universität wien

DISSERTATION

Titel der Dissertation

„Chloroplast-localized protein kinases and
a peek at the impact of protein acylation on chloroplast import"

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer. nat.)

Verfasser: Mag. Roman Bayer

Dissertationsgebiet (lt. Studienblatt): Genetik - Mikrobiologie

Betreuer: Dr. Markus Teige

Wien, im Juli 2010

Unendlich viele Wege, du kannst sie alle geh'n,

sie alle sind von Zufällen gesäumt.

Wähl' aus und überlege, um endlich einzuseh'n,

daß dich das Schicksal dahin, wo es dich haben will, räumt.

Reinhard Mey – Das Leben ist…

Mein Dank gilt

ABSTRACT

Chloroplasts are the fundamental organelles enabling the photoautotrophic life style of plants. Besides their outstanding physiological role in fixating atmospheric CO_2, chloroplasts harbor many important processes such as the biosynthesis of amino acids, vitamins or hormones. It is crucial that these processes are tightly regulated and coordinated in response to environmental changes. Thus, the chloroplast is integrated into the cellular network of signal transduction with protein kinases as the key mediators. Based on targeting prediction at least 70 protein kinases are expected to be present inside the chloroplast. However, so far only very few chloroplast protein kinases have been reported in the literature including the "state transition" kinases STN7 and STN8 and the plastid transcription kinase CKII.

This thesis aimed at the identification of novel chloroplast-localized protein kinases in higher plants. To this end, three different strategies were developed: a candidate, a phylogenetic and a proteomic approach. In the course of the candidate approach protein kinases were selected based on chloroplast targeting prediction and their subcellular localization was investigated by YFP (yellow fluorescent protein)-fusion analysis. The phylogenetic approach was based on the hypothesis that protein kinases derived from the cyanobacterial plastid ancestor could have been maintained in the chloroplast. Hence, evolutionary conserved proteins between *Arabidopsis thaliana* and cyanobacteria were determined and the localization of identified protein kinases was examined by YFP-fusion analysis. The proteomic approach aimed at the mass spectrometric identification of protein kinases enriched from extracts of isolated chloroplasts by various chromatographic techniques. Detected protein kinases were subjected to verification by YFP localization studies.

Taken together, of all novel protein kinases identified, only one unusual protein kinase could be confirmed to be chloroplast-localized. Nevertheless, the proteomic approach led to the identification of novel chloroplast proteins with important metabolic or regulatory functions. And furthermore, a comparison of affinity-purified stromal proteomes of *Pisum sativum* and Arabidopsis revealed unexpected developmental state- and/or species-specific differences.

Last but not least, the regulation of the subcellular localization of proteins by co- and posttranslational N-terminal acylation was analyzed. The Arabidopsis calcium-dependent protein kinase CDPK16 was shown to be plasma membrane attached via N-terminal myristoylation and palmitoylation, but upon removal of the myristic acid moiety the protein was relocated to chloroplasts. Vice versa, the chloroplast import of the known Arabidopsis chloroplast proteins FNR and Rubisco activase, which lack N-terminal lipid modifications, could be inhibited by artificial introduction of myristoylation and palmitoylation.

ZUSAMMENFASSUNG

Chloroplasten sind jene Organellen, die durch CO_2-Fixierung die photoautotrophe Lebensweise von Pflanzen ermöglichen. Außerdem verrichten sie viele wichtige Prozesse wie zum Beispiel die Biosynthese von Aminosäuren, Vitaminen oder Hormonen. Zur Regulierung dieser Prozesse ist der Chloroplast in das zelluläre Netzwerk der Signaltransduktion integriert, in dem Proteinkinasen eine zentrale Rolle spielen. Basierend auf Lokalisierungsvorhersagen werden im Chloroplasten zumindest 70 Proteinkinasen erwartet, bisher wurden jedoch nur wenige beschrieben, wie zum Beispiel die „state transition" Kinasen STN7 und STN8, oder die Plastid-Transkriptionskinase CKII.

Diese Dissertation hatte die Identifizierung neuer chloroplastenlokalisierter Proteinkinasen in höheren Pflanzen als Ziel. Dazu wurde ein Kandidaten-, ein phylogenetischer und ein proteomischer Ansatz angewandt. Im Zuge des Kandidatenansatzes wurden Proteinkinasen aufgrund von Chloroplastenvorhersage ausgewählt und deren Lokalisierung mittels YFP (yellow fluorescent protein)-Fusionsanalyse untersucht. Der phylogenetische Ansatz basierte auf der Hypothese, dass vom cyanobakteriellen Plastidenvorfahren abstammende Proteinkinasen eventuell im Chloroplasten beibehalten wurden. Daher wurden die zwischen *Arabidopsis thaliana* und Cyanobakterien evolutionär konservierten Proteine bestimmt und die Lokalisierung der dabei entdeckten Proteinkinasen mittels YFP-Fusionsanalyse untersucht. Im proteomischen Ansatz wurde Proteinkinasen, die aus Proteinextrakten von isolierten Chloroplasten durch verschiede chromatographische Methoden angereichert wurden, mittels Massenspekrometrie identifiziert und anschließend YFP-Lokalisierungsstudien unterzogen.

Von allen neu identifizierten Proteinkinasen konnte nur die Chloroplastenlokalisierung einer einzigen ungewöhnlichen Proteinkinase bestätigt werden. Trotzdem führte der proteomische Ansatz zur Identifzierung neuer Chloroplastenproteine, und ein Vergleich der affinitätsaufgereinigten stromalen Proteome von *Pisum sativum* und Arabidopsis offenbarte unerwartete entwicklunszustands- und/oder artspezifische Unterschiede.

Zu guter Letzt wurde die Regulierung der subzellulären Lokalisierung von Proteinen durch co- und posttranslationale N-terminale Acylierung untersucht. Es konnte gezeigt werden, dass die calciumabhängige Proteinkinase CDPK16 aus Arabidopsis durch N-terminale Myristoylierung und Palmitoylierung in der Plasmamembran verankert ist. Bei Entfernung der Myristoylierung wurde CDPK16 jedoch in den Chloroplasten eingeschleust. Umgekehrt konnte der Chloroplastenimport der Arabidopsis-Proteine FNR und Rubisco-Aktivase, die normalerweise keine N-terminalen Lipidmodifizierungen aufweisen, durch künstliche Einführung von Myristoylierung und Palmitoylierung verhindert werden.

