Published in final edited form as:
J Exp Bot. 2012 February ; 63(4): 1713–1723. doi:10.1093/jxb/err377.

Chloroplast-localized protein kinases: a step forward towards a complete inventory

Roman G. Bayer[1], **Simon Stael**[1], **Agostinho G. Rocha**[2], **Andrea Mair**[1], **Ute C. Vothknecht**[2,3], and **Markus Teige**[1,*]

[1]Department of Biochemistry and Cell Biology, MFPL, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria

[2]Department of Biology I, Botany, LMU Munich, Großhaderner Str. 2, D-82152 Planegg-Martinsried, Germany

[3]Center for Integrated Protein Science (Munich) at the Department of Biology of the LMU Munich, D-81377 Munich, Germany

Abstract

In addition to redox regulation, protein phosphorylation has gained increasing importance as a regulatory principle in chloroplasts in recent years. However, only very few chloroplast-localized protein kinases have been identified to date. Protein phosphorylation regulates important chloroplast processes such as photosynthesis or transcription. In order to better understand chloroplast function, it is therefore crucial to obtain a complete picture of the chloroplast kinome, which is currently constrained by two effects: first, recent observations showed that the bioinformatics-based prediction of chloroplast-localized protein kinases from available sequence data is strongly biased; and, secondly, protein kinases are of very low abundance, which makes their identification by proteomics approaches extremely difficult. Therefore, the aim of this study was to obtain a complete list of chloroplast-localized protein kinases from different species. Evaluation of protein kinases which were either highly predicted to be chloroplast localized or have been identified in different chloroplast proteomic studies resulted in the confirmation of only three new kinases. Considering also all reports of experimentally verified chloroplast protein kinases to date, compelling evidence was found for a total set of 15 chloroplast-localized protein kinases in different species. This is in contrast to a much higher number that would be expected based on targeting prediction or on the general abundance of protein kinases in relation to the entire proteome. Moreover, it is shown that unusual protein kinases with differing ATP-binding sites or catalytic centres seem to occur frequently within the chloroplast kinome, thus making their identification by mass spectrometry-based approaches even more difficult due to a different annotation.

Keywords

Casein kinase; chloroplast protein kinase; organellar proteomics; photosynthesis; STN7; STN8; subcellular localization; YFP fusion protein

*To whom correspondence should be addressed. markus.teige@univie.ac.at.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Localization analysis of predicted chloroplast-localized protein kinases.

Table S1. Overview of predicted chloroplast-localized protein kinases.

Europe PMC Funders Author Manuscripts

Introduction

Chloroplasts are essential plant organelles of endosymbiotic origin that carry out a wide range of important metabolic pathways such as photosynthesis, and the biosynthesis of amino acids, vitamins, and lipids. These processes require tight regulation and coordination with the metabolic state of the whole plant. To this end, chloroplasts are integrated into the signalling network of the entire cell. In addition to the predominant redox regulation in chloroplasts (Montrichard *et al.*, 2009), reversible protein phosphorylation is a key mechanism for the regulation of cellular processes and for signal transduction in response to environmental changes (Cohen, 2000). Phosphorylation can influence the activity, the subcellular localization, or the stability of target proteins (Stone and Walker, 1995; Cohen, 2000; Schliebner *et al.*, 2008). Plant protein kinases are involved in the regulation of metabolism, cell division, growth, and differentiation, and they mediate cellular responses to biotic and abiotic stress including changing light conditions, altered temperatures, and pathogen invasion (Stone and Walker, 1995; Halford *et al.*, 2004). In eukaryotic genomes it is estimated that 1–3% of all genes encode protein kinases (Stone and Walker, 1995). In the human genome, 518 out of the ~22 200 proteins are annotated as protein kinases (2.3%) (Manning *et al.*, 2002; Orchard *et al.*, 2005), and the nuclear genome of the model plant *Arabidopsis thaliana* (~27 400 protein-coding genes) encodes ~1050 different protein kinases (3.8%) (Gribskov *et al.*, 2001; Wang *et al.*, 2003; Martin *et al.*, 2009). This slightly higher number in *Arabidopsis* is probably a result of multiple gene duplications that are generally found in plant genomes (Chevalier and Walker, 2005). No protein kinases are encoded in the chloroplast genome (TAIR; www.arabidopsis.org).

Historically, the first report of protein phosphorylation within chloroplasts dates back to the 1970s, when phosphorylation of light-harvesting complex (LHC) proteins was shown (Bennett, 1977). Stromal protein phosphorylation was discovered in 1983, when pyruvate, orthophosphate dikinase was found to be inactivated by phosphorylation in *Zea mays* chloroplasts (Ashton and Hatch, 1983). Since that time, more and more targets of protein phosphorylation have been identified in chloroplasts, using different techniques such as incubation of chloroplast protein extracts with radioactively labelled ATP or detection with phosphothreonine-specific antibodies (Laing and Christeller, 1984; Foyer, 1985; Rintamaki *et al.*, 1997). Given that ~2100 proteins are estimated to be imported into the chloroplast based on targeting prediction (Richly and Leister, 2004) and 3.8% of all *Arabidopsis* proteins encode protein kinases, at least 80 chloroplast protein kinases could be expected (i.e. 3.8% of 2100). However, to date, only a handful of chloroplast-localized protein kinases have been reported in the literature. The most thoroughly described examples are the protein kinases Stt7 and Stl1 from *Chlamydomonas reinhardtii* and their *Arabidopsis* orthologues STN7 (At1g68830) and STN8 (At5g01920) (Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005). These kinases were first discovered in a genetic screen in *Chlamydomonas* aiming at the identification of regulatory factors for photosynthetic acclimation under changing light conditions, a process called state transition (Depege *et al.*, 2003). These kinases are localized within the thylakoid membrane and phosphorylate photosystem II (PSII) core and LHCII proteins resulting in the redistribution of LCHIIs. Analysis of fluorescence recovery after photobleaching revealed that a small portion of chlorophyll (~15%) could move between grana stacks within a 10 min time scale in an STN7-dependent manner (Goral *et al.*, 2010). The phosphorylation of PSII also leads to visible macroscopic changes in the folding of thylakoid membranes. The lack of phosphorylation in the *stn7/stn8* double mutant results in an increased size of appressed thylakoid membranes (Fristedt *et al.*, 2009). STN7 was recently shown to provide a link between short-term (state transition) and long-term (nuclear gene expression) acclimation to fluctuating light conditions (Pesaresi *et al.*, 2009). This long-term acclimation also includes a readjustment of distinct metabolic states (Brautigam *et al.*, 2009). All together these processes optimize photosynthesis under

fluctuating light conditions, and, accordingly, the phenotype of *stn7* mutants becomes particularly obvious under such growth conditions (Brautigam *et al.*, 2009; Tikkanen *et al.*, 2010).

Another well-described chloroplast protein kinase is the chloroplast casein kinase 2 (cpCK2), which was first identified in *Sinapis alba*. Later, the *Arabidopsis* homologue (At2g23070) was found to be associated with the plastid RNA polymerase and to phosphorylate parts of the transcription machinery and RNA-binding proteins (Ogrzewalla *et al.*, 2002; Salinas *et al.*, 2006). The plastid sigma factor AtSIG6 is phosphorylated by cpCK2 at different sites. This results in specific and efficient promoter binding of the polymerase, which leads to altered expression of chloroplast genes (Schweer *et al.*, 2010). Recently, the chloroplast sensor kinase CSK (AT1G67840), which is a prokaryotic-like two-component histidine kinase, was shown to couple photosynthesis to chloroplast gene expression via redox signals (Puthiyaveetil *et al.*, 2008) and was identified as an interaction partner of cpCK2 (Puthiyaveetil *et al.*, 2010). Furthermore, studies in *Pisum sativum* and *Spinacia oleracea* provided evidence for the presence of protein kinases in the outer envelope of chloroplasts (Soll, 1985, 1988; Soll *et al.*, 1988; Siegenthaler and Bovet, 1993). The importance of phosphorylation in chloroplasts is further highlighted by the results of a recent phosphoproteomic study in *Arabidopsis* (Reiland *et al.*, 2009). In this study, a total of 353 unique phosphosites was identified in 174 proteins, which were annotated as chloroplast proteins with high confidence. These proteins included LHC proteins, proteins involved in RNA binding and carbohydrate metabolism, photosystem core subunits, and STN7.

