.*, 2002; Salinas *et al.*, 2006). Notably, whereas nucleo-cytoplasmic CKII from different systems assembles into a hetrotetramer consisting of α -, α' -, and two β -subunits (Pinna, 2002; Montenarh, 2010), chloroplasts lack the α' - and the β -subunits (Salinas *et al.*, 2006). Nonetheless, size exclusion chromatography with extracts from mustard (*Sinapis alba*) detected at least two different pCKII α -subunit-comprising complexes with native masses of 100–150 kDa and >700 kDa (Baginsky *et al.*, 1997) suggesting that the α -subunit probably does not act as a monomer in chloroplasts *in vivo*. In mammalian systems, the association between the catalytic α -subunits and the regulatory β -subunit determines the substrate specificity of the CKII holoenzyme (Pinna, 2002; Venerando *et al.*, 2014). It is currently not clear whether different pCKII α -subunits comprising complexes in chloroplasts have different substrates *in vivo*. A set of pCKII substrates was identified by biochemical assays and motif prediction without distinguishing between different pCKII complexes (Reiland *et al.*, 2009; Schonberg and Baginsky, 2012). In the initial experiments, an RNA-binding protein was identified as a pCKII substrate in pea chloroplasts *in vitro* (Kanekatsu *et al.*, 1993). The list of targets was extended by experiments with partially fractionated

extracts from mustard chloroplasts, in which components of the RNA polymerase as well as sigma factors were identified as pCKII substrates (Baginsky *et al.*, 1997; Baginsky *et al.*, 1999). Using large-scale phosphoproteomics, several candidate pCKII targets were identified that were found phosphorylated at predicted CKII phosphorylation motifs (Reiland *et al.*, 2009).

At present, the regulation of protein phosphorylation by pCKII is largely unknown. Plastid CKII responds to changes in redox poise with a change in its *in vitro* phosphorylation activity, but it is not known whether this regulation is relevant for the *in vivo* situation (Baginsky *et al.*, 1999; Turkeri *et al.*, 2012). In fact, recent phosphoproteome analyses at the end-of-day and end-of-night revealed only few changes in the chloroplast phosphoproteome, suggesting an unexpectedly minor regulation of the phosphorylation status of many chloroplast proteins (Reiland *et al.*, 2009). An exception is the light-induced phosphorylation of RNP29 during de-etiolation of rice plants (Kleffmann *et al.*, 2007). In case of its spinach homologue RNP24, phosphorylation increases the RNA-binding affinity to the *petD* and *psbA* mRNA 3'-UTRs (Loza-Tavera *et al.*, 2006). Rice RNP29 and Spinach RNP24 are homologues of the first reported CKII substrate in pea (see above) (Kanekatsu *et al.*, 1993), suggesting that pCKII may be involved in the regulation of plastid differentiation in both dicots and monocots. However, whereas pCKII is well characterized in dicots, it is not known whether a casein kinase II homologue is active in the plastids of monocotyledonous species such as rice or maize.

Here we report the identification of rice plastid CKII and its substrate protein spectrum. We extracted plastid proteins that are phosphorylated at canonical pCKII phosphorylation motifs from our own and previously published rice phosphoproteomics data (Nakagami *et al.*, 2010). Identification of pCKII phosphorylation motifs used *in vivo* provides a footprint of kinase activity, and previous analyses have shown that the assembly of phosphorylation networks solely based on motif-prediction can yield reliable results (Linding *et al.*, 2007). Prerequisite for high accuracy of a predicted kinase network is 'context' information; for example, on the co-localization of kinase and phosphoprotein in the same organelle. Chloroplasts represent a compartmentalized and therefore highly constrained phosphoproteome, which allows defining kinase/substrate relationships with high accuracy (Linding *et al.*, 2007; Reiland *et al.*, 2009). On this basis, we identified several putative pCKII targets in rice plastids. Our analysis shows that analogous to the situation in dicots, a pCKII alpha subunit is active in the plastids of monocots but the details of pCKII-mediated regulation seem to differ even between closely related species within the dicotyledonous clade.

Materials and methods

Isolation of rice chloroplasts

For plastid isolation, 180 g of rice (*Oryza sativa* cv. japonica) seeds were washed in 5% sodium hydrochloride solution for 10 min, rinsed four times with deionized water, and swollen overnight at 29 °C. Seeds were transferred to wet Vermiculite supplemented with

half-strength concentrated Murashige and Skoog medium (3:2 (v/v) Vermiculite/medium). Seedlings were grown in a 16h/8h light/dark cycle at a constant temperature of 29 °C for 10 days. Plastid isolation was performed by Percoll step gradient centrifugation. All isolation procedures were carried out at 4 °C. One-hundred and fifty grams of rice seedlings (excluding the roots) were cut into small pieces (0.5 cm) and homogenized with a Waring blender in 2000 ml of isolation buffer (10 mM HEPES/KOH, pH 7.8, 2 mM EDTA, 2 mM MgCl₂, 1 mM tetrasodium pyrophosphate, and 600 mM sorbitol) supplemented with 0.2% bovine serum albumin. The homogenate was subsequently filtered through two and four layers of Miracloth. The filtrate was subsequently centrifuged for 4 min at 200 g to remove cellular debris. The supernatant was re-centrifuged for 10 min at 8000 g. Pellets containing chloroplasts were carefully resuspended in isolation buffer with 1 mM DTT and subjected to Percoll step gradient centrifugation. Approximately 4 ml of resuspended chloroplasts were loaded onto Percoll step gradients that were composed of 5 ml 75% Percoll and 15 ml 35% Percoll in gradient buffer (40 mM Tricine, pH 8.5, 5 mM EDTA, 10 mM MgCl₂, 600 mM Sorbitol). The gradient was centrifuged for 30 min at 8000 g. The band of intact rice plastids between 35% and 75% Percoll was collected and washed with isolation buffer containing 1 mM DTT. After centrifugation at 8000 g for 10 min, the pellet containing intact chloroplasts was frozen in liquid nitrogen and stored at -80 °C.

Preparation and characterization of protein fractions after heparin-Sepharose chromatography

The isolated chloroplasts were lysed in extraction buffer (50 mM Tris-HCl, 1 mM DTT, 1× Protein Inhibitor Cocktail (Roche Applied Science, Indianapolis, USA)), homogenized on ice, and centrifuged at 16 000 g for 10 min at 4 °C. The supernatant was loaded onto a heparin-Sepharose column (2 ml bed-volume) (GE-Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was equilibrated and washed with buffer (50 mM Tris-HCl, 0.3 M KCl, 1 mM DTT and 10% (v/v) glycerol), and bound proteins were eluted in a single step with a buffer containing 50 mM Tris-HCl, 1.8 M KCl, 1 mM DTT, and 10% (v/v) glycerol. The eluted fractions were dialysed overnight in 50 mM Tris-HCl, 1 mM DTT, 10% glycerol, and 1× Protease Inhibitor Cocktail. Kinase activity was assayed at 30 °C for 30 min in 20 mM Tris, pH 7.4, 5 mM MgCl₂, 5 mM CaCl₂, 50 μM ATP, 1 μCi γ [³²P]ATP, and 5 μg of purified recombinant 29RNP-Strep tagged protein. SDS sample buffer (5×) (0.312 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 25% (v/v) glycerol, 5% (v/v) mercaptoethanol, 0.05% Bromphenolblue) was added to each reaction, and the samples were heated at 70 °C for 5 min to stop the reaction. The reaction mixtures were loaded onto a 10% SDS-polyacrylamide gel. The incorporation of radioactive phosphate into RNP29 was determined by autoradiography (Fuji X-ray film RX). The fractions containing RNP29 kinase activity were pooled and concentrated by centrifugation in Amicon® Ultra-4 centrifugal filter units. The protein concentration was determined by the Bradford method (Bradford, 1976).