TABLE OF CONTENTS

1. Introduction

This thesis focused on the identification of novel chloroplast-localized protein kinases in higher plants. Furthermore, the impact of N-terminal acylation (myristoylation and palmitoylation) of proteins on chloroplast import was investigated.

In the introduction all relevant aspects on the issues of chloroplasts, protein kinases and acylation will be covered.

1.1. Chloroplasts

Chloroplasts are semi-autonomous organelles of endosymbiotic origin that are found in all plants and algae as well as in some kleptoplastic sea slugs and parasites. Chloroplasts belong to the diverse family of plastids. There are several types of plastids, which all have specialized roles and are derived from undifferentiated proplastids. The most important types of plastids are chloroplasts, which contain chlorophyll and conduct photosynthesis, leucoplasts, which have no chlorophyll and are found mostly in roots and non-photosynthetic tissue and chromoplasts which are responsible for the coloring of fruits and flowers. Furthermore, plastids are also involved in gravity perception and the opening and closure of stomata (Wise and Hoober, 2007).

Chloroplasts cannot be synthesized *de novo* but they divide via fission and are equally distributed among the daughter cells after cell division. Besides photosynthesis they have essential roles in processes such as biosynthesis of amino acids and vitamins, lipid synthesis or storage of starch (Wise and Hoober, 2007).

The chloroplast itself is surrounded by two membranes: the outer and the inner envelope.

Fig. 1. Chloroplast structure. 1 - outer envelope. 2 - interenvelope space. 3 - inner envelope. 4 – stroma. 5 - thylakoid lumen. 6 - thylakoid membrane. 7 - grana stack of thylakoids. 8 - stroma thylakoid. 9 – starch. 10 – ribosome. 11 - plastidial DNA. 12 – plastoglobule. (http://de.wikipedia.org/wiki/Chloroplast)

The membranous structures inside the chloroplast are called thylakoids, which are arranged in grana stacks and connected by lamellar stroma thylakoids. Further, the chloroplast harbors three aqueous compartments: the stroma, the interenvelope space and the thylakoid lumen. Within the stroma plastoglobules reside, which are lipoprotein particles thought to be storage compartments for vitamin E, lipids and quinones (Fig.1)(Austin et al., 2006).
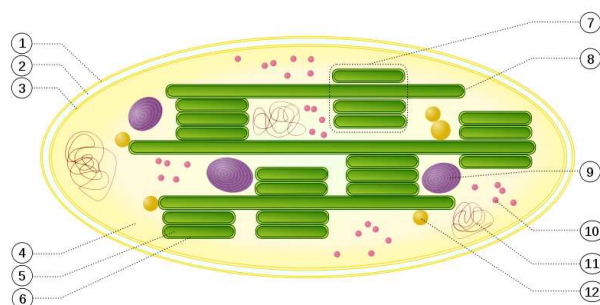
Evolutionary origin of chloroplasts

Nowadays it is widely accepted that plastids emerged through an event called endosymbiosis (Cotton, 1993). The endosymbiotic theory was first postulated by Mereschkowsky based on the work of Schimper (Schimper, 1885; Mereschkowsky, 1905). According to this theory plastids are derived from a photosynthetic cyanobacterium that has been incorporated by a heterotrophic host cell around 1.5 billion years ago. But still it is not clear from which cyanobacterial ancestor plastids arose. Evidences from the comparison of genes of Arabidopsis, rice, *Chlamydomonas reinhardtii* and red algae to cyanobacterial genomes point to a heterocyst-forming ancestor such as *Anabaena variabilis* or Nostoc (Deusch et al., 2008).

After the ancestral plastid had been taken up by the host cell, it transformed into a contemporary organelle and reorganized in order to adapt to the new environment and to cope with the new requirements. In the course of evolution most of the cyanobacterial genes were transferred into the nuclear genome of the new host. It is suggested that 4300-4500 Arabidopsis proteins were acquired from the ancestral plastid (Fig.2)(Martin et al., 2002; Jarvis, 2004). According to CyanoBase *Anabaena variabilis* and *Nostoc punctiforme* contain 5105 and 6191
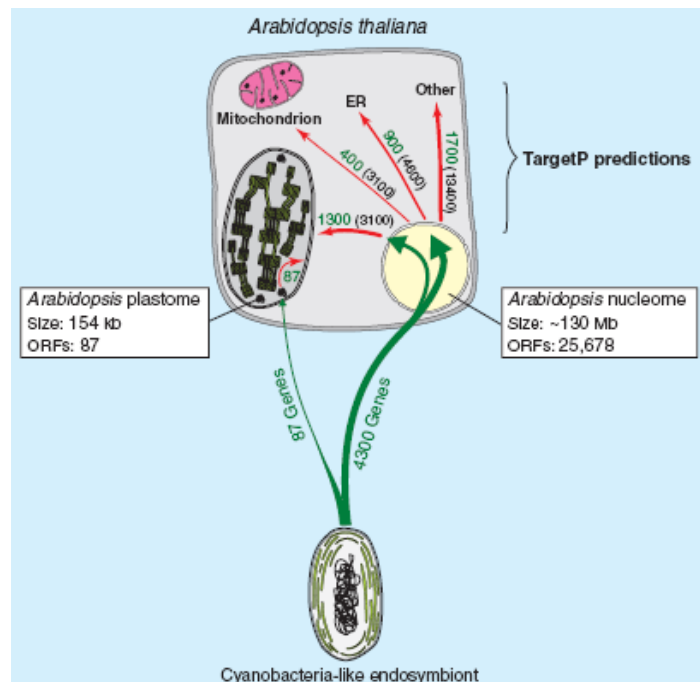
Fig. 2. Fate of cyanobacterial genes (Jarvis, 2004). After endosymbiosis the vast majority of cyanobacterial genes had been transferred to the nucleus (~4300). Their gene products are targeted to chloroplasts, mitochondria, the ER or other compartments. Only 88 genes (latest TAIR release) have been maintained in the chloroplast.

genes, respectively, which shows that the majority of ancestral genes had been transferred to the nuclear genome (Nakao et al., 2009). In contrast, according to TAIR only 88 protein-encoding genes have been maintained in the Arabidopsis chloroplast genome including genes for ribosomal proteins, the large subunit of Rubisco, RNA polymerase subunits and genes involved in photosynthesis (Wakasugi et al., 2001; Swarbreck et al., 2008). The CORR (*co-location for redox regulation*) hypothesis provides an answer for the question, why genes have been retained in the chloroplast genome. CORR proposes that chloroplast co-location of proteins and their encoding genes facilitates redox regulatory coupling of gene expression (Allen, 2003).