In order to understand chloroplast function fully it is crucial to obtain a complete list of protein kinases involved in the regulation of chloroplast processes. Here, the aim is to compile a complete inventory of chloroplast-localized protein kinases according to the current state of knowledge. To this end, the literature was screened for all experimentally verified chloroplast protein kinases, and experimental reports of protein kinases with assigned chloroplast localization were evaluated by analysis of yellow fluorescent protein (YFP) fusion proteins, including also candidates which have been identified only with low confidence in proteomics studies, and candidates selected from bioinformatics-based approaches.

Materials and methods

Plant growth and chloroplast preparations

Arabidopsis thaliana (var. Columbia Col-0) was grown on soil at 22 °C under a 16 h/8 h photoperiod at 150 μmol photons m^{-2} s^{-1} for 7 weeks. Chloroplasts were purified as described previously (Seigneurin-Berny *et al.*, 2008) and stored at −80 °C until further use.

Pisum sativum (cultivar Arvika) plants were grown under a 16 h light/8 h dark photoperiod at 21/16 ° (light/dark) at 250 μmol photons m^{-2} s^{-1}. Chloroplasts were isolated from leaves of 10- to 12-day-old peas as described previously (Waegemann and Soll, 1995).

Preparation of membrane and soluble Arabidopsis chloroplast proteins

Chloroplasts were disrupted by lysis in kinase buffer [20 mM Tricine pH 7.6, 10% glycerol, 0.5% dithiothreitol (DTT)], supplemented with protease inhibitors (complete EDTA free; Roche) and phosphatase inhibitors (Phospho-Stop; Roche). After incubation on ice for 15 min, membranes and soluble components were separated by centrifugation at 60 000 *g* for 10 min. To remove loosely associated proteins, the membrane fraction was washed with lysis buffer containing 0.8 M NaCl and re-suspended in lysis buffer. The 60 000 *g* supernatants of the first and second centrifugation were combined, desalted via a PD10

column (GE Healthcare), and concentrated using Vivaspin 500 spin columns (3 kDa cut-off, GE Healthcare). All operations were carried out either on ice or at 4 °.

Protein phosphorylation assays in Arabidopsis

A standard phosphorylation assay (50 μl), using soluble or total membrane proteins, was carried out in lysis buffer supplemented with 2 mM EGTA and 10 mM $MgCl_2$. For the measurement of ATP-dependent phosphorylation, 5 μM cold ATP and 2–5 μCi of [γ-^{32}P]ATP (3000 Ci mmol $^{-1}$, Perkin Elmer, Waltham, MA, USA) were included in the assay. Control assays for ATP-dependent phosphorylation were carried out using recombinant CPK4 (At4g09570) with histone (S3, Sigma) as substrate. Due to the calcium dependency of CPKs (calcium-dependent protein kinases), the assay included 5 mM $CaCl_2$ instead of EGTA. For the measurement of GTP-dependent phosphorylation the assay contained 5 μM cold GTP and 2–5 μCi of [γ-^{32}P]GTP (6000 Ci mmol $^{-1}$, Perkin Elmer). Control assays for GTP-dependent phosphorylation were performed using recombinant CK2 (α2, human, Calbiochem) with dephosphorylated casein (from bovine milk, Sigma Aldrich) as substrate. All reactions were carried out for 25 min at room temperature and stopped by the addition of 12 μl of 4× SDS sample buffer.

For the analysis of calcium-dependent phosphorylation in chloroplasts, stromal extracts were incubated with 0.1 μl of [γ-^{32}P]ATP (6000 Ci mmol $^{-1}$, 10 Ci ml $^{-1}$; Perkin Elmer), 4 μl of 5× kinase buffer, and 5 mM $CaCl_2$ or 5 mM EGTA in a total volume of 20 μl. The kinase reactions were incubated for 20 min at room temperature and subsequently analysed by SDS–PAGE. The incorporation of γ-^{32}P into proteins was analysed using a Storage Phosphor Screen and a Typhoon Trio Imager by the software ImageQuant (GE Healthcare).

Total leaf protein extraction from pea seedlings

Leaves of 7-day-old *P. sativum* seedlings were homogenized in extraction buffer [330 mM sorbitol, 20 mM MOPS, 13 mM TRIS, 0.1% bovine serum albumin (BSA), 3 mM $MgCl_2$] using a Waring blender. After filtration through four layers of Miracloth (Merck) and centrifugation for 2 min at 2314 *g* and 4 °C, the supernatant was buffer exchanged to buffer A (50 mM TRIS pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$) using PD-10 Desalting Columns (GE Healthcare). The final protein extract was stored at 4 °C until further usage.

Stromal protein extraction from pea chloroplasts

Stromal protein extraction from isolated chloroplasts of pea seedlings and subsequent depletion of RuBisCO by size exclusion chromatography was carried out as previously described (Bayer *et al.*, 2011).

Protein phosphorylation assays and inhibitor studies in pea

For the analysis of calcium-dependent phosphorylation, stromal extracts were incubated with 0.1 μl of [γ-^{32}P]ATP (6000 Ci mmol $^{-1}$, 10 Ci ml $^{-1}$; Perkin Elmer), 4 μl of 5× kinase buffer, and $CaCl_2$ (0.010–10 mM) or 5 mM $MgCl_2$ in the presence or absence of 5 mM EGTA in a total volume of 20 μl. For protein kinase inhibitor experiments, total pea leaf or stromal protein extracts were mixed with 0.1 μl of [γ-^{32}P]ATP, 4 μl of 5× kinase buffer (100 mM HEPES, 75 mM $MgCl_2$, 1 mM DTT, pH 7.5 with KOH), and 2.5 μl of 800 μM purvalanol B (PurB; in dimethylsulphoxide; Tocris Bioscience) in a total volume of 20 μl. All kinase reactions were incubated for 20 min at room temperature. Subsequently, 8 μl of 4× SDS-PAGE buffer (Laemmli, 1970) were added to each reaction mix and 8 μl were separated on a 12% SDS gel. After Coomassie staining/destaining and drying of the gel, the incorporation of γ-^{32}P into proteins was analysed using a Storage Phosphor Screen and a Typhoon Trio Imager by the software ImageQuant (GE Healthcare).

Subcellular localization analysis of candidate proteins

All candidate protein kinases were cloned in front of YFP in the expression vector pBIN19, expressed in tobacco epidermal cells, and subsequently examined for their subcellular localization by confocal laser scanning microscopy as described previously (Benetka *et al.*, 2008).

Results and Discussion

Selection and localization analysis of candidate protein kinases

First, the aim was to identify novel chloroplast-localized protein kinases by using a bioinformatics-based approach. Therefore, 10 protein kinases with clear prediction for chloroplast localization by different prediction methods (Supplementary Table S1 available at *JXB* online) were cloned and their subcellular localization as YFP fusion proteins in infiltrated tobacco leaves was investigated. Unexpectedly, a chloroplast localization could not be confirmed for any kinase tested (Supplementary Fig. S1), thus underpinning the previous observation that the prediction of chloroplast targeting is particularly biased for protein kinases, which is in many cases due to interference with N-terminal protein acylation (Mehlmer *et al.*, 2010;Stael *et al.*, 2011). Similar results were previously reported in an analysis of protein kinases, which were also predicted for chloroplast targeting by several prediction programs (Schliebner *et al.*, 2008). Also, in that study, chloroplast localization could only be verified for two out of the nine tested candidates. Combining both studies, in total only two out of 18 protein kinases with a firm prediction for chloroplast localization by different programs could be experimentally confirmed. This result is in strong contrast to the known specificity of ~80% of those targeting prediction programs (Emanuelsson and von Heijne, 2001;Richly and Leister, 2004; Zybailov *et al.*, 2008).