SDS-polyacrylamide gel electrophoresis and in-gel tryptic digest

Approximately 50 μg of heparin-Sepharose-enriched protein was loaded onto 10 cm homogeneous 12% SDS-polyacrylamide gels. After electrophoresis, the gel was cut into five segments along the lanes. Every segment was cut into small pieces (1 mm³) and subjected to in-gel tryptic digest as previously described (Shevchenko *et al.*, 1996), using sequencing grade modified trypsin (Promega, USA) at a ratio of one part trypsin per ten parts protein. Proteins were digested overnight at 37 °C. After desalting with a Sep-Pak C18 column (Waters, USA), the tryptic peptides were dried and stored at -80 °C until analysis.

Protein identification by LTQ-FT-ICR-MS/MS

Tryptic peptides from each fraction were resuspended in 20 μl of 5% acetonitrile (ACN)/0.2% formic acid (FA) in water and loaded

onto laboratory-made silica-capillary columns (inner diameter of 75 μm, length of 9 cm; BGB Analytik AG, Böckten, Switzerland) packed with C-18 reverse phase material (Magic C18 resins; 3 μm, 100 Å pore; Michrom BioResources, Auburn, CA, USA). The peptide mixture was separated and eluted by a gradient elution from 5–65% ACN/0.2% FA over 1 h followed by an increase of up to 80% ACN during an additional 7 min period. The flow rate was set to 200 nl min⁻¹. LC was coupled to a FT-ICR LTQ-MS (2D ion trap and Fourier transform ion cyclotron resonance mass spectrometer) (Thermo Scientific, Germany) equipped with a nanospray source. Mass spectrometry analysis was performed in data-dependent mode by one MS full scan followed by three data-dependent MS/MS scans of the three most intense precursor ions or by an *m/z* inclusion list. The *m/z* inclusion list containing all predicted tryptic peptides of the different rice CKII gene models was generated with MS-Digest software (ProteinProspector 3.0, <http://prospector.ucsf.edu>). The dynamic exclusion function was enabled to allow two measurements of the same precursor ion for 1 min followed by exclusion of measurements for 1 min. MS/MS spectra were extracted with DeconMSn software (Mayampurath *et al.*, 2008). MS/MS spectra were searched with MASCOT v2.4 and PeptideProphet and ProteinProphet using the Trans-Proteomic Pipeline (TPP v.4.7_SQUALL; <http://tools.proteomecenter.org/TPP.php>) against the *Oryza sativa* protein database (download from <ftp://ftp.plantbiology.msu.edu>, February 2014) supplemented with contaminants (<ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta>). The search parameters were as follows: requirement for tryptic ends, one missed cleavage allowed, and mass tolerance ±10 ppm. We chose a PeptideProphet cutoff of 0.90 for peptides that were used for the ProteinProphet analysis. To filter the ProteinProphet data, a 0.95 probability cutoff value was used for protein identification. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino *et al.*, 2014) via the PRIDE partner repository with the dataset identifier PXD000951 and DOI 10.6019/PXD000951.

Localization of rice chloroplast CKII

The CKII alpha gene LOC_Os03g55490 (N terminal 184aa) was amplified by PCR using the following primers: 5'-TCTAGAG GAAGAGAGGTACATGGCCTG-3' and 5'-GGATCCTCGATTC ATCATCCCTGACAAC-3'. PCR products were ligated into the green fluorescent protein (GFP) fusion vector pBI221-GFP, which encodes GFP under the constitutive control of the cauliflower mosaic virus 35S promoter. The empty pBI221-GFP plasmid served as a control for intracellular targeting. Protoplasts were isolated from 10-day-old rice seedling stems and leaves according to previously published methods (Chen *et al.*, 2006; Miao and Jiang, 2007), with slight modifications. The rice seedling stems and leaves were cut into 0.5 mm pieces and incubated for 4 h at room temperature in protoplast culture media (PCM) [1× MS basal salt with vitamins (Duchefa, Haarlem, The Netherlands)], 0.4 M sucrose, 2 mM MES, pH 5.7, 250 mg l⁻¹ NH₄NO₃ with 2.5% cellulase Onozuka R-10 and 0.3% Macerozyme R-10 (Yakult Honsa). After incubating in W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7) for 1 h at room temperature, the released protoplasts were filtered through a 100 μm nylon net and collected by centrifugation at 200 g for 5 min. The isolated protoplasts were washed once with W5 buffer and resuspended in buffer (0.4 M mannitol, 20 mM CaCl₂, 5 mM MES, pH 5.7). Plasmid DNA (approximately 20 μg) was mixed with 100–200 μl of resuspended protoplasts in an Eppendorf tube. The same volume of PEG buffer (40% PEG4000, 0.4 M mannitol, 100 mM Ca(NO₃)₂) was added to the mixture of DNA and protoplasts and subsequently incubated for 30 min at room temperature. Then, 800 μl of PCM was added to the tube. After 20 h of incubation at 28 °C under continuous light (100 μE·s⁻¹·m⁻²), green fluorescence and chlorophyll red autofluorescence were monitored with a confocal laser scanning microscope (Leica TCS SP5, Germany). GFP was excited with a 488 nm laser, and its emission was detected using a

505–550 band pass filter. The chlorophyll red auto-fluorescence was monitored using a 600 nm long pass filter.

Identification of the RNP29 phosphorylation site

To identify the RNP29 phosphorylation site, the phosphorylation reaction was performed *in vitro* as described above. RNP29 was subsequently loaded onto a SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The RNP29 bands were excised and in-gel digested with trypsin. The phosphorylated peptides were enriched with magnetic TiO₂ beads (Thermo Scientific, Germany). Dried peptides were resuspended in 5% ACN and 0.3% FA and analysed on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nano-electrospray ion source. Peptides were separated using an Eksigent Nano LC system (Eksigent Technologies) equipped with a 12 cm fused silica emitter (75 μm i.d.) packed with reverse phase C18 resin (3 μm particles, 120 Å pore size, SunChrom, Friedrichsdorf, Germany). The flow rate was 600 nl min⁻¹. The peptide mixture was separated and eluted by a 5–65% ACN/0.3% FA gradient over 1 h followed by an increase of up to 80% ACN for an additional 7 min. Nano-ESI was accomplished at a spray voltage of 2.5 kV and a heated capillary temperature of 230 °C. A cycle of one full-scan mass spectrum (400–2000 amu) followed by five data-dependent MS/MS spectra was recorded continuously. All MS/MS spectra were acquired at normalized collision energy 35% and an ion selection threshold of 1e⁵ counts. Selected ions were dynamically excluded for 30 s. MS/MS spectra were extracted with DeconMSn software (Mayampurath et al., 2008). MS/MS spectra were searched against the rice database (reference) containing common contaminants (ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta), such as keratin, using the MASCOT v2.4 software (Matrix Science). Carbamylation of Cys was set as fixed modification and oxidation of Met and phosphorylation of Ser, Thr, and Tyr was set as variable modification. We performed database searches using MASCOT with the following parameters: restricted to tryptic peptides with a maximum of one missed cleavage, allowing for doubly and triply charged peptides, a precursor mass error tolerance of 5 ppm, and a fragment ion error tolerance of 0.6 Da. Identifications were accepted if the MASCOT ion score was 40 or greater and if the MASCOT expect value was less than 0.01. The identified phosphopeptides were also analysed by PhosCalc based on the software manual's instructions for validation (MacLean et al., 2008). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the dataset identifier PXD000857 and DOI: 10.6019/PXD000857.