Although most cyanobacterial genes had been transferred to the nuclear genome, many of their gene products are still needed in the plastid. In order to establish an effective "back-transport" to their original location, most chloroplast proteins evolved an N-terminal signal sequence called cTP (chloroplast transit peptide), which is recognized by a plastid import machinery that had evolved simultaneously (Soll and Schleiff, 2004; Li and Chiu, 2010).

Chloroplast protein import

The vast majority of nuclear-encoded chloroplast proteins are translated as precursor proteins equipped with a cTP, which mediates post-translational chloroplast import through the TOC (*T*ranslocon at the *O*uter envelope membrane of *C*hloroplasts) and TIC (*T*ranslocon at the *I*nner envelope membrane of *C*hloroplasts) protein complexes (Fig.3). The TOC complex consists of several subunits including TOC159, which recognizes precursor proteins in dependence of GTP and TOC75 which forms the import-channel in the outer envelope. The subunit TOC34 may control recognition of precursor proteins or may possess regulatory function (Soll and Schleiff, 2004). In Arabidopsis different isoforms of TOC159 and TOC34 have been identified and there are evidences that these isoforms are acting substrate-specifically. The proteins AtTOC159 and AtTOC33 are proposed to be involved in the chloroplast import of abundant photosynthetic precursor proteins whereas AtTOC132 and AtTOC34 seem to be specific for non-photosynthetic precursor proteins (Jarvis, 2008). The import channel in the inner envelope is formed by TIC110, which is part of the multi-protein complex TIC. Import is completed by the activity of ATP-dependent stromal chaperones and eventually the cTP is removed by a stromal processing peptidase (SPP). Subsequently stromal proteins are folded into their mature form and proteins destined for thylakoids are introduced into a further internal sorting pathway (Fig.3)(Soll and Schleiff, 2004). Thylakoid lumenal proteins are carrying an additional signal peptide and they are targeted to their final destination via the Sec (Secretory) or the Tat (Twin-arginine translocase) pathway. Thylakoid membrane proteins either integrate spontaneously into the membrane or they employ a SRP (signal recognition particle)-dependent pathway (Jarvis, 2008). Post-translational targeting of proteins to the thylakoid via chloroplastic SRP is related to the co-translational targeting of secretory proteins to the ER via cytoplasmic SRP (Nussaume, 2008). Furthermore, while interenvelope and inner envelope targeting of proteins involves recognition of canonical cTPs, most outer envelope proteins in contrast are targeted via a non-cleavable signal sequence (Fig.3).
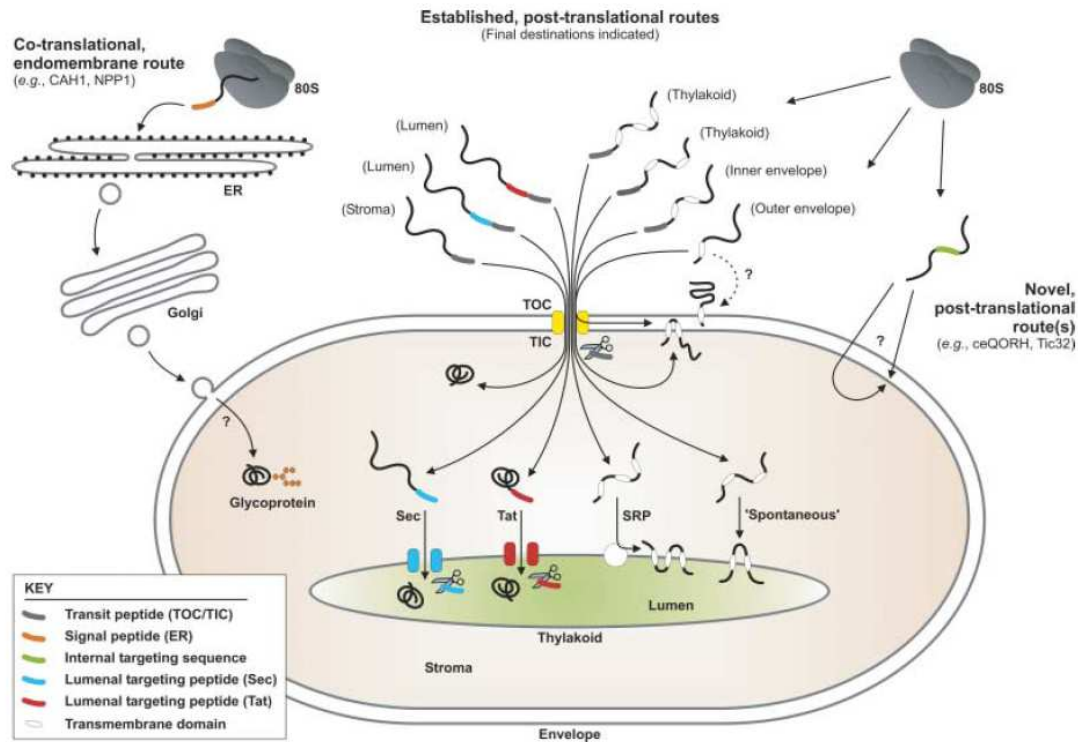
Fig. 3. Protein targeting to and protein sorting within chloroplasts (Jarvis, 2008). All chloroplast import and internal sorting pathways known to date are outlined.