Secondly, available organellar proteomic databases and the literature were searched for experimental evidence of chloroplast-localized protein kinases from *Arabidopsis* and other plant species, and verification of the localization of the *Arabidopsis* proteins was sought by YFP fusion analysis. cpCK2 was used as positive control and the following 10 protein kinases were selected: At2g01210, At3g44610, At4g32250, ABC1K1 (At1g71810), ABC1K2 (At1g79600), ABC1K3 (At4g31390), AtDSK1 (At3g13690), AtSK41 (At1g09840), AKIN10 (At3g01090), and AKIN11 (At3g29160) (Fig. 1). At2g01210 and At4g32250 were assigned to chloroplasts in mass spectrometric studies (Dunkley *et al.*, 2006; Zybailov *et al.*, 2008; Ferro *et al.*, 2010), and At3g44610 is listed in the plastid database plprot (Kleffmann *et al.*, 2006). In the Plant Proteome Database (PPDB) six members of the ABC1 family are annotated as putative plastid protein kinases (ABC1K1–ABC1K6) in addition to the known chloroplast protein kinases STN7, STN8, and cpCK2 (Sun *et al.*, 2008). However, according to the protein domain database PROSITE (www.expasy.org/prosite), ABC1K4 (At5g05200) and ABC1K5 (At5g64940) do not contain a protein kinase domain and were therefore excluded from further studies. The ABC1 kinases were identified in proteomic studies of plastoglobules (Ytterberg *et al.*, 2006; Zybailov *et al.*, 2008). These are lipoprotein particles inside chloroplasts, which function in the storage and synthesis of vitamin E, lipids, and quinones (Vidi *et al.*, 2006). In addition to the protein kinase domain, these kinases contain an ABC1 domain, which was first described in the yeast mitochondrial ABC1 protein in the regulation of the bc1 complex (Bousquet *et al.*, 1991).

Further reports of chloroplast protein kinases from other organisms include MsK4 from *Medicago sativa* and NtDSK1 from *Nicotiana tabacum*. MsK4 was found to localize to starch granules and to be involved in the regulation of carbohydrate metabolism in response to salt stress (Kempa *et al.*, 2007). NtDSK1 is a dual-specificity protein kinase and its

expression is regulated in response to light, suggesting a role in chloroplast light signalling (Cho *et al.*, 2001). The closest *Arabidopsis* homologues of MsK4 and NtDSK1, AtSK41 and AtDSK1, respectively, were analysed. Further, the SNF1-related protein kinases AKIN10 and AKIN11, which were recently reported to be chloroplast localized (Fragoso *et al.*, 2009), were also analysed. Finally, YFP alone was included as a negative control.

Chloroplast localization, indicated by an overlap of the YFP signal and the chlorophyll autofluorescence, could only be confirmed for the positive control cpCK2 and the three ABC1 family protein kinases. In contrast, At2g01210, At3g44610, and At4g32250 seemed to localize to the plasma membrane, and AtDSK1 and AtSK41 to the plasma membrane and the cytoplasm. AKIN10 and AKIN11 were found in the cytoplasm as well as in the nucleus (Fig. 1), which is in perfect agreement with their proposed function as central regulators of energy metabolism in response to starvation and stress (Baena-Gonzalez *et al.*, 2007), and with the localization of their substrates (Baena-Gonzalez *et al.*, 2007; Jossier *et al.*, 2009). The negative control YFP was observed in the nucleus and in the cytoplasm.

A current survey of chloroplast-localized protein kinases

To summarize the current state of knowledge on chloroplast-localized protein kinases, a revised survey was created by integrating the data obtained in this study and data extracted from the current literature. The search was extended to all chloroplast-containing organisms so far investigated (Table 1). It is important to note that the chloroplast localization of a protein kinase was only considered to be true if compelling experimental evidence such as immunolocalization or fluorescent protein fusion studies, or chloroplast import assays were found. Sole identification in a chloroplast proteomic study was not accepted as a proof of localization, due to the high risk of detecting contaminations with the sensitive mass spectrometry instruments (Baginsky, 2009).

In a similar survey from 2008 (Schliebner *et al.*, 2008), the seven protein kinases STN7, STN8, cpCK2, AtRP1 (At4g21210), TAK1 (At4g02630), At1g51170, and CIPK13 (At2g34180) were listed. AtRP1 was classified as a protein phosphatase, exhibiting both protein kinase and phosphatase activity on pyruvate, orthophosphate dikinase (Chastain *et al.*, 2008). TAK1 was originally purified from thylakoids using a specific antiserum (Snyders and Kohorn, 1999), and its chloroplast localization has also been confirmed by chloroplast import assays in an independent study (Bonardi, 2006). TAK1 is part of a family of protein kinases together with TAK2 (At1g01540) and TAK3 (At4g01330), which both have initially been suggested to localize to thylakoids due to sequence homology with TAK1 (Snyders and Kohorn, 1999). However, in the meantime, TAK2 and TAK3 have been found localized at the plasma membrane according to the PPDB database. The protein kinases At1g51170 and CIPK13 were both identified due to the presence of a predicted chloroplast targeting peptide, and their localization was verified by red fluorescent protein fusion analysis (Schliebner *et al.*, 2008). Whereas nothing is known about At1g51170, CIPK13 belongs to the well-characterized family of calcineurin B-like protein (CBL)-interacting protein kinases (CIPKs) (Batistic and Kudla, 2009).

Based on YFP analyses carried out in this study, the ABC1 protein kinases ABC1K1, ABC1K2, and ABC1K3 could now be added to the complement of chloroplast-localized protein kinases. It should be noted, however, that ABC1K6 (At3g24190), which exhibits high sequence homology (e-value $3e^{-113}$) with the other kinases of the ABC1 kinase family, is most probably chloroplast localized as well, as it has been identified with high confidence in a chloroplast proteomic study (Zybailov *et al.*, 2008). Furthermore, the chloroplast-localized nucleoside diphosphate kinase 2 (NDPK2; At5g63310), which has been shown to undergo autophosphorylation (Shen *et al.*, 2006), was included. NDPK2 had originally been implicated in phytochrome-mediated signalling in the cytoplasm (Choi *et al.*,

1999), and in salt stress and H_2O_2 signalling involving SOS2 and two mitogen-activated protein kinases (MPK3 and MPK6) (Moon *et al.*, 2003;Verslues *et al.*, 2007). However, recently, NDPK2 was clearly shown to be localized in chloroplasts by green fluorescent protein analysis, import assay, and immunoblotting (Bolter *et al.*, 2007). Moreover, the protein kinases MsK4 and NtDSK1, the chloroplast sensor kinase CSK, and the protein kinase PPK (At5g16810), which has recently been identified in a directed proteomic study (Bayer *et al.*, 2011), could be included (Table 1).

In contrast, contradictory evidence was obtained for the localization of the protein kinases AKIN10 and AKIN11, and therefore their localization was considered as ambiguous. Similarly, the *Arabidopsis* MAPKK MKK4 (At1g51660) was shown to be targeted to chloroplasts by an *in vitro* import assay (Samuel *et al.*, 2008). However, this is in contradiction to the nuclear and cytoplasmic localization observed in a previous study (Koroleva *et al.*, 2005) and to its function in the cytoplasmic- and nuclear-localized MAPK signalling cascade. Therefore, the chloroplast localization of MKK4 is also contested. Finally, AtSK41 and AtDSK1, the closest *Arabidopsis* homologues of *Medicago* MsK4 and tobacco NtDSK1, respectively, could not be verified inside the chloroplast by YFP fusion analysis. However, MsK4 appeared to be localized in chloroplasts only when starch was present, which might explain the difference. It could also be that AtSK41 is not the real *Arabidopsis* orthologue of MsK4, because it belongs to the family of GSK3/shaggy-like protein kinases consisting of at least 10 members with overall high sequence homology (Jonak and Hirt, 2002; Wang *et al.*, 2003). In contrast, NtDSK1 seems to be a tobacco-specific protein kinase, because *Arabidopsis* AtDSK1 shares only 41% sequence identity, and this is most probably due to the well-conserved protein kinase domain. Summarizing, experimental evidence was found for 15 chloroplast-localized protein kinases from all organisms, of which 13 orthologues could be assigned in *Arabidopsis*.