Recombinant expression of OspCKII and RNP29 for in vitro phosphorylation assays

The pCK2α gene LOC_Os03g55490 (C terminal 325aa, without chloroplast transit peptide) was cloned and ligated into vector pASK-IBA3plus. The pCK2α was expressed in *E. coli* (BL21) under 0.2 μg/l anhydrotetracycline with 4 h induction. The recombinant CK2α was purified with the Strep-Tag Starter Kit according to manufacturer instructions (IBA GmbH, Goettingen, Germany). The 29RNP gene LOC_Os07g43810 (C terminal 226aa, without chloroplast transit peptide) was cloned and ligated into vector pASK-IBA3plus. The 29RNP was expressed in *E. coli* (BL21) under 0.2 μg l⁻¹ anhydrotetracycline with 3 h induction. The recombinant 29RNP protein was purified with the Strep-Tag Starter Kit according to manufacturer instructions (IBA GmbH, Goettingen, Germany).

Rice leaf phosphoproteome analysis

The rice leaf phosphoproteome screen was performed with rice plants grown as described above. Proteins were extracted from frozen material in a buffer containing 40 mM Tris, 4% (w/v) SDS, 40 mM DTT, and phosphatase inhibitors [PhosSTOP, according to manufacturer instructions (Roche Applied Science, Indianapolis,

USA)] and solubilized proteins were precipitated by methanol/chloroform (Wessel and Flugge, 1984). Finally, the protein pellets were resolved in a small volume of resuspension buffer (20 mM Tris-HCl, pH 8.3, 3 mM EDTA, 8 M Urea). For tryptic digestion the protein solution was diluted to 1 M urea by adding 20 mM Tris-HCl, pH 8.3, 3 mM EDTA. Phosphopeptide enrichment was performed as described previously by a combined strong cation exchange and TiO₂ affinity chromatography (Reiland et al., 2009). Dried peptides were resuspended in 5% ACN, 0.1% formic acid and analysed on a LTQ-Orbitrap mass spectrometer (Thermo Scientific, Germany) interfaced with a nanoelectrospray ion source. Data acquisition and interpretation was performed as described previously (Reiland et al., 2009). The samples were acquired using internal lock mass calibration on *m/z* 429.088735 and 445.120025. MS and MS/MS data were searched against the *Arabidopsis* database (TAIR10, ftp://ftp.Arabidopsis.org) containing common contaminants (ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta) using MASCOT v2.4 (Matrix Science, London, UK). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the dataset identifier PXD001168 and DOI: 10.6019/PXD001168.

Results

Enrichment, characterization, and identification of rice plastid CKII (OspCKII)

In dicotyledonous species such as mustard and *Arabidopsis*, plastid CKII co-purifies with the chloroplast transcription system, e.g. with the TAC and subcomplexes thereof (Pfannschmidt and Link, 1994; Baginsky et al., 1997; Ogrzewalla et al., 2002). We prepared soluble stroma fractions from rice chloroplasts and enriched the TAC and TAC-associated proteins using heparin-Sepharose chromatography. Bound proteins were eluted in a single step with 1.8 M KCl, and the elution fraction (eluate) as well as the input, flow-through, and the wash fractions were assayed for kinase activity. We chose RNP29 as a standard substrate for pCKII (see materials and methods). The soluble stroma extract contains a RNP29 phosphorylation activity that is significantly enriched in the protein fraction bound to heparin-Sepharose (Fig. 1A, B). This phosphorylation activity is dependent on the presence of divalent ions such as Mg²⁺ or Mn²⁺ and it is highly active on casein. Further characterization of the RNP29 phosphorylation activity revealed the characteristic features of casein kinase II such as inhibition by heparin and utilization of GTP as phosphate donor (Fig. 1B). Poly-lysine has a substrate-specific inhibitory effect on the CKII alpha subunit, whereas it stimulates the activity of the CKII holoenzyme (Pinna, 2002). In our assays, poly-lysine inhibits RNP29 phosphorylation. Our data suggest that the RNP kinase observed here is a CKII alpha subunit (Kanekatsu et al., 1998).

We next identified the protein kinase in the heparin-Sepharose eluate fraction by mass spectrometry using an FT-ICR-LTQ mass spectrometer (Supplementary Table S1). Among the identified proteins are the four RPO subunits (αββ'β'') of the plastid-encoded RNA polymerase (PEP) and 20 proteins with a function in DNA/RNA-binding or RNA processing, including seven annotated TAC subunits (2, 3, 6, 7, 10, 12, 14). Furthermore, several proteins were identified that have a homologue in *Arabidopsis* and that is an established TAC subunit, such as TRX-Z, FLN1, FLN2, FSD2, and FSD3