Chloroplast transit peptides

The majority of chloroplast proteins are rendered import-competent by the presence of a cTP. Hence, it is expected that this cTP must be conserved to a certain extent. However, analysis of hundreds of experimentally verified chloroplast proteins revealed that cTPs vary in length between 13-146 amino acids (average: 58 amino acids) and further exhibit no significant primary sequence conservation (Zhang and Glaser, 2002). cTPs only appear to be rich in hydroxylated and deficient in acidic amino acid residues resulting in an overall positive charge. Furthermore, they are very similar to mitochondrial targeting peptides (mTPs). In this context it is still unclear how specific import into mitochondria and chloroplasts is accomplished. Additionally, reports of dually targeted proteins that are specifically localized to both organelles accumulate (Carrie et al., 2009). Recently, the small membrane protein SMP2 from Arabidopsis was even shown to be targeted to three compartments, namely mitochondria, chloroplasts and peroxisomes (Abu-Abied et al., 2009). However, despite lacking a conserved primary structure, cTPs were shown to exhibit an alpha-helical secondary structure in a hydrophobic environment mimicking the chloroplast envelope (Bruce, 2000). Thus, it is very difficult to identify cTPs, but prediction programs such as TargetP have already been developed based on similarities of the N-terminal sequences of already known chloroplast proteins (Emanuelsson et al., 2000; Richly and Leister, 2004).

12

It is clear that most of the nuclear-encoded chloroplast-localized proteins are carrying a canonical cTP and are transported through the TOC/TIC complexes. Nevertheless, several proteins targeted to the chloroplast via non-canonical pathways have already been described (Fig.3). For example, the carbonic anhydrase CAH1 from Arabidopsis, the rice α-amylase AmyI-1 and the rice nucleotide pyrophosphatase/phosphodiesterase NPP1 have been shown to be transported to the plastid through the secretory pathway (Villarejo et al., 2005; Nanjo et al., 2006; Kitajima et al., 2009). Furthermore, the envelope proteins TIC32 and ceQORH are chloroplast-localized despite lacking a canonical cTP (Miras et al., 2002; Nada and Soll, 2004). ceQORH was shown to contain an internal signal element which is important for energy-consumptive chloroplast targeting through a proteinaceous import complex other than TOC/TIC (Jarvis, 2008). A completely different mechanism has been described for the NADPH:protochlorophyllide oxidoreductase PORA, which is supposed to be chloroplast-imported only when bound to its substrate protochlorophyllide (Reinbothe et al., 1997).

1.2. The chloroplast proteome

Analyzing the chloroplast proteome is important in order to uncover chloroplast functioning by providing information about protein composition and compartmentalization of metabolic pathways (Jan van Wijk, 2000; Jarvis, 2004; Lilley and Dupree, 2007; Baginsky, 2009).

Beginning with the completion of the genome sequence of *Arabidopsis thaliana* in the year 2000 efforts were made to estimate the size of the chloroplast proteome based on the prediction of cTPs. There are various prediction programs publicly available with the most frequently used ones being TargetP and ChloroP (Emanuelsson et al., 1999; Emanuelsson et al., 2000). The Arabidopsis Genome Initiative calculated an overall number of ~3600 chloroplast proteins using TargetP (AGI, 2000). In the same year the group of Dario Leister analyzed the chloroplast prediction of a subset of ~14000 already identified Arabidopsis proteins using ChloroP. Extrapolation to the whole genome gave rise to ~1900-2500 predicted chloroplast proteins (Abdallah et al., 2000). The difference in prediction of more than 1000 proteins compared to TargetP can be explained by the fact that cTPs are remarkable in their diversity as previously mentioned (Jarvis, 2008). Therefore an improved prediction strategy was applied where cTPs were accepted only when they were identified by at least three of four different programs (Richly and Leister, 2004). This resulted in the prediction of ~2100 proteins which probably fits best to the actual size of the chloroplast proteome. Due to the poor specificity of prediction programs it is indispensable to experimentally analyze the

chloroplast proteome because reliable information on the subcellular localization of proteins cannot be deduced from genome sequences (Lilley and Dupree, 2007; Baginsky, 2009).

One alternative to bioinformatic prediction of the chloroplast proteome is the direct experimental detection of proteins in isolated chloroplasts. In plants, whose genomes are already sequenced, mass spectrometry (MS) coupled proteomic approaches are routinely employed (Haynes and Roberts, 2007). Since the first plant genomes were published several large-scale proteome surveys of plant organelles have been conducted. In this way the proteome of all major organelles such as plastids, mitochondria, peroxisomes or vacuoles was analyzed and the obtained protein data were integrated into databases. MS-based organelle specific databases are for example plprot for plastids, AMPDB for mitochondria, and AraPerox for peroxisomes (Heazlewood et al., 2004; Reumann et al., 2004; Kleffmann et al., 2006). Further localization databases are SUBA and PPDB, which are dedicated to all proteins and include data not only from MS studies but from all available sources such as fluorescent fusion protein or immunolocalization analyses (Heazlewood et al., 2005; Sun et al., 2008). But whereas SUBA only provides links to publications, PPDB tries to integrate and manually curate all available localization data. PPDB also includes data of a recently published chloroplast study which claims to be the most comprehensive chloroplast proteome analysis to date (Zybailov et al., 2008). Altogether PPDB provides by far the most extensive curated resource for experimentally verified chloroplast-localized proteins. It contains ~1200 proteins which were assigned to the chloroplast only if there was sufficient and compelling evidence from available localization and functional data. Recently, another extensive chloroplast proteomic study was released (Ferro et al., 2010). In total 1323 proteins were identified and integrated into the novel database AT_CHLORO but only envelope proteins were curated. Nevertheless together with all curated chloroplast proteins extracted from PPDB, both databases make up for a total of ~1700 unique chloroplast-localized proteins (Ferro et al., 2010). This number probably reflects the amount of chloroplast proteins that is accessible with current MS technologies and traditional preparation techniques.