Ca^{2+}-dependent protein kinase activity in chloroplasts

The presence of CIPK13 in chloroplasts would provide a first mechanism to decode Ca^{2+} signals, which are known to occur inside chloroplasts (Sai and Johnson, 2002). CIPKs require CBLs as regulatory proteins that interact with CIPKs upon calcium binding via internal EF-hand motifs (Batistic *et al.*, 2008; Batistic and Kudla, 2009). However, none of the 10 CBLs known in *Arabidopsis* has been reported or predicted to be chloroplast localized. Nevertheless, it was possible to demonstrate calcium-dependent protein kinase activity in pea chloroplasts by incubation of stromal extracts with radioactively labelled ATP in the presence or absence of free calcium (Fig. 2A). Increasing calcium levels did not change the phosphorylation pattern much as compared with the control without addition of Ca^{2+} or EGTA (middle lane in Fig. 2A). Only the intensity of an ~50 kDa band increased slightly. This weak effect is most probably due to the presence of residual amounts of Ca^{2+} in the stromal extracts. In contrast, the full depletion of calcium by EGTA resulted in the complete loss of phosphorylation of this 50 kDa band. Moreover, phosphorylation could only be restored by addition of calcium but not by addition of magnesium (see inset box next to Fig. 2A), thus indicating a true calcium-dependent phosphorylation event. Importantly, this effect is not limited to pea because a similar Ca^{2+}-dependently phosphorylated band of ~50 kDa was also visible in *Arabidopsis* chloroplast extracts (Fig. 2B).

In previous attempts to identify chloroplast-localized protein kinases an affinity purification was used on the immobilized canonical protein kinase inhibitor PurB (Bayer *et al.*, 2011). Interestingly, PurB was also able to inhibit the phosphorylation of the 50 kDa band (Fig. 2C), indicating that this activity is caused by a canonical protein kinase and might be attributed to CIPK13. Therefore, future experiments should include the careful localization

analysis of all CBLs and the analysis of the calcium-dependent phospho-rylation pattern in chloroplasts of CIPK13 knock-out lines.

Unusual protein kinases in chloroplasts

When different protein kinase inhibitors were tested for their ability to inhibit protein kinase activities in stromal extracts it was noticed that addition of PurB to RuBisCO-depleted stromal protein extracts from pea had a very weak effect besides the inhibition of the 50 kDa band (Fig. 2C). In contrast, PurB, which has been shown to interact with >80 protein kinases from total human cell extracts (Wissing *et al.*, 2007), proved to be the most active protein kinase inhibitor of plant protein kinases in a whole leaf extract (Fig. 2D). This observation suggested that unusual protein kinases might be present in chloroplasts.

The conserved protein kinase catalytic domains range from 250 to 300 amino acids and have been divided into 11 major subdomains (I–XI) as depicted in Fig. 3A (Hanks *et al.*, 1988). Many of those subdomains directly participate in ATP binding and phosphotransfer like the so-called ‘phosphate gripper’ consensus GXGXXG, which is also found in many other nucleotide-binding proteins (Saraste *et al.*, 1990;Knowles, 1991). A multiple sequence alignment of the subdomains I and II and VI and VII, which harbour critical amino acids for ATP binding and phosphotransfer, shows that chloroplast protein kinases can be divided into two categories: conventional and unusual protein kinases (Fig. 3B). Conventional protein kinases, such as STN7/8 or cpCK2, contain a conserved protein kinase domain consisting of a complete ATP-binding region as shown for subdomains I and II, and an active site signature according to PROSITE as shown for subdomains VI and VII. In contrast, the unusual protein kinase PPK lacks a characteristic aspartate residue in subdomain VI, which usually constitutes the kinase active centre (Fig. 3B). Moreover, AtRP1, NDPK2, and CSK lack the complete protein kinase domain, but all were shown to retain protein kinase activity (Shen *et al.*, 2006; Chastain *et al.*, 2008;Puthiyaveetil *et al.*, 2008). Furthermore, the alignment of subdomain I of all four ABC1 kinases with the conventional serine/threonine-specific protein kinases STN7 and cpCK2 revealed that despite an overall high conservation, two crucial glycine residues within the ATP-binding site are missing (Fig. 3B). This differing structure of the ATP-binding pocket of ABC1 kinases in comparison with conventional protein kinases could indicate a different mode of nucleotide binding.

The mystery of chloroplast-localized protein kinases

Based on phosphoproteomic studies and experiments with phospho-specific antibodies and radioactively labelled ATP, there is unequivocal evidence for extensive phosphorylation of chloroplast proteins in the stroma as well as at the thylakoids (Laing and Christeller, 1984; Foyer, 1985; Rintamaki *et al.*, 1997; Reiland *et al.*, 2009; Tikkanen *et al.*, 2010). However, while at least 80 chloroplast-localized protein kinases could be expected in *Arabidopsis* based on targeting predictions, to date only 13 (1.2%) out of all ~1050 *Arabidopsis* protein kinases can be assigned to the chloroplast with high confidence. This clearly shows that targeting prediction fails in the case of protein kinases and that they seem to be under-represented in chloroplasts. It rather appears that only a relatively small number of protein kinases is responsible for the vast majority of chloroplast phosphorylation events. This would be in accordance with recent results of a chloroplast phosphoproteomic study, which showed that cpCK2 phosphorylation motifs are strongly over-represented among 174 identified chloroplast phosphoproteins (Reiland *et al.*, 2009). This suggests that cpCK2 functions as a central regulator of chloroplast processes. To test this hypothesis experimentally, use was made of the unique feature of CK2 to use GTP as co-substrate as efficiently as ATP (Niefind *et al.*, 1999). The phosphorylation patterns of stromal extracts and thylakoids were compared after the removal of nucleotides by gel filtration and subsequent incubation with either ATP or GTP (Fig. 4). The calcium-dependent protein

kinase CPK4 served as control for an ATP-dependent kinase, and commercial CK2 served as control for cpCK2 activity. It was found that indeed, the activity of CPK4 towards histone as substrate was completely dependent on ATP, whereas CK2 did phosphorylate casein equally efficiently in the presence of both ATP and GTP (Fig. 4, left panels). Looking at the chloroplast samples it was found that the phosphorylation patterns in stromal extracts were very similar, thus indicating that cpCK2 presents the dominating protein kinase activity in the stroma (Fig. 4, right panels). In the thylakoid samples, the LHCs (~29 kDa) presented the main phosphorylation targets, and their phosphorylation was strictly dependent on ATP as expected for the phosphorylation by STN7/8. In line with these results, the analysis of phosphorylation patterns in wild-type and *stn7stn8* double knock-out mutants revealed that all major phosphorylation events in isolated thylakoid membranes could be attributed to STN7 and STN8 (Tikkanen *et al.*, 2010).

In contrast, out of all 217 *Arabidopsis* protein phosphatases, already 10 (4.6%) are known to be chloroplast localized (Schliebner *et al.*, 2008; Pribil *et al.*, 2010). Therefore, they seem to be over-represented in chloroplasts compared with kinases, implicating an important regulatory role for chloroplast phosphatases.

Furthermore, considering the ABC1 family protein kinases, AtRP1, NDPK2, CSK, and PPK and the unexpected results from protein kinase inhibitor experiments, evidence for unusual chloroplast protein kinases accumulates. Seven out of the 13 experimentally verified chloroplast protein kinases in *Arabidopsis* contain unusual kinase domains, thus suggesting that unusual protein kinases are responsible for many of the remaining protein phosphorylation events occurring inside the chloroplast.