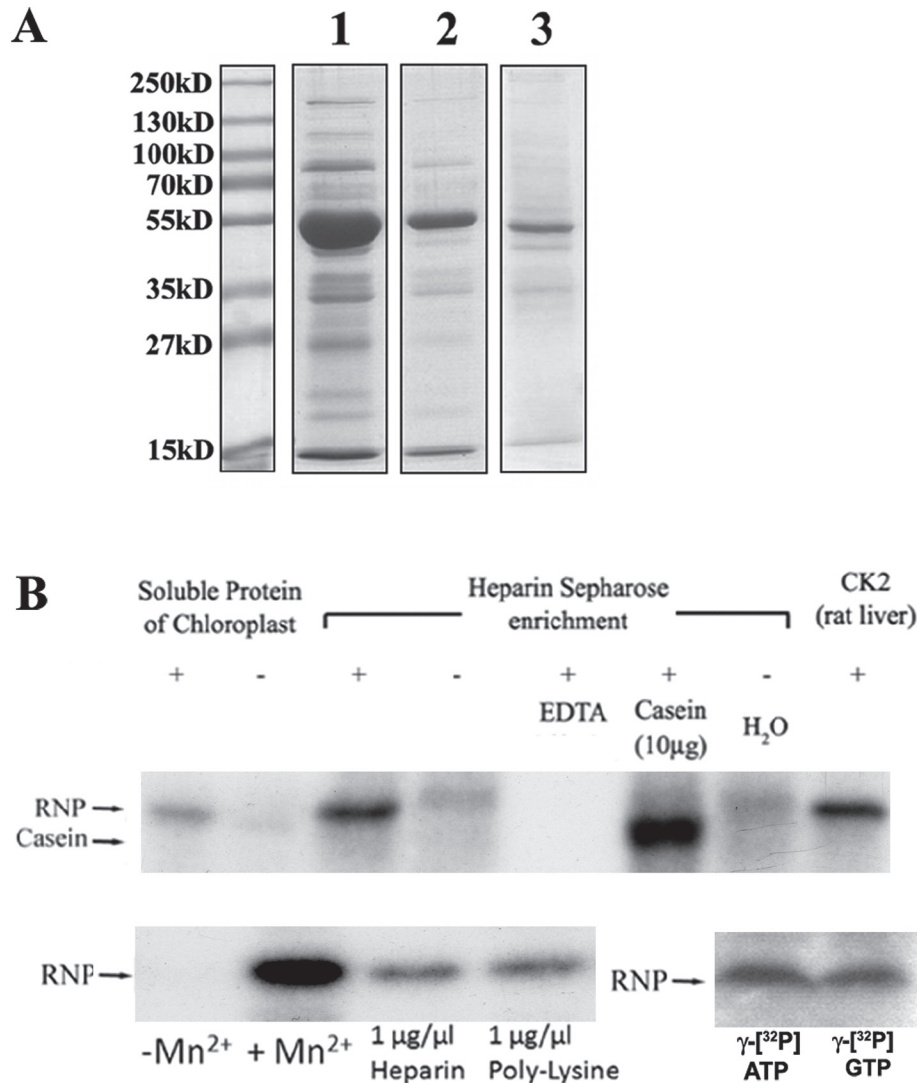


Fig. 1. Enrichment and characterization of the rice chloroplast RNP29 kinase. (A) Coomassie brilliant blue stained SDS gel of the input (1), the flow-through (2), and the elution fraction (3). Bound proteins were eluted in a single step using equilibration buffer supplemented with 1.8M KCl. (B) Biochemical characterization of the 29RNP kinase. Kinase activity assays were performed with either casein or RNP29 as externally added substrate, as indicated. Control assays contained either EDTA, poly-lysine, heparin, Mn^{2+} instead of Mg^{2+} , or $\gamma\text{-}^{32}\text{P}\text{-ATP}$ or $\gamma\text{-}^{32}\text{P}\text{-GTP}$ as indicated. Samples were subjected to SDS-PAGE and visualized by autoradiography.

(Supplementary Table S1). These data show that the structurally stable transcriptional core of the chloroplast TAC (as defined by Pfalz and Pfannschmidt, 2013) can be enriched by heparin-Sepharose chromatography also in rice (Pfalz and Pfannschmidt, 2013). Together, casein kinase II activity in rice plastids co-elutes with the transcription system in heparin-Sepharose chromatography (Baginsky *et al.*, 1997).

We could not identify any protein kinase directly in the eluate fractions. Plastid CKII has a very low abundance and most proteomics studies conducted so far failed to detect pCKII in TAC-enriched fractions (Olinares *et al.*, 2010; Schröter *et al.*, 2010). We therefore decided to use a targeted proteomics approach by directly screening the kinase active fractions for peptides of the different CKII alpha subunits in the rice genome. Similar to the situation in *Arabidopsis*, rice contains four CKII alpha subunits with high sequence identity (Table 1). We generated an m/z inclusion list from all four

CKII alpha subunits using MS-Digest (ProteinProspector 3.0, <http://prospector.ucsf.edu>). With this inclusion list, we re-analysed the kinase active fraction using FTICR mass spectrometry. This analysis identified two peptides, $_{201}\text{VLYPTLLDYDIR}_{212}$ and $_{162}\text{ILQONLYGGPNIVK}_{174}$, which unambiguously identify LOC_Os03g55490 as the rice plastid CKII alpha subunit (Table 2). Plastid CKII was not identified in the unsupervised MS scan because of its low abundance. The peptide VLYPTLLDYDIR (m/z 740.9074) was identified at the retention time 51.54 min with a signal intensity of 1.12×10^3 . This signal intensity is roughly 100-times lower than that of other peptide peaks co-eluting in a similar time frame (e.g. between 45 and 55 min; Fig. 2A, B). With this signal intensity, m/z 740.9074 is the 40th highest peak in the full MS scan at 51.54 min (Fig. 2 C). Similarly, the peptide ILQONLYGGPNIVK (m/z 714.9160) was identified at the retention time 39.59 min, where its precursor ion has the 20th

highest peak intensity in the MS full scan. Thus, data-dependent scans that consider the three to five most intense ions only will miss these peptides (see Materials and methods).

Our data suggest that LOC_Os03g55490 represents the plastid CKII alpha subunit in rice. This protein has an N-terminal extension that could function as a chloroplast transit peptide (Supplementary Fig. S1). The prediction of its sub-cellular localization is inconclusive, with WoLF_PSORT predicting a chloroplastic localization (Horton et al., 2007), whereas TargetP (Emanuelsson et al., 2007), iPSORT (Bannai et al., 2002), and Predotar (Small et al., 2004) predict a mitochondrial localization. To confirm the chloroplastic localization of this CKII alpha subunit, we fused the N-terminal 184 amino acids of LOC_Os03g55490 to GFP and transiently expressed the construct in rice protoplasts. Confocal microscopy revealed the chloroplastic localization of the GFP fusion protein indicated by the co-localization of GFP fluorescence and chlorophyll auto-fluorescence. The GFP control localizes to the cytosol (Fig. 3). Thus, our data show that the N-terminal extension of LOC_Os03g55490 functions as a plastid transit peptide *in vivo* and validate this protein as the rice plastid CKII that we henceforth refer to as OspCKII.

Identification of the RNP29 phosphorylation site used by OspCKII in vitro

We determined the phosphorylation site of OspCKII in RNP29 to assess the target motif preference for this enzyme in rice plastids. Recombinant RNP29 was expressed in

Table 1. Casein kinase II alpha subunits encoded in the rice genome

The subcellular localization for the different rice casein kinase II alpha sequences are based on: TargetP (Emanuelsson et al., 2007), WoLF PSORT (Horton et al., 2007), iPSORT (Bannai et al. 2002), and Predotar (Small et al., 2004).

Protein No.	TargetP	WoLF PSORT (KNN=14)	iPSORT	Predotar
LOC_Os07g02350.1	-	-	-	-
LOC_Os03g10940.1	-	-	-	-
LOC_Os03g55490.1	M	C (12)	M	M
LOC_Os03g55389.1	-	2	-	-

M, Mitochondrial; C, Chloroplast

Table 2. Identification of LOC_Os03g55490 as plastid casein kinase II alpha subunit in the heparin-Sepharose eluate fractions by inclusion mass scanning

Provided are the scoring details for the protein identification (upper panel) as well as the identification scores for the two identified peptides.

ProteinProphet Probability	Coverage	Unique peptides	Tot num. peps	MASCOT score
1	11.2%	2	2	61
Weight	Ion Charge	Peptide sequence	Nsp adj. prob.	MASCOT score
wt-1.00	2	VLYPTLLDYDIR	0.9714	39
wt-1.00	2	ILQNLYGGPNIVK	0.9719	36

E. coli and purified by Strep-Tag affinity chromatography. Isolated RNP29 was incubated with recombinant OspCKII, the reaction mixture was loaded onto an SDS gel, the RNP29 band was cut out and phosphopeptides were enriched with TiO₂ beads after tryptic digest. Using an LTQ-Orbitrap mass spectrometer, we identified two phosphopeptides with the same phosphorylation site assignment and MASCOT scores of 60.63 and 92.58 (Table 3). Figure 4 shows the spectra for the triply charged peptide ₅₁RLSPVAVAVSSEVEEEEGGAEpSEGFEFAEDLK₈₁ (Fig. 4). As expected for a phosphopeptide, the neutral loss of phosphate [M+3H-H₃PO₄]³⁺ with an *m/z* of 1078.2095 represents the predominant ion in the MS/MS spectra. To further support the phosphorylation site assignment, we used PhosCalc to evaluate the confidence level of the phosphorylation site identification. The PhosCalc scores for different phosphorylation site scenarios allow a clear distinction between the phosphorylation site determined by MASCOT and other potential phosphorylation sites within this peptide (Table 4) (MacLean et al., 2008). Both MASCOT and PhosCalc suggest Ser72 as the phosphorylation site used by recombinant OspCKII. This residue is located in a canonical CKII consensus motif E/D-S-E/D-X-E/D (Meggio and Pinna, 2003).