Up to date neither the proteome of an organism nor an organelle has been experimentally identified completely. On the one hand not all proteins are equally accessible for proteomic techniques as a consequence of their physicochemical properties. On the other hand the low coverage of proteomic studies is probably due to the dynamic range of proteins (10^6 magnitudes) leading to a repeated detection of abundant proteins while low-abundant ones are missed. The development of more sensitive mass spectrometers could enhance the detection of low-abundant proteins but to overcome the dynamic range problems it is necessary to

modify the fractionation techniques prior to MS (Baginsky, 2009). In accordance with Ferro et al. I think that classical large scale chloroplast proteomic approaches have reached their limit and only directed approaches have the potential to unveil low-abundant proteins (Ferro et al., 2010). To date there are only very few reports about studies aiming on the targeted identification of organellar proteins present in the literature. Examples are the identification of thioredoxin interacting proteins in the stroma of chloroplasts by using immobilized thioredoxin affinity columns and the analysis of ATP binding proteins in chloroplast membranes or in the mitochondrial matrix by ATP-affinity chromatography (Motohashi et al., 2001; Balmer et al., 2003; Kishimoto et al., 2003; Ito et al., 2006).

1.3. Signaling by protein kinases

Plants have to sense changes in their environment in order to adapt their cellular metabolism and to ensure survival. In a biochemical process called signal transduction mechanical or chemical stimuli are linked with cellular reactions (Fig.4)(Campbell and Reece, 2009). Key players in signal transduction are protein kinases and calcium-binding proteins that decode calcium signals elicited under various conditions (Baginsky and Gruissem, 2009; Bussemer et al., 2009). Since this thesis focuses on protein kinases they will be discussed now.
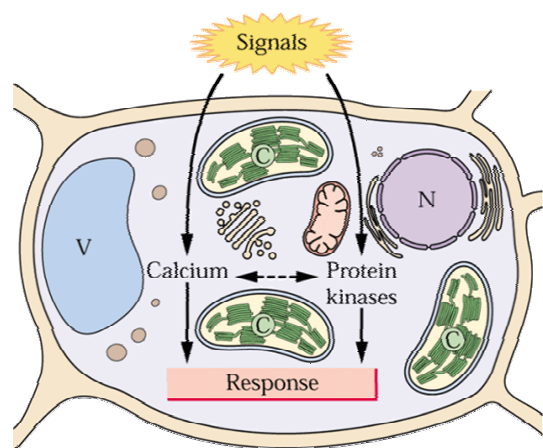
Fig. 4. Scheme of signal transduction (Buchanan et al., 2000). In response to an extracellular signal an intracellular response is triggered via calcium and/or protein kinases. C=chloroplast; N=nucleus; V=vacuole.

Protein kinases are enzymes that catalyze the phosphorylation of target proteins, a reaction that can be reversed by protein phosphatases. Protein phosphorylation is a mechanism for the regulation of protein kinase substrates resulting in their activation or deactivation.

Protein phosphorylation was first discovered in 1955 when it was shown that ATP was required for the activation of the enzyme phosphorylase. This phosphorylase kinase was the first protein kinase to be purified and characterized (Sutherland and Wosilait, 1955; Krebs, 1983).

Protein kinases can only phosphorylate specific amino acid residues within their target molecules and thus, can be divided into basically four different groups according to their specificity: serine/threonine-specific protein kinases (STKs), tyrosine-specific protein kinases (TKs), dual-specificity protein kinases (DSKs) and histidine protein kinases (HKs). While

STKs phosphorylate substrates at serine and/or threonine residues and TKs at tyrosine residues, DSKs are able to phosphorylate substrates at either serine and/or threonine as well as tyrosine residues (Hunter, 1991). STKs, TKs and DSKs have a well-conserved primary structure consisting of 12 subdomains (I-V, VIA, VIB and VII-XI) of a total size of ~280 amino acids (Fig.5). Subdomains I-IV are building the ATP-binding site, the substrate binding site and the catalytic center are found within subdomains VIA-XI and subdomain V serves as a linker. Furthermore, many protein kinases contain additional domains involved in various processes such as protein-protein interactions, transcription or protein degradation (Hanks and Hunter, 1995; Zhang et al., 2007).
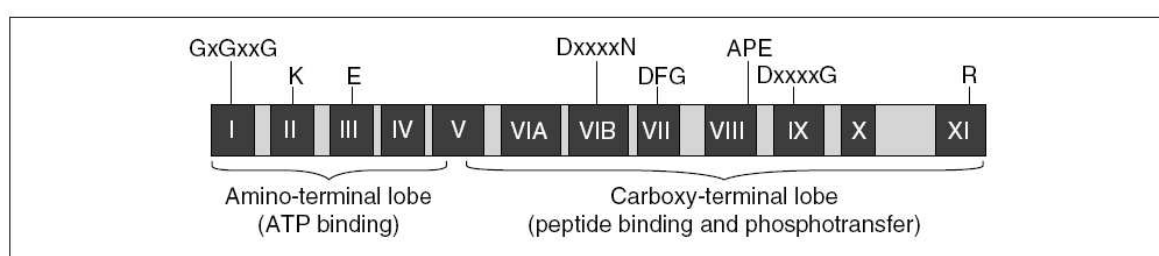
Fig. 5. The 12 subdomains of the conserved protein kinase motif (Hanks, 2003). While the ATP binding domain of STKs and TKs is identical, they slightly differ in their active site according to Prosite (http://www.expasy.org/prosite).

In contrast, histidine kinases (HKs) are structurally distinct from other protein kinases and are usually part of two-component signal transduction systems. A two-component system consists of a so called sensor histidine kinase and a response regulator (RR). Upon recognition of an extracellular signal the HK autophosphorylates at a histidine residue (Grefen and Harter, 2004). Subsequently the phosphate group is transferred to an aspartate residue within the RR leading to an activation of a cellular response (Puthiyaveetil et al., 2008). HKs are ubiquitous in prokaryotes but they are also found in yeast and plants. In yeast a HK was shown to be involved in the HOG-pathway which modulates gene expression in response to osmolarity changes. In plants phytochromes and ethylene as well as cytokinin receptors have already been identified as HKs (Hwang et al., 2002).