Conclusions

Chloroplast phosphorylation has already been known for >30 years, and the importance of chloroplast protein kinases has already been demonstrated, for example in the case of STN7, STN8, or cpCK2. Considering the experimental reports of extensive phosphorylation and based on targeting prediction, numerous protein kinases are generally assumed to be present inside chloroplasts. However, in contrast to this expectation, the complement of chloroplast protein kinases seems to be much smaller than initially anticipated. It seems that STN7, STN8, and cpCK2 represent the major protein kinase activities in chloroplasts. In addition to those three, a number of other protein kinases, including unusual protein kinases such as the ABC1 protein kinases or PPK, most probably have more specialized functions, which need to be elucidated in the future. For this reason, the identification of novel chloroplast protein kinases cannot rely solely on directed approaches involving mass spectrometry, as this depends on the correct functional annotation of proteins. It is therefore suggested rather to use known chloroplast protein kinase substrates in order to identify their interacting protein kinases as has been done for the identification of a protein kinase that phosphorylates chloroplast precursor proteins (Martin *et al.*, 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been funded by the Austrian GEN-AU program in the ERA-PG project *CROPP* (Project no. 818514), the Austrian Science Foundation FWF (P19825-B12), and by the EU in the Marie-Curie ITN *COSI* (ITN 2008 GA 215-174). The authors thank Helga Waltenberger for technical assistance, Gustav Ammerer and all members of the mass spectrometry facilities of the MFPL for their good collaboration, Felix Kessler (University of Neuchatel) for discussion about ABC kinases, and Giulio Superti-Furga and Uwe Rix (CeMM, Vienna) for

providing protein kinase inhibitors. The purified recombinant CPK4 was a kind gift of Dr Norbert Mehlmer (LMU Munich).

Abbreviations

AGI	*Arabidopsis* gene identifier
CPK	calcium-dependent protein kinase
PurB	purvalanol B
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
YFP	yellow fluorescent protein

References

Ashton AR, Hatch MD. Regulation of C4 photosynthesis: regulation of pyruvate, Pi dikinase by ADP-dependent phosphorylation and dephosphorylation. Biochemical and Biophysical Research Communications. 1983; 115:53–60. [PubMed: 6311212]

Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J. A central integrator of transcription networks in plant stress and energy signalling. Nature. 2007; 448:938–942. [PubMed: 17671505]

Baginsky S. Plant proteomics: concepts, applications, and novel strategies for data interpretation. Mass Spectrometry Reviews. 2009; 28:93–120. [PubMed: 18618656]

Batistic O, Kudla J. Plant calcineurin B-like proteins and their interacting protein kinases. Biochimica et Biophysica Acta. 2009; 1793:985–992. [PubMed: 19022300]

Batistic O, Sorek N, Schultke S, Yalovsky S, Kudla J. Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK Ca^{2+} signaling complexes in Arabidopsis. The Plant Cell. 2008; 20:1346–1362. [PubMed: 18502848]

Bayer RG, Stael S, Csaszar E, Teige M. Mining the soluble chloroplast proteome by affinity chromatography. Proteomics. 2011; 11:1287–1299. [PubMed: 21365755]

Bellafiore S, Barneche F, Peltier G, Rochaix JD. State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature. 2005; 433:892–895. [PubMed: 15729347]

Benetka W, Mehlmer N, Maurer-Stroh S, Sammer M, Koranda M, Neumuller R, Betschinger J, Knoblich JA, Teige M, Eisenhaber F. Experimental testing of predicted myristoylation targets involved in asymmetric cell division and calcium-dependent signalling. Cell Cycle. 2008; 7:3709–3719. [PubMed: 19029837]

Bennett J. Phosphorylation of chloroplast membrane polypeptides. Nature. 1977; 269:344–346.

Bolter B, Sharma R, Soll J. Localisation of Arabidopsis NDPK2—revisited. Planta. 2007; 226:1059–1065. [PubMed: 17562072]

Bonardi, V. Molecular-genetic characterization of thylakoid protein phosphorylation in *Arabidopsis thaliana*. Ludwig-Maximilians-Universität München; Munich: 2006. PhD thesis

Bonardi V, Pesaresi P, Becker T, Schleiff E, Wagner R, Pfannschmidt T, Jahns P, Leister D. Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. Nature. 2005; 437:1179–1182. [PubMed: 16237446]

Bousquet I, Dujardin G, Slonimski PP. ABC1, a novel yeast nuclear gene has a dual function in mitochondria: it suppresses a cytochrome b mRNA translation defect and is essential for the electron transfer in the bc 1 complex. EMBO Journal. 1991; 10:2023–2031. [PubMed: 1648478]

Brautigam K, Dietzel L, Kleine T, et al. Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in Arabidopsis. The Plant Cell. 2009; 21:2715–2732. [PubMed: 19737978]

Burnell JN, Chastain CJ. Cloning and expression of maize-leaf pyruvate, Pi dikinase regulatory protein gene. Biochemical and Biophysical Research Communications. 2006; 345:675–680. [PubMed: 16696949]

Europe PMC Funders Author Manuscripts

Chastain CJ, Xu W, Parsley K, Sarath G, Hibberd JM, Chollet R. The pyruvate, orthophosphate dikinase regulatory proteins of Arabidopsis possess a novel, unprecedented Ser/Thr protein kinase primary structure. The Plant Journal. 2008; 53:854–863. [PubMed: 17996018]

Chevalier D, Walker JC. Functional genomics of protein kinases in plants. Briefings in Functional Genomics and Proteomics. 2005; 3:362–371. [PubMed: 15814026]

Cho HS, Yoon GM, Lee SS, Kim YA, Hwang I, Choi D, Pai HS. A novel dual-specificity protein kinase targeted to the chloroplast in tobacco. FEBS Letters. 2001; 497:124–130. [PubMed: 11377426]

Choi G, Yi H, Lee J, Kwon YK, Soh MS, Shin B, Luka Z, Hahn TR, Song PS. Phytochrome signalling is mediated through nucleoside diphosphate kinase 2. Nature. 1999; 401:610–613. [PubMed: 10524631]

Cohen P. The regulation of protein function by multisite phosphorylation—a 25 year update. Trends in Biochemical Sciences. 2000; 25:596–601. [PubMed: 11116185]

Depege N, Bellafiore S, Rochaix JD. Role of chloroplast protein kinase Stt7 in LHCII phosphorylation and state transition in Chlamydomonas. Science. 2003; 299:1572–1575. [PubMed: 12624266]

Dunkley TP, Hester S, Shadforth IP, et al. Mapping the Arabidopsis organelle proteome. Proceedings of the National Academy of Sciences, USA. 2006; 103:6518–6523.

Emanuelsson O, von Heijne G. Prediction of organellar targeting signals. Biochimica et Biophysica Acta. 2001; 1541:114–119. [PubMed: 11750667]

Ferro M, Brugiere S, Salvi D, et al. AT_CHLORO: s comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Molecular and Cellular Proteomics. 2010; 9:1063–1084. [PubMed: 20061580]

Foyer CH. Stromal protein phosphorylation in spinach (Spinacia oleracea) chloroplasts. Biochemical Journal. 1985; 231:97–103. [PubMed: 4062895]

Fragoso S, Espindola L, Paez-Valencia J, Gamboa A, Camacho Y, Martinez-Barajas E, Coello P. SnRK1 isoforms AKIN10 and AKIN11 are differentially regulated in Arabidopsis plants under phosphate starvation. Plant Physiology. 2009; 149:1906–1916. [PubMed: 19211700]

Fristedt R, Willig A, Granath P, Crevecoeur M, Rochaix JD, Vener AV. Phosphorylation of photosystem II controls functional macroscopic folding of photosynthetic membranes in Arabidopsis. The Plant Cell. 2009; 21:3950–3964. [PubMed: 20028840]

Goral TK, Johnson MP, Brain AP, Kirchhoff H, Ruban AV, Mullineaux CW. Visualizing the mobility and distribution of chlorophyll proteins in higher plant thylakoid membranes: effects of photoinhibition and protein phosphorylation. The Plant Journal. 2010; 62:948–959. [PubMed: 20230505]

Gribskov M, Fana F, Harper J, Hope DA, Harmon AC, Smith DW, Tax FE, Zhang G. PlantsP: a functional genomics database for plant phosphorylation. Nucleic Acids Research. 2001; 29:111–113. [PubMed: 11125063]

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Zhang Y, Paul MJ. Highly conserved protein kinases involved in the regulation of carbon and amino acid metabolism. Journal of Experimental Botany. 2004; 55:35–42. [PubMed: 14645392]

Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science. 1988; 241:42–52. [PubMed: 3291115]

Jonak C, Hirt H. Glycogen synthase kinase 3/SHAGGY-like kinases in plants: an emerging family with novel functions. Trends in Plant Sciences. 2002; 7:457–461.