Rice phosphoproteomics and a meta-analysis of existing data shows the frequency of CKII motif utilization in vivo and OspCKII target proteins

Based on the identified OspCKII phosphorylation motif, we obtained information about the frequency at which this and similarly acidic motifs are used *in vivo*. We extracted plastid proteins from existing rice phosphoproteomics data and analysed their phosphorylation sites for CKII phosphorylation motifs using Motif-X (Nakagami et al., 2010). As the published rice phosphoproteomics studies were performed with rice cell cultures, we decided to generate an additional phosphoproteomics dataset from 10-day-old rice leaves to identify *in vivo* substrates of OspCKII under conditions of active photosynthesis. In this experiment, we identified 511 different phosphoproteins and 1152 spectra for phosphopeptides (Supplementary Table S2).

To extract plastid proteins from both datasets, we generated a chloroplast proteome reference table for rice using the *Arabidopsis* plastid reference proteome for a BLAST search against the rice genomedatabase (van Wijk and Baginsky, 2011).

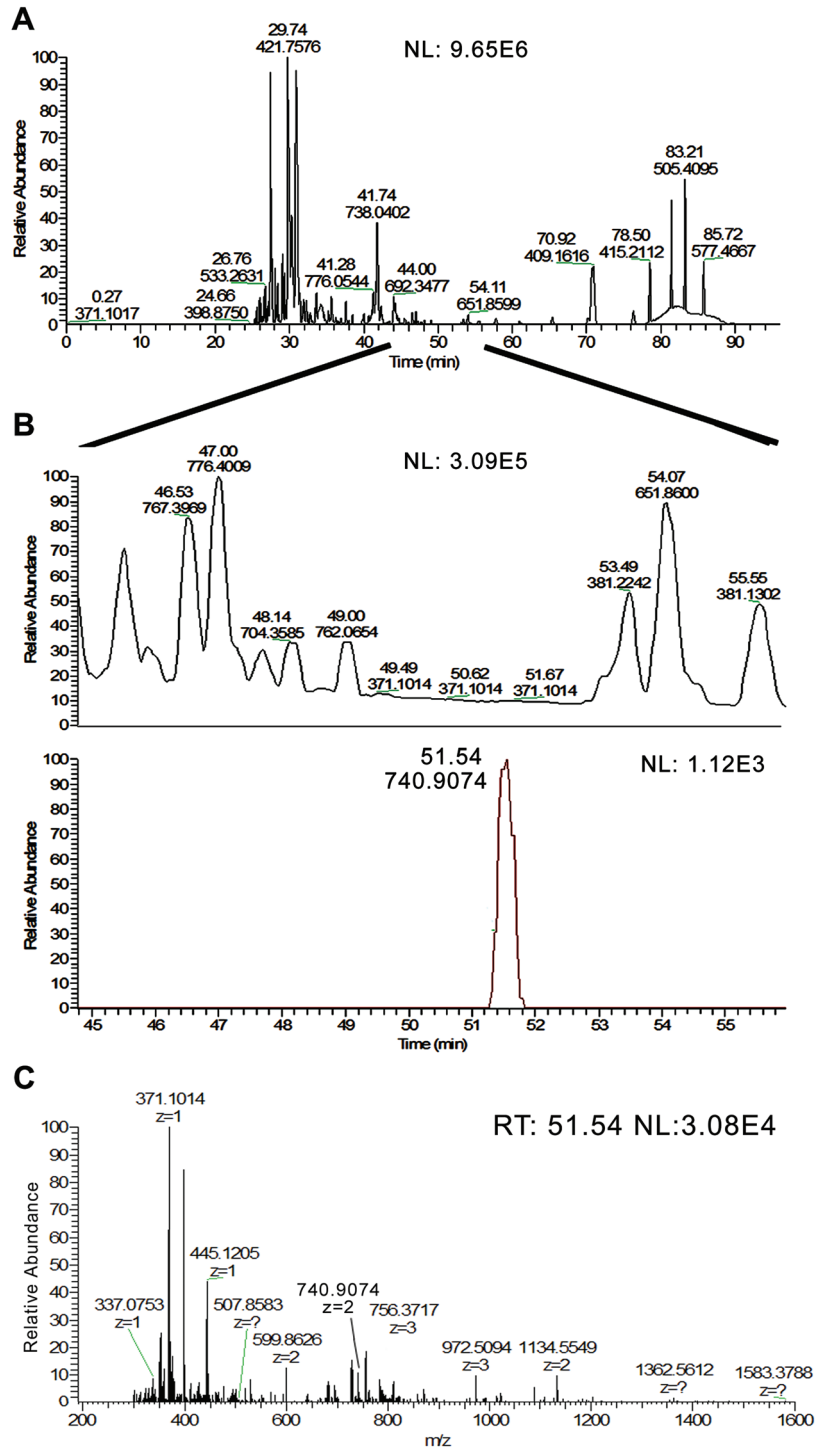


Fig. 2. Low-abundance of pCKII in the heparin-Sepharose eluate fraction. (A) Base-peak intensity chromatogram of peptide mixture. (B) Zoom-in on the base-peak chromatogram at the retention time window 45–55 min (upper panel). Plastid CKII was identified by low abundance peptide peaks such as the one illustrated here at m/z 740.9074 (lower panel). This peak is not visible in the base-peak chromatogram because it has background intensity (compare both panels). (C) The pCKII peptide at m/z 740.9074 is a minor peak at the retention time 51.54 minute. NL, the absolute intensity of the base peak.

We accepted all hits with an e -value below e^{-30} . The rice homologues for *Arabidopsis* plastid proteins were further analysed by TargetP for the presence of a plastid transit peptide. Only proteins with a predicted transit peptide were accepted for the rice proteome reference table. We are aware that TargetP may underestimate the number of rice chloroplast proteins because of the relatively high false-negative rate

of transit peptide prediction (between 15% and 30%), but for this dataset we prefer high specificity to high sensitivity (e.g. OspCKII is not represented in this list because TargetP predicts a mitochondrial transit peptide). This combined search resulted in a list of 1528 putative plastid proteins. We added to this list the plastome-encoded proteins and the established etioplast proteins identified in rice etioplast proteomic