Arabidopsis contains in total ~1050 different protein kinases (Wang et al., 2003; Martin et al., 2009). There are no *bona fide* tyrosine protein kinases but 61 DSKs that are able to phosphorylate tyrosine residues. DSKs can be divided into two families which are structurally different: the STY family with 51 annotated genes, and the MAPKK (mitogen-activated protein kinase kinase) family with 10 members (Ichimura, 2002; Rudrabhatla et al., 2006). Furthermore, Arabidopsis contains 54 two-component proteins, which besides HKs also include RRs (Hwang et al., 2002). Thus, STKs account for the vast majority of protein kinases within the Arabidopsis kinome. The prevalence and importance of serine/threonine

phosphorylation is further highlighted by a phosphoproteomic study on whole cell lysates of Arabidopsis (Sugiyama et al., 2008). Analysis of 2172 unique phosphorylation sites in 1346 proteins resulted in a distribution of phosphoserine, phosphothreonine and phosphotyrosine sites of 85%, 10.7% and 4.3%, respectively.

Plant protein kinases are involved in the regulation of many cellular processes such as cell division, cell growth and cell differentiation. They are also mediating cellular responses to various biotic and abiotic stresses for instance changing light conditions, altered temperatures and pathogen invasion (Stone and Walker, 1995; Laurie and Halford, 2001).

A classic example for signal transduction by protein kinases is the mitogen-activated protein kinase (MAPK) pathway. In response to a certain extracellular stimulus elicited by a biotic or abiotic stress factor a MAPKKK is engaged that phosphorylates a MAPKK, which subsequently phosphorylates a MAPK. Eventually, the MAPK activates a transcription factor by phosphorylation leading to an altered gene expression and thereby to an adaptation of the cell to the changed environmental condition (Seger and Krebs, 1995).

Since protein kinases have such profound effects on cells their activity must be highly regulated, which can be achieved for example by phosphorylation, by binding of regulatory proteins, by regulation of their subcellular localization or via the second messenger Ca^{2+} (Hunter, 2000).

It is known that in response to various stresses such as cold, drought or high salinity, the cytoplasmic Ca^{2+} concentration is increased from the normal level of 0.1µM up to 2.2µM by release of Ca^{2+} from intracellular storages and/or from the apoplast (Knight et al., 1996, 1997; White and Broadley, 2003). Furthermore, fluxes of Ca^{2+} in the cytoplasm as well as in the chloroplast stroma have been observed upon onset of darkness reaching a peak of ~5-10µM in the stroma (Sai and Johnson, 2002).

In plants basically two families of protein kinases are linking calcium fluxes with protein phosphorylation: calcium-dependent protein kinases (CDPKs) and calcineurin B-like protein (CBL)-interacting protein kinases (CIPKs). Upon Ca^{2+}-binding CBLs are able to interact with CIPKs thereby regulating their activity. In Arabidopsis 10 CBLs are forming an interaction network with 25 CIPKs (Batistic and Kudla, 2009). CDPKs contain a calmodulin consisting of usually 4 EF-hands fused to the protein kinase domain and thus, can be directly activated by calcium-binding. They are unique for plants, green algae and some protozoa. In the Arabidopsis genome 34 CDPKs are encoded (Hrabak et al., 2003).

1.4. Protein phosphorylation in chloroplasts

Protein phosphorylation by protein kinases is a key mechanism to transduce signals within a cell and to regulate processes according to environmental changes. The chloroplast is integrated into the cellular signaling network because photosynthesis as well as all other metabolic processes clearly require tight regulation and coordination with the metabolic state of the whole plant.

Historically, the first reports of protein phosphorylation within chloroplasts date back to the 1970s when phosphorylation of light-harvesting complex (LHC) proteins was demonstrated (Bennett, 1977). In contrast, stromal protein phosphorylation was first reported in 1983, when pyruvate, orthophosphate dikinase was shown to be inactivated by phosphorylation in *Zea mays* chloroplasts (Ashton and Hatch, 1983). But so far only a handful chloroplast-localized protein kinases have been reported in the literature.

The most thoroughly described examples are the "state transition" kinases STN7 and STN8, which were first discovered in a genetic screen in *Chlamydomonas reinhardtii*, but homologs exist in all higher plants (Rochaix, 2007). They are localized within the thylakoid membrane and are involved in the photosynthetic acclimation under changing light conditions by phosphorylation of LHC proteins (Bonardi et al., 2005). The three protein kinases TAK1, TAK2 and TAK3 have also been suggested to localize to thylakoids (Snyders and Kohorn, 1999). The chloroplast α subunit of casein kinase II (CKII) was first identified in mustard, and the Arabidopsis homolog has been shown to be chloroplast-localized as well (Ogrzewalla et al., 2002; Salinas et al., 2006). This protein kinase is associated with the plastidiar RNA polymerase and phosphorylates parts of the transcription machinery and RNA-binding proteins. Recently, the chloroplast sensor kinase CSK was described. It is a prokaryotic-like two-component histidine kinase, which couples photosynthesis to chloroplast gene expression via a redox regulatory system (Puthiyaveetil et al., 2008). Furthermore, four members of the ABC1 family of protein kinases have been identified in chloroplast proteomic studies, whereas three of them were found exclusively in plastoglobules (Vidi et al., 2006; Ytterberg et al., 2006; Zybailov et al., 2008). In addition to the protein kinase domain, they contain an ABC1 domain, which was first discovered in the yeast ABC1 protein that is targeted to mitochondria and involved in the regulation of the activity of the bc1 complex (Bousquet et al., 1991). In Arabidopsis the ABC1 protein kinases are supposed to be involved in the regulation of quinone synthesis, but their localization has not been verified yet. Further reports of chloroplast protein kinases from other organisms include MSK4 from *Medicago sativa* and NtDSK1 from *Nicotiana tabacum*. MSK4 was reported 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 since its expression is regulated in response to light, it is suggested to have a role in chloroplast light signaling (Cho et al., 2001). Furthermore, studies in *Pisum sativum* provided evidences for the presence of protein kinases in the outer envelope of chloroplasts but envelope-localized protein kinases are beyond the scope of this PhD thesis (Soll, 1988; Soll et al., 1988).