Jossier M, Bouly JP, Meimoun P, Arjmand A, Lessard P, Hawley S, Grahame Hardie D, Thomas M. SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in Arabidopsis thaliana. The Plant Journal. 2009; 59:316–328. [PubMed: 19302419]

Kempa S, Rozhon W, Samaj J, et al. A plastid-localized glycogen synthase kinase 3 modulates stress tolerance and carbohydrate metabolism. The Plant Journal. 2007; 49:1076–1090. [PubMed: 17319843]

Kleffmann T, Hirsch-Hoffmann M, Gruissem W, Baginsky S. plprot: a comprehensive proteome database for different plastid types. Plant and Cell Physiology. 2006; 47:432–436. [PubMed: 16418230]

Knowles JR. Enzyme catalysis: not different, just better. Nature. 1991; 350:121–124. [PubMed: 2005961]

Koroleva OA, Tomlinson ML, Leader D, Shaw P, Doonan JH. High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. The Plant Journal. 2005; 41:162–174. [PubMed: 15610358]

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680–685. [PubMed: 5432063]

Laing WA, Christeller JT. Chloroplast phosphoproteins: distribution of phosphoproteins within spinach chloroplasts. Plant Science Letters. 1984; 36:99–104.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002; 298:1912–1934. [PubMed: 12471243]

Martin DM, Miranda-Saavedra D, Barton GJ. Kinomer v. 1.0: a database of systematically classified eukaryotic protein kinases. Nucleic Acids Research. 2009; 37:D244–D250. [PubMed: 18974176]

Martin T, Sharma R, Sippel C, Waegemann K, Soll J, Vothknecht UC. A protein kinase family in Arabidopsis phosphorylates chloroplast precursor proteins. Journal of Biological Chemistry. 2006; 281:40216–40223. [PubMed: 17090544]

Mehlmer N, Wurzinger B, Stael S, Hofmann-Rodrigues D, Csaszar E, Pfister B, Bayer R, Teige M. The Ca^{2+}-dependent protein kinase CPK3 is required for MAPK-independent salt-stress acclimation in Arabidopsis. The Plant Journal. 2010; 63:484–498.

Montrichard F, Alkhalfioui F, Yano H, Vensel WH, Hurkman WJ, Buchanan BB. Thioredoxin targets in plants: the first 30 years. Journal of Proteomics. 2009; 72:452–474. [PubMed: 19135183]

Moon H, Lee B, Choi G, et al. NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proceedings of the National Academy of Sciences, USA. 2003; 100:358–363.

Niefind K, Putter M, Guerra B, Issinger OG, Schomburg D. GTP plus water mimic ATP in the active site of protein kinase CK2. Nature Structural Biology. 1999; 6:1100–1103.

Ogrzewalla K, Piotrowski M, Reinbothe S, Link G. The plastid transcription kinase from mustard (Sinapis alba L.). A nuclear-encoded CK2-type chloroplast enzyme with redox-sensitive function. European Journal of Biochemistry. 2002; 269:3329–3337. [PubMed: 12084075]

Orchard S, Hermjakob H, Apweiler R. Annotating the human proteome. Molecular and Cellular Proteomics. 2005; 4:435–440. [PubMed: 15691850]

Pesaresi P, Hertle A, Pribil M, et al. Arabidopsis STN7 kinase provides a link between short- and long-term photosynthetic acclimation. The Plant Cell. 2009; 21:2402–2423. [PubMed: 19706797]

Pribil M, Pesaresi P, Hertle A, Barbato R, Leister D. Role of plastid protein phosphatase TAP38 in LHCII dephosphorylation and thylakoid electron flow. PLoS Biology. 2010; 8:e1000288. [PubMed: 20126264]

Puthiyaveetil S, Ibrahim IM, Jelicic B, Tomasic A, Fulgosi H, Allen JF. Transcriptional control of photosynthesis genes: the evolutionarily conserved regulatory mechanism in plastid genome function. Genome Biology and Evolution. 2010; 2:888–896. [PubMed: 21071627]

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF. The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proceedings of the National Academy of Sciences, USA. 2008; 105:10061–10066.

Reiland S, Messerli G, Baerenfaller K, Gerrits B, Endler A, Grossmann J, Gruissem W, Baginsky S. Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiology. 2009; 150:889–903. [PubMed: 19376835]

Richly E, Leister D. An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of Arabidopsis and rice. Gene. 2004; 329:11–16. [PubMed: 15033524]

Rintamaki E, Salonen M, Suoranta UM, Carlberg I, Andersson B, Aro EM. Phosphorylation of light-harvesting complex II and photosystem II core proteins shows different irradiance-dependent regulation *in vivo*. Application of phosphothreonine antibodies to analysis of thylakoid phosphoproteins. Journal of Biological Chemistry. 1997; 272:30476–30482. [PubMed: 9374540]

Sai J, Johnson CH. Dark-stimulated calcium ion fluxes in the chloroplast stroma and cytosol. The Plant Cell. 2002; 14:1279–1291. [PubMed: 12084827]

Salinas P, Fuentes D, Vidal E, Jordana X, Echeverria M, Holuigue L. An extensive survey of CK2 alpha and beta subunits in Arabidopsis: multiple isoforms exhibit differential subcellular localization. Plant and Cell Physiology. 2006; 47:1295–1308. [PubMed: 16926165]

Samuel MA, Chaal BK, Lampard G, Green BR, Ellis BE. Surviving the passage: non-canonical stromal targeting of an Arabidopsis mitogen-activated protein kinase kinase. Plant Signaling Behavior. 2008; 3:6–12. [PubMed: 19516975]

Saraste M, Sibbald PR, Wittinghofer A. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends in Biochemical Sciences. 1990; 15:430–434. [PubMed: 2126155]

Schliebner I, Pribil M, Zuhlke J, Dietzmann A, Leister D. A survey of chloroplast protein kinases and phosphatases in. Arabidopsis thaliana. Current Genomics. 2008; 9:184–190. [PubMed: 19440515]

Schweer J, Turkeri H, Link B, Link G. AtSIG6, a plastid sigma factor from Arabidopsis, reveals functional impact of cpCK2 phosphorylation. The Plant Journal. 2010; 62:192–202. [PubMed: 20088902]

Seigneurin-Berny D, Salvi D, Joyard J, Rolland N. Purification of intact chloroplasts from Arabidopsis and spinach leaves by isopycnic centrifugation. Current Protocols in Cell Biology. 2008 Chapter 3, Unit 3 30.

Shen Y, Kim JI, Song PS. Autophosphorylation of Arabidopsis nucleoside diphosphate kinase 2 occurs only on its active histidine residue. Biochemistry. 2006; 45:1946–1949. [PubMed: 16460041]

Siegenthaler PA, Bovet L. A unique protein-kinase activity is responsible for the phosphorylation of the 26- and 14-kDa proteins but not of the 67-kDa protein in the chloroplast envelope membranes of spinach. Planta. 1993; 190:231–240.