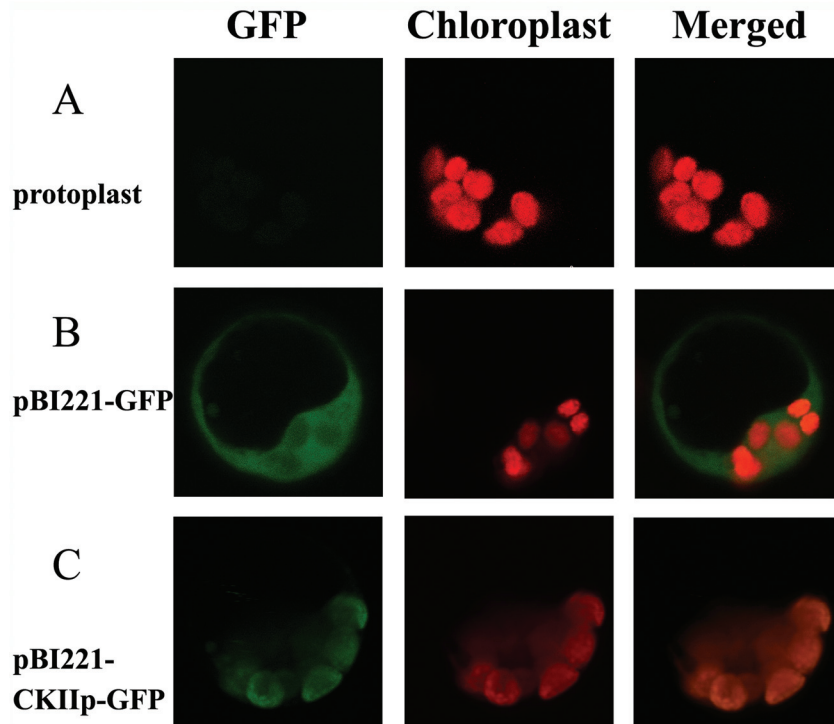


Fig. 3. Subcellular Localization of LOC_Os03g55490. The 184 N-terminal amino acids were fused to GFP and the construct was transformed into rice protoplasts. On the left, the green channel for the GFP, at the centre the red channel for chlorophyll autofluorescence and on the right, the merged images are shown. Here, orange indicates the co-localization of GFP and chlorophyll fluorescence. The following transformations were employed: (A) non-transformed rice protoplast, (B) GFP control (i.e. blank vector pBI221-GFP), (C) CKII alpha N-terminal 184 amino acids fused with GFP.

Table 3. Identification of phosphorylated peptides in the 29RNP preparation

Search results were obtained from MASCOT; the details of the identification are provided in the table. Abbreviations: m/z, mass over charge; z, charge state of the identified peptide; Δ mass, deviation between measured and predicted peptide mass.

m/z	z	Δ mass	Score	Expect	Peptide sequence
1587.1987	2	-0.003	60.63	2.3E-06	LSPVAVAVSSEVEEEEEGGAEpSEGEFAEDLK
1110.5029	3	-2E-04	92.58	1.9E-08	RLSPVAVAVSSEVEEEEEGGAEpSEGEFAEDLK

studies (Kleffmann *et al.*, 2007; von Zychlinski *et al.*, 2005). Altogether, the rice plastid reference proteome comprises 1806 protein entries (Supplementary Table S3).

Using this reference list, we identified 128 different plastid phosphoproteins in both datasets, 40 in the new dataset generated here and 105 in the previously published dataset (Supplementary Table S4) (Nakagami *et al.*, 2010). Thus, protein identifications in both sets are nearly complementary indicating differences in the phosphorylation of plastid proteins in cultured cells and leaf tissue. For example, our analysis identified several phosphoproteins with a role in photosynthesis that are missing from the cell culture dataset. Among these are several chlorophyll-binding proteins (LOC_Os03g39610, LOC_Os01g41710, LOC_Os01g52240, LOC_Os07g37240, LOC_Os09g17740), photosystem I reaction centre subunit II (LOC_Os08g44680), photosystem II 10 kDa polypeptide (LOC_Os07g05360), and phosphoglycerate kinase (LOC_Os05g41640). In sum, 205 different phosphopeptides were identified. Motif-X analysis identified the canonical CKII motif -S-D-X-E- at a 0.001 significance threshold in nine

phosphopeptides. The motif -S-E-X-D/E- is not significantly enriched but present in seven additional peptides. These two acidic motifs represent suitable CKII phosphorylation sites and we therefore suggest that these are potentially targeted by OspCKII *in vivo*. The putative OspCKII target peptides are derived from 13 proteins. Several of the proteins are involved in plastid gene expression such as FLN1 and FLN2, which are components of the TAC, the DEAD-box RNA helicase RH3, a PPR protein, and the chloroplast group IIA intron splicing facilitator CRS1 (Table 5). With the exception of FLN2 and RH3, none of the other OspCKII targets has a homologue in *Arabidopsis* that is phosphorylated at CKII motifs according to PhosPhAT (<http://phosphat.uni-hohenheim.de/>). Although the phosphorylation sites of FLN2 in rice and *Arabidopsis* are similar (S132/133 in *Arabidopsis* versus S159 in rice), the phosphorylation of RH3 occurs in different positions of the protein, i.e. S80 in *Arabidopsis* and S670 in rice.

We matched the list of putative pCKII targets in rice with the list of their *Arabidopsis* homologue(s) and assessed the conservation of the pCKII phosphorylation site in these two

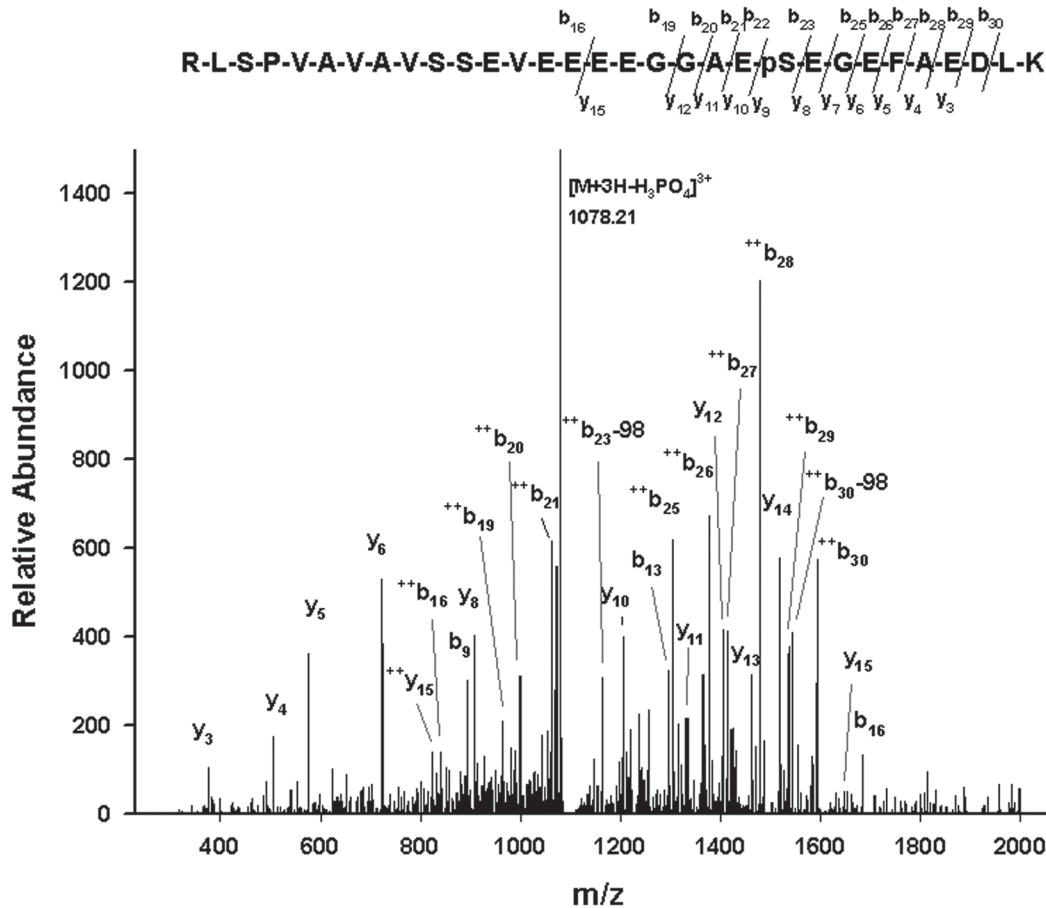


Fig. 4. Identification of the RNP29 phosphopeptide and the phosphorylation site used by recombinant OspCKII. Shown is the MS/MS spectrum of peptide ${}_{51}\text{RLSPVAVAVSSEVEEEE}GGA\text{E}_p\text{SEGEFAEDLK}_{81}$. The neutral loss of phosphate from the triply charged precursor ion at an m/z ratio of 1110.5029 is the base peak in the spectrum; other b- and y-ions are indicated.