The importance of phosphorylation in chloroplasts was revealed by a recent phosphoproteomic study in Arabidopsis (Reiland et al., 2009). In total 353 unique phosphosites were identified in 174 high-confidence chloroplast proteins including LHC proteins, proteins involved in RNA binding and carbohydrate metabolism, photosystem core subunits and STN7. The phosphorylation site distribution for serine, threonine and tyrosine residues was 72%, 27% and 1%, respectively.

1.5. Myristoylation and palmitoylation

The correct subcellular localization of proteins is crucial for their physiological function (Scott et al., 2005). Generally, proteins can be targeted to soluble compartments of the cell such as the cytoplasm, the mitochondrial matrix, or the chloroplast stroma or they can be targeted to cellular membranes such as the plasma membrane or the ER. It is estimated that ~30% of all cellular proteins are targeted to membranes (Kleinschmidt, 2003).

Membrane attachment of proteins can be achieved via hydrophobic transmembrane domains, electrostatic interaction with membrane components or lipid modifications (Batistic et al., 2008). The two major mechanisms of lipid modification are acylation and prenylation. Prenylation is the covalent attachment of a farnesyl or geranylgeranyl moiety to a cysteine residue within a consensus motif located at the C-terminus of target proteins (Zhang and Casey, 1996).

The two major forms of acylation, which is known to effect numerous proteins involved in signal transduction, are myristoylation and palmitoylation (Towler et al., 1988; Taniguchi, 1999). N-myristoylation is the irreversible, co-translational attachment of myristic acid (C14:0) to an N-terminal glycine that is required at position 2 of a protein. During translation, following removal of the N-terminal methionine residue by a methionylaminopeptidase, myristic acid is linked to the glycine on position 2 via an amide bond by a N-myristoyltransferase (NMT)(Farazi et al., 2001). NMT recognizes a certain consensus motif – in many cases MGXXX(S/T) – which can be predicted by various programs (Maurer-Stroh et al., 2002; Bologna et al., 2004; Podell and Gribskov, 2004; Sorek et al., 2009). In contrast,

palmitoylation is the post-translational attachment of palmitic acid (C16:0) to N-terminal or internal cysteine residues of proteins via a reversible thioester bond catalyzed by a protein palmitoyltransferase (PPT). PPTs are much likely located at membranes but the mechanism of their action is still unclear (Yalovsky et al., 1999; Weber, 2006). Internal palmitoylation of proteins is myristoylation-independent, whereas N-myristoylation is a prerequisite for N-terminal palmitoylation in most cases. Furthermore, palmitoylation is not restricted to the presence of a specific consensus motif (Sorek et al., 2009).

Myristoylation facilitates only reversible membrane binding of proteins because the energy provided by myristate-lipid interaction is too low for stable membrane attachment (Peitzsch and McLaughlin, 1993). In contrast, palmitoylation is suggested to mediate a stable membrane anchoring, which corresponds to the fact that palmitoylated proteins are almost exclusively found in membrane preparations whereas myristoylated proteins are also present in soluble protein extracts (Towler et al., 1988; Taniguchi, 1999). Stable membrane attachment of myristoylated proteins can only be achieved by additional factors that support membrane binding such as palmitoylation, interaction of a polybasic amino acid stretch with acidic phospholipids or interaction with a membrane protein (Murray et al., 1997; Weber, 2006).

Acylation of proteins can influence their membrane targeting, their structure and activity or their interaction with other proteins (Beven et al., 2001). The physiological relevance of N-myristoylation has already been demonstrated for example in the case of the Arabidopsis proteins SOS3 (salt overly sensitive) and CBL1.

During salt stress the plasma membrane Na^+/H^+ exchanger SOS1 is regulated in a calcium-dependent manner via the joint action of SOS3 (a CBL protein) and SOS2 (a CIPK protein). It was shown that in salt-hypersensitive *sos3-1* mutant plants SOS1 activity was impaired. This could only be complemented by expression of wild-type SOS3 but not SOS3G2A, which has the glycine on position 2 exchanged for alanine and as a consequence cannot be myristoylated anymore (Ishitani et al., 2000; Qiu et al., 2002). Similarly to SOS3, mutation of the calcineurin B-like protein CBL1 results in a salt-sensitive phenotype. Again, only wild-type CBL1 but not CBL1G2A was able to partially complement the defect of the *cbl1* mutant. Furthermore, CBL1C3S, which has the cysteine on position 3 exchanged for serine resulting in prevention of palmitoylation, was not able to complement the salt-sensitive phenotype. This indicated an influence of palmitoylation as well on the physiological function of CBL1 (Batistic et al., 2008).

1.6. Detailed objectives of this thesis

1) Identification of novel chloroplast-localized protein kinases in higher plants by
   - a candidate approach
   - a phylogenetic approach
   - a proteomic approach

2) Localization analysis of selected proteins identified via the phylogenetic and the proteomic approach

3) Comparison of the chloroplast stromal proteomes of *Pisum sativum* and *Arabidopsis thaliana*

4) Investigation of the effects of N-terminal acylation, namely myristoylation and palmitoylation, on the chloroplast import competence of selected proteins

2. Material and Methods

2.1. Used clones and vectors

cDNA clones

b5Rsol   in vector pGEM3; obtained from Sara Colombo (Institute of Neuroscience, University of Milan, Italy); used as control for SRP assay

b5RWT   in vector pGEM3; obtained from Sara Colombo; used as control for SRP assay

DQ446901   Full-length cDNA clone for At4g36070 (CPK18) from ABRC (http://www.arabidopsis.org/abrc/)

MsMSK4   Full-length cDNA clone obtained from Wilfried Rozhon

RAFL21-73-A21   Full-length cDNA clone for At3g57810 (OTL) from Riken BioResourceCenter (Seki et al., 1998; Seki et al., 2002; Sakurai et al., 2005)

Plant organelle markers

All marker clones were created by Nelson and co-workers (Nelson et al., 2007) and obtained from ABRC (http://www.arabidopsis.org/abrc/). They immediately could be used for agrobacterium-mediated transfection of tobacco leaf epidermal cells.