Snyders S, Kohorn BD. TAKs, thylakoid membrane protein kinases associated with energy transduction. Journal of Biological Chemistry. 1999; 274:9137–9140. [PubMed: 10092584]

Soll J. Phosphoproteins and protein-kinase activity in isolated envelopes of pea (*Pisum sativum L.*) chloroplasts. Planta. 1985; 166:394–400. [PubMed: 24241523]

Soll J. Purification and characterization of a chloroplast outer-envelope-bound, ATP-dependent protein kinase. Plant Physiology. 1988; 87:898–903. [PubMed: 16666242]

Soll J, Fischer I, Keegstra K. A guanosine 5′-triphosphate-dependent protein kinase is localized in the outer envelope membrane of pea chloroplasts. Planta. 1988; 176:488–496. [PubMed: 24220945]

Stael S, Bayer RG, Mehlmer N, Teige M. Protein *N*-acylation overrides differing targeting signals. FEBS Letters. 2011; 585:517–522. [PubMed: 21219905]

Stone JM, Walker JC. Plant protein kinase families and signal transduction. Plant Physiology. 1995; 108:451–457. [PubMed: 7610156]

Sun Q, Zybailov B, Majeran W, Friso G, Olinares PD, van Wijk KJ. PPDB, the Plant Proteomics Database at Cornell. Nucleic Acids Research. 2008; 37:D969–D974. [PubMed: 18832363]

Tikkanen M, Grieco M, Kangasjarvi S, Aro EM. Thylakoid protein phosphorylation in higher plant chloroplasts optimizes electron transfer under fluctuating light. Plant Physiology. 2010; 152:723–735. [PubMed: 19965965]

Verslues PE, Batelli G, Grillo S, Agius F, Kim YS, Zhu J, Agarwal M, Katiyar-Agarwal S, Zhu JK. Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H_2O_2 signaling in Arabidopsis thaliana. Molecular and Cellular Biology. 2007; 27:7771–7780. [PubMed: 17785451]

Vidi PA, Kanwischer M, Baginsky S, Austin JR, Csucs G, Dormann P, Kessler F, Brehelin C. Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. Journal of Biological Chemistry. 2006; 281:11225–11234. [PubMed: 16414959]

Waegemann K, Soll J. Characterization and isolation of the chloroplast protein import machinery. Methods in Cell Biology. 1995; 50:255–267. [PubMed: 8531798]

Wang D, Harper JF, Gribskov M. Systematic trans-genomic comparison of protein kinases between Arabidopsis and. Saccharomyces cerevisiae. Plant Physiology. 2003; 132:2152–2165. [PubMed: 12913170]

Wissing J, Jansch L, Nimtz M, Dieterich G, Hornberger R, Keri G, Wehland J, Daub H. Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. Molecular and Cellular Proteomics. 2007; 6:537–547. [PubMed: 17192257]

Ytterberg AJ, Peltier JB, van Wijk KJ. Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiology. 2006; 140:984–997. [PubMed: 16461379]

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ. Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS One. 2008; 3:e1994. [PubMed: 18431481]

Europe PMC Funders Author Manuscripts

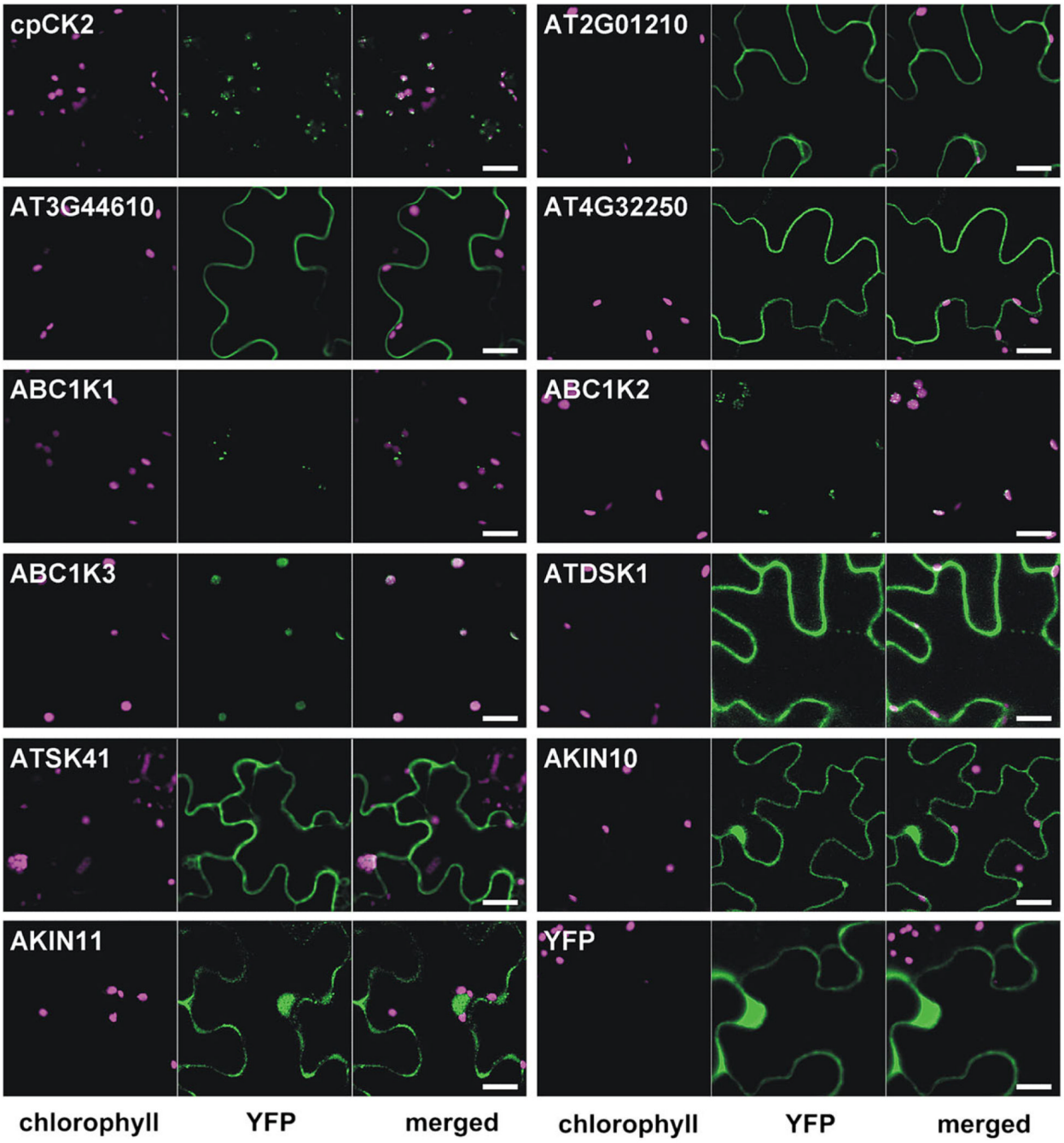

Fig. 1.

Localization analysis of selected protein kinases. Tobacco leaves infiltrated with genes of interest fused in front of YFP in the plant expression plasmid pBIN19 were analysed by confocal laser scanning microscopy 2 d after infiltration. Chlorophyll autofluorescence (magenta) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Scale bar=20 μm.

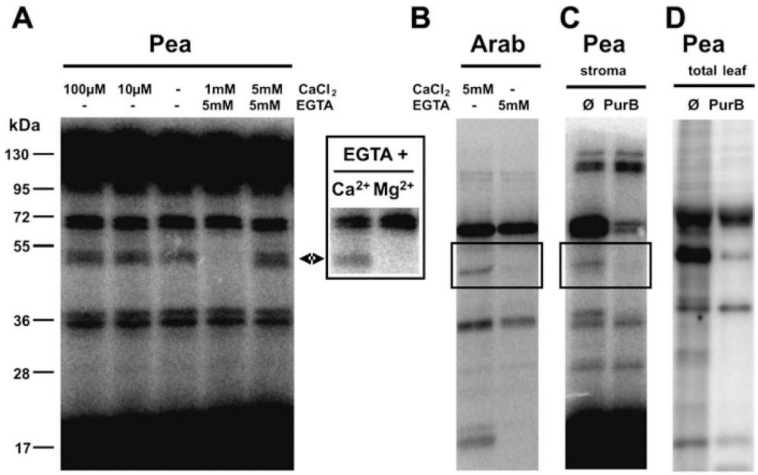

Fig. 2.