Table 4. PhosCalc Results for the phosphorylation site assignment of RNP29

The highest PhosCalc score is given to the most likely phosphorylation site assignment.

Phosphosite variant	Ions	Matched ions	P-value	Score
RLSPVAVAVSSEVEEEE $GGA\text{E}_p\text{SEGEFAEDLK}$	37	18	5.59e-016	153
RLSPVAVAVS[S]EVEEEE $GGA\text{E}G\text{EFAEDLK}$	36	10	9.22e-007	60
RL[S]PVAVAVSSEVEEEE $GGA\text{E}S\text{EGEFAEDLK}$	36	6	2.34e-003	26
RLSPVAVAV[S]SEVEEEE $GGA\text{E}S\text{EGEFAEDLK}$	36	10	9.22e-007	60
LSPVAVAVSSEVEEEE $GGA\text{E}_p\text{SEGEFAEDLK}$	37	18	5.59 e-016	153
LSPVAVAVS[S]EVEEEE $GGA\text{E}S\text{EGEFAEDLK}$	37	10	1.21e-006	59
LSPVAVAV[S]SEVEEEE $GGA\text{E}S\text{EGEFAEDLK}$	37	9	1.04e-005	50
L[S]PVAVAVSSEVEEEE $GGA\text{E}S\text{EGEFAEDLK}$	37	7	4.96e-004	33

'Ions', the number of ions predicted from the peptide sequence (b and y); 'Matched ions', the number of predicted ions that have a mass that matched the masses in the data file; 'P-value', the likelihood of this number of matches occurring by chance; 'Score', phosphorylation site score.

species. This analysis revealed that pCKII phosphorylation sites are not strictly conserved, the chloroplast group IIA intron splicing facilitator CRS1 being the exception (Fig. 5). As pCKII phosphorylates components of the transcription machinery, we furthermore compared identified pCKII phosphorylation sites in TAC5, TAC10, and FLN2 in these two plant species. As discussed above, the TAC subunit FLN2 is phosphorylated at CKII sites in rice and *Arabidopsis* at similar positions (Fig. 5). In *Arabidopsis*, putative pCKII targets

are TAC10 and TAC5, which were not found phosphorylated in rice (Reiland *et al.*, 2009). We aligned the TAC5, TAC10, and FLN2 protein sequences of the rice and *Arabidopsis* homologues to assess whether these subunits can potentially be phosphorylated by pCKII in other species. In case of TAC5 and TAC10, the established *Arabidopsis* CKII phosphorylation sites are not conserved, not even between the two dicots pea and *Arabidopsis* (Fig. 6). In case of FLN2, the potential phosphorylation site is also not strictly conserved

Table 5. *Rice plastid proteins phosphorylated at canonical CKII motifs*

The phosphorylation site assignment was based on MASCOT (this study, [Nakagami et al., 2010](#)).

Identifier	Annotation	Peptide
LOC_Os01g63220	Kinase, pfkB family, FLN1	NTQE ρ SD ρ SEGE ρ EEEEPPK
LOC_Os03g24180	Pleckstrin homology	VHADD ρ SDAERSEDAK
LOC_Os03g32630	ABC transporter	APASSYMGDIDLPP ρ SDEEEEDADLVAMATKPK; APASSYMGDIDLPP ρ SDEEEEDADLVAoxMATKPK
LOC_Os03g40550	Kinase, pfkB family, FLN2	ρ TVEELSDNEGEDLGEDLVPSNDR
LOC_Os03g50120	Zinc finger family protein	SFDDSFREDDDG ρ SDPDEGER; SR ρ SFDDSFREDDDGSDPDEGER; SR ρ SFDD ρ SFREDDDGSDPDEGER; SRSFDD ρ SFREDDDGSDPDEGER
LOC_Os03g61220	DEAD-box RNA helicase 3 (RH3)	GGWD ρ SDGEDRFR
LOC_Os03g61890	Toc159	FGLGD ρ SDDEFHDDVEE ρ EEGEVNGK
LOC_Os04g33300	Amino acid kinase	ALVA ρ SDDDNKPR
LOC_Os04g43140	DEAD-box RNA helicase	NAESMGLLLET ρ SD ρ SEEEERVQGHK
LOC_Os04g50204	Transport protein-related	DGATNGSHKEESNVKGD ρ SEDDEPVMVAK; DGATNGSHKEESNVKGD ρ SEDDEPVoxMVAK; EESNVKGD ρ SEDDEPVMVAK
LOC_Os05g05480	OsDegp6	ESQV ρ SDFEDLEEDK
LOC_Os05g22870	PPR protein	FLGQDDAD ρ SDADEEE ρ E ρ EW
LOC_Os11g37990	CRS1	LDSAY ρ S ρ SDEEDVEDEDDEAYLR; LDSApY ρ S ρ SDEEDVEDEDDEAYLR



Fig. 5. Alignment of different rice plastid proteins that are phosphorylated at putative pCKII phosphorylation sites with their *Arabidopsis* homologue. The alignment was performed with ClustalOmega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), shown is an excerpt of the sequence containing the determined phosphorylation site.

TAC10

Arabidopsis	ILGDY EANPAKYEGKKLSELSDDE * DFDEQKDIEYGEAYYKKTLPKVIKTS-VKELDLE	472
Pea	ILDDMA INPDKYEGKKLSDLVDEE	471
Rice	ILDHEEENPDKFKDTTYESTVDSSIVHEENRVQHTEGYFKDTLLKKEVVISRSLIWMLPV	464
Maize	ILDYEEQNPDKFKNTTYESTVDAP	468
	** ** ** * . . . * . . * . . : : : : * . * * : : : *	

FLN2

Arabidopsis	EEISEFLVDNDDVLDKESIV-SALKPKKTRTRKKA AASSDV--- * EEVKTEKKVRRKRTV	150
Pea	GDTDD-----ASIEGSSAAASDSSKTRTRTRKKGASSAGLEEKKEVKEKKVRRRKKT	147
Rice	GETQ-----EGQVSTEEDESPEGTKKIKRRGRKKAATTASSEEKDKAKEPKKRGRRKVK	153
Maize	DVGE-----EIEVIT-EQTAAEETKEVKRRGRKKAATSASSEEEKDKAKEPKKRGRRKVK	218
 * **.* : : : . . . * ** * : : .	