ER   clone CD3-959; ER marker gene fused to the fluorescent protein mCherry (Shaner et al., 2004)

mitochondrion   clone CD3-991; mitochondrial marker gene fused to mCherry

peroxisome   clone CD3-983; peroxisomal marker gene fused to mCherry

Used vectors

pBAT   pBluescript derivate; carries rabbit myoglobin leader sequence; used for *in vitro* translation of proteins (Annweiler et al., 1991)

pBIN-Basta   pBIN19 derivate (Bevan, 1984); plant kanamycin resistance marker exchanged with BASTA resistance marker and one ApaI restriction site deleted by Norbert Mehlmer; binary plant transformation vector

pCR-Blunt   Invitrogen (Paisley, UK); used as a carrier vector for the cloning of PCR products

pGEM3   Promega (Madison, WI, USA); used for SRP assay

pGEX4T-3   GE Healthcare (Chalfont St. Giles, England); used for GST (glutathione S-transferase)-mediated protein purification

2.2. Bacteria strains

Escherichia coli (*E.coli*)

(bacterial cultures were grown at 37°C under vigorous shaking)

strain DH5α   F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1

strain ER2566   from NEB (Ipswich, MA, USA); F– λ– fhuA2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73::miniTn10–TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]

Agrobacterium tumefaciens

(bacterial cultures were grown at 30°C under vigorous shaking)

strain AGL1   is a derivate of AGL0; *recA::bla* pTiBo542ΔT Mop$^+$ Cb^R (Lazo et al., 1991)

2.3. Plant material

Arabidopsis thaliana

Col-0   ecotype Columbia

SALK_047737   T-DNA insertion line for AT5G16810 (Alonso et al., 2003)

Nicotiana tabacum

cv. Petite Havana SR1

Pisum sativum

cv. Arvika ZS (BSV-Bayrische Futtersaatbau GmbH, Ismaning, Germany)

2.4. Radiolabeled chemicals

ATP-[γ-^{32}P]: 6000Ci/mmol; 10mCi/ml (PerkinElmer, Waltham, MA, USA)

L-methionine-[^{35}S]: 1175Ci/mmol; 10.2mCi/ml (PerkinElmer, Waltham, MA, USA)

Myristic acid-[9, 10-^{3}H]: 60Ci/mmol; 1mCi/ml (ARC, St. Louis, MO, USA)

2.5. Antibody

α-FSBA   from mouse; purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); used for immunoprecipitation

2.6. Media

(all media were prepared with deionized water and autoclaved for 20min at 120°C)

Lysogeny broth (LB) medium

For 1 litre: 5g Yeast extract; 10g tryptone; 5g NaCl; optionally add 3% Bacto Agar for plates

LB+AMP medium
Add 1ml of 1000x ampicillin stock (100mg/ml in 50% ethanol; stored at -20°C) to 1l LB after autoclaving; optionally add 3% Bacto Agar for plates

LB+KAN medium
Add 1ml of 1000x kanamycin stock (50mg/ml; stored at -20°C) to 1l LB after autoclaving; optionally add 3% Bacto Agar for plates

½ Murashige-Skoog (MS) medium for plates
For 1 litre: 2.2g Murashige & Skoog medium (Duchefa, Haarlem, Netherlands); 7g Plant Agar (Duchefa, Haarlem, Netherlands); 10g saccharose; adjust pH to 5.8 with 0.1M KOH

2.7. Buffers/Solutions

(for liquids: % in v/v; for solid substances: % in w/v; all buffers/solutions were stored at RT unless otherwise noted; buffers and solutions are listed alphabetically)

ATP buffer
Buffer S200-A + 100mM NaCl; 0.05% NP-40

Coomassie staining solution
for 1 litre: 2.5g Coomassie Brilliant Blue R-250; 45% isopropanol; 10% acetic acid

Coomassie destaining solution
10% isopropanol; 10% acetic acid

DNA loading buffer
for 100ml: 250mg bromophenol blue; 250mg xylene cyanol; 50mM Tris-HCl (pH 7.6); 60% glycerol ; prior to use mix 300µl of loading buffer with 700µl of ddH$_2$O

DNA Quick-Prep buffer 1
25mM Tris; 10mM Na$_2$EDTA; adjust pH to 8 with HCl; 250µg/ml RNase A; store at 4°C

DNA Quick-Prep buffer 2
0.2M NaOH; 1% SDS

DNA Quick-Prep buffer 3

5M KCH$_3$COO; 11.5% CH$_3$COOH

GE buffer

10mM reduced glutathione; 50mM Tris-HCl (pH 8); store at -20°C

Genomic DNA extraction buffer

200mM Tris-HCl (pH 7.5); 250mM NaCl; 0.5% SDS; 25mM EDTA (pH 8)

GST buffer

50mM Tris; 20mM MgSO$_4$; 5mM EDTA; pH to 8 with HCl; add 2mM DTT prior to use

10x HMS buffer

3.3M sorbitol; 500mM HEPES-KOH (pH 7.6); 30mM MgSO$_4$

HOMO buffer

450mM sorbitol; 20mM Tricine-KOH (pH 8.4); 10mM EDTA; 5mM NaHCO$_3$; 0.1% BSA; add 10mM isoascorbate and 1mM reduced glutathione prior to use; readjust pH to 8.4 (KOH)

IP buffer

50mM Tris-HCl (pH 7.5); 50mM NaCl; 10mM MgCl$_2$

IP-W buffer

50mM Tris-HCl (pH 7.5); 250mM NaCl; 0.1% NP-40; 0.05% deoxycholic acid

5x kinase buffer

100mM HEPES; 75mM MgCl$_2$; 1mM DTT; pH 7.5 with KOH; store at -20°C

4x Laemmli buffer (for SDS-PAGE)

0.25 M Tris-HCl (pH 6.8); 8% SDS; 40% glycerine; 20% β-Mercaptoethanol; 0.016% bromophenol blue

MQ-A

20mM Tris-HCl (pH 7.8 or 8); filter buffer through a 0.22μm membrane and degas

MQ