Protein kinase activity in chloroplasts: Ca^{2+} dependency and the effect of the protein kinase inhibitor purvalanol B (PurB). (A) Ca^{2+}-dependent phosphorylation of RuBisCO-depleted stromal proteins from pea chloroplasts. The extracted proteins were incubated with radioactively labelled ATP in the absence or presence of $CaCl_2$ and/or EGTA. The insert shows a protein kinase assay in the presence of 5 mM EGTA and 5 mM $CaCl_2$ or $MgCl_2$, respectively. (B) Ca^{2+}-dependent phosphorylation of stromal proteins from *Arabidopsis* chloroplasts. (C and D) The effect of the protein kinase inhibitor PurB on pea protein kinases. A RuBisCO-depleted stromal extract and a total leaf extract were incubated with radioactively labelled ATP in the absence or presence of 100 µM PurB. Ø=control reaction without inhibitor.

Europe PMC Funders Author Manuscripts

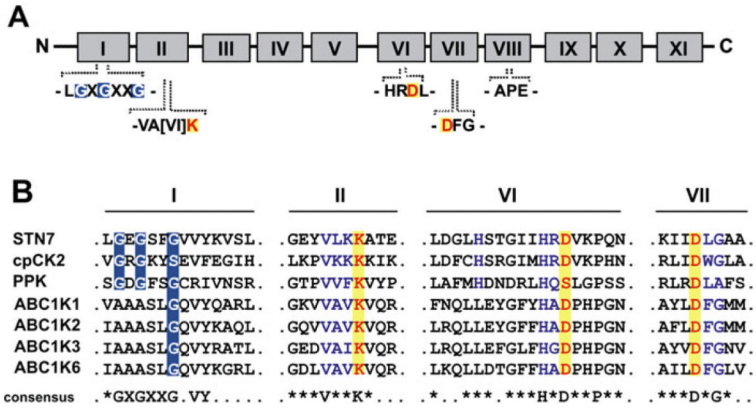

Fig. 3.

Conserved protein kinase domains and their presence in chloroplast-localized protein kinases. (A) Illustration of the 11 highly conserved protein kinase domains according to Hanks *et al.* (1988), highlighting the ATP-binding motifs in subdomain I and II and essential catalytic residues in subdomains II, VI, and VII. (B) Alignment of the ‘classical’ protein kinases STN7 and cpCK2 with the unusual kinases PPK and the ABC1 kinases. The ATP-binding sites are highlighted in subdomain I and the essential catalytic residues are indicated in subdomains II, VI, and VII, respectively. A consensus sequence for homologous regions is displayed below.

Europe PMC Funders Author Manuscripts

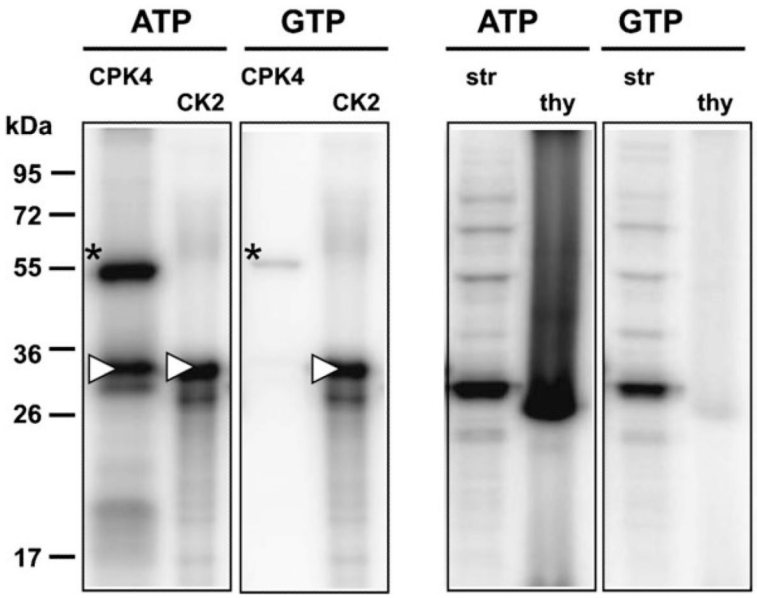

Fig. 4.

The effect of using ATP versus GTP as co-substrate for phosphorylation assays. Protein kinase assays were performed as described in the Materials and methods using purified protein kinases and their substrates (left panel) or chloroplast extracts (right panel) with ATP or GTP as co-substrate. The calcium-dependent protein kinase CPK4 and histone as substrate served as control for a strictly ATP-dependent kinase, and commercial CK2 and casein as substrate were used as a positive control. The autophosphorylation of CPK4 is indicated by an asterisk and the substrate phosphorylation of histone and casein is indicated by a white triangle. Gel-filtrated stromal extracts (str) incubated with ATP and GTP are shown in lanes 5 and 7, respectively and the phosphorylation of thylakoid membranes (thy) by stromal extracts in the presence of either ATP or GTP is shown in lanes 6 and 8, respectively.

Europe PMC Funders Author Manuscripts

Table 1

Survey of chloroplast-localized protein kinases with conventional and unusual ATP binding or active sites

		Name	AGIcode (or GenBank)	Organism(s)	PROSITE kinase domain	Reference(s)
Chloroplast localized	Conventional	Stt7/STN7	AT1G68830	*Chlamydomonas; Arabidopsis*	Complete	Depege *et al.* (2003); Bellafiore *et al.* (2005); Bonardi *et al.* (2005)
		Stl1/STN8	AT5G01920	*Chlamydomonas; Arabidopsis*	Complete	Depege *et al.* (2003); Bonardi *et al.* (2005)
		cpCK2	AT2G23070	*Sinapis alba; Arabidopsis*	Complete	Ogrzewalla *et al.* (2002); Salinas *et al.* (2006)
		TAK1	AT4G02630	*Arabidopsis*	Complete	Snyders and Kohorn (1999)
		CIPK13	AT2G34180	*Arabidopsis*	Complete	Schliebner *et al.* (2008)
		At1g51170	AT1G51170	*Arabidopsis*	Complete	Schliebner *et al.* (2008)
		MsK4	AF432335.1	*Medicago sativa*	Complete	Kempa *et al.* (2007)
		NtDSK1	AF106957.1	*Nicotiana tabacum*	Complete	Cho *et al.* (2001)
	Unusual	ABC1K1	AT1G71810	*Arabidopsis*	Unusual ATP site	Ytterberg *et al.* (2006); Zybailov *et al.* (2008); this study
		ABC1K2	AT1G79600	*Arabidopsis*	Unusual ATP site	Vidi *et al.* (2006); Ytterberg *et al.* (2006); Zybailov *et al.* (2008); this study
		ABC1K3	AT4G31390	*Arabidopsis*	Unusual ATP site	Ytterberg *et al.* (2006); Zybailov *et al.* (2008); this study
		AtRP1	AT4G21210	*Zea mays; Arabidopsis*	Absent	Burnell and Chastain (2006)
		CSK	AT1G67840	*Arabidopsis*	Absent	Puthiyaveetil *et al.* (2008)
		NDPK2	AT5G63310	*Arabidopsis*	Absent	Bolter etal. (2007); Reiland *et al.* (2009)
		PPK	AT5G16810	*Arabidopsis*	Single D absent	Bayer etal. (2011)
Localization ambiguous	Conventional	AKIN10	AT3G01090	*Arabidopsis*	Complete	Fragoso *et al.* (2009); this study
		AKIN11	AT3G29160	*Arabidopsis*	Complete	Fragoso *et al.* (2009); this study
		MKK4	AT1G51660	*Arabidopsis*	Complete	Samuel *et al.* (2008)
		AtSK41	AT1G09840	*Arabidopsis*	Complete	This study
		AtDSK1	AT3G13690	*Arabidopsis*	Complete	This study