TAC5

Arabidopsis	VMTAELLQRLFMD EDVETD * -----KDEASTMKKEEAGNGAVFSTS ^Q VPEKKQSIVKDQS	287
Pea	IMTAELLERLYLEIRNKDTGSATQDKQSTTVLPKEVENGAAVASVAENSEGQQKDV--KS	281
Rice	IMTSDLLDMLFTGQVG-----QDVKTGDINGAAIPSITEIAEIQQTVVKGNG	266
Maize	VMTSELLEWLFSGKTG-----EEVKTKDGTNGAAVPAVTGIAEVKRKTAVAENG	241
	: ** : : ** : * : : : : ** . . : : : * : : . * : :	

Fig. 6. Multiple sequence alignment of three TAC subunits that are pCKII substrates in *Arabidopsis* chloroplasts. We aligned the *Arabidopsis* sequences of FLN2, TAC10, and TAC5 with their closest homologues from pea, rice, and maize with ClustalOmega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Shown is an excerpt of the sequences containing the pCKII phosphorylation sites in *Arabidopsis*.

but a serine residue surrounded by acidic amino acids is found in similar sequence positions in the two monocots rice and maize but not in pea. Thus, our comparison shows that CKII phosphorylation sites within TAC components are not conserved suggesting that the details of phosphorylation-mediated transcriptional regulation are species-specific.

Discussion

Protein identification in heparin-Sepharose enriched fractions

We identified rice plastid CKII (OspCKII) in heparin-Sepharose-enriched protein fractions by inclusion mass scanning (Fig. 2, Table 2). OspCKII co-purifies with the TAC, similar to what has been reported for mustard (Baginsky *et al.*, 1997). Despite our sensitive analysis, we could not directly identify the OspCKII enzyme and therefore applied inclusion mass scanning to specifically screen for its peptides. This mass spectrometry technique is also known as accurate inclusion mass scanning (AIMS) and has been used successfully for the identification of low-abundance proteins in different species, including the detection of phosphorylated peptides and proteins (Courcelles *et al.*, 2012; Jaffe *et al.*, 2008). Using our mass spectrometry data, we show that OspCKII is of very low abundance even after heparin-Sepharose chromatography enrichment. This conclusion is based on the comparison between the enrichment of OspCKII activity and the low peptide intensity in the MS full scan (Figs 1 and 2). Thus, an untargeted approach will most likely miss the identification of OspCKII peptides, explaining why pCKII has not been detected in other proteomics approaches (Olinares *et al.*, 2010). Targeted proteomics approaches such as the AIMS method applied here or the more widely applied selected reaction monitoring (SRM) are the key to increasing proteome coverage and the quality of proteomics data (Picotti *et al.*, 2013).

At the same time, however, targeted methods are so sensitive that minor contaminants may be detected in apparently pure organelle preparations. We are aware that this could result in a wrong interpretation of the localization of LOC_Os03g55490. However, our data support the plastid localization of OspCKII at different levels. First, we enriched CKII activity from chloroplast extracts by heparin-Sepharose chromatography. Second, LOC_Os03g55490 is imported into chloroplasts as a GFP fusion protein (Fig. 3) suggesting that its N-terminal extension (Supplementary Fig. S1) functions as chloroplast transit peptide (Fig. 3). Third, chloroplast phosphoproteomics shows that canonical CKII target motifs are phosphorylated in rice chloroplasts *in vivo*, providing an indirect argument for the localization of LOC_Os03g55490 in chloroplasts. None of the points raised above is fully convincing by itself, but in combination, our data clearly support the chloroplastic localization of OspCKII.

The target protein spectrum of pCKII is largely species specific

OspCKII activity has the same properties as the homologous enzyme from dicotyledonous species. For example, it uses GTP as phosphate donor and its activity is inhibited by low concentrations of heparin (Fig. 1). The fact that poly-lysine inhibits its activity suggest that rice plastid CKII is a α -subunit that does not associate with a regulatory β -subunit, because the holoenzyme complex (α , α' , 2 β) is stimulated by poly-lysine rather than inhibited (Pinna, 2002; Montenarh, 2010; Venerando *et al.*, 2014). In *Arabidopsis*, none of the four nucleus encoded β -subunits localizes to plastids (Salinas *et al.*, 2006). The rice genome encodes for two β -subunit homologues that clearly lack an N-terminal extension that could function as a plastid transit peptide (Supplementary Fig. S2). Together these data suggest that plastid CKII occurs as α -subunit in both monocots and dicots.

A comparison of pCKII target proteins in rice and *Arabidopsis* suggests that the details of pCKII regulation differ between these two species and in many cases, pCKII phosphorylation sites are not conserved. In case of the rice Deg6 pCKII phosphorylation site, Ser is exchanged by Thr and the acidic amino acid in the +3 position is missing in *Arabidopsis* (Fig. 5). In CRS1, the CKII phosphorylation site seems to be conserved but S801/802 were not found phosphorylated in *Arabidopsis* phosphoproteomics experiments, despite much higher sampling depth. An interesting case is FLN2 because the *Arabidopsis* enzyme carries glutamic acid in the position of the phosphorylated serine in rice. Acidic amino acids are considered phosphomimetic, suggesting that this position may be of regulatory importance (Beltrao *et al.*, 2013; Facette *et al.*, 2013; Rocha *et al.*, 2014). The *Arabidopsis* pCKII substrates TAC5 and TAC10 were not found phosphorylated in rice or maize and the *Arabidopsis* phosphorylation sites are not conserved, not even in the dicotyledonous plant pea (Facette *et al.*, 2013) (Fig. 6). Similarly, TAC12 was found phosphorylated in maize but not in *Arabidopsis* or rice (Facette *et al.*, 2013). Although none of the phosphoproteome analyses is saturated and more proteins will be found phosphorylated under different conditions at different positions, our analysis nevertheless provides initial information on the details of phosphorylation control by pCKII in different plant species.

The examples discussed above illustrate the complexity during the evolution of phosphorylation signalling. Different scenarios must be distinguished such as the utilization (vs. non-utilization) of candidate phosphorylation sites, the substitution of phosphomimetic acidic amino acids by amino acids that can be phosphorylated, the phosphorylation stoichiometry, and the use of phosphorylation sites under different conditions. An increasing number of reports suggest that the evolutionary constraints on phosphorylation sites are rather small and that their divergence between different species seems to be the rule rather than the exception (Beltrao *et al.*, 2013; Nakagami *et al.*, 2010). It is possible to interpret the 'lack of conservation' as 'lack of functionality', but this is a simplification of a significantly more complex biological process. For example, phosphomimetic amino acids in positions of phosphorylated amino acids in different species may be particularly indicative of functional relevance as demonstrated recently for plastid transketolase (Rocha *et al.*, 2014). Furthermore, phosphorylation sites may occur in other protein positions in different species. Comparative analysis of phosphorylation site utilization in different species and under different environmental conditions are important next steps for understanding signal transduction in a cellular context.

Supplementary data

Supplementary data are available at *JXB* online

[Table S1](#). Identification of proteins in the heparin-Sepharose eluates

[Table S2](#). Phosphoproteomics Data obtained with protein extracts from rice leaf tissue

[Table S3](#). Rice plastid reference proteome

[Table S4](#). Chloroplast phosphoproteins in rice

[Fig. S1](#). Alignment of rice CKII α -subunits

[Fig. S2](#). Alignment of rice CKII β -subunits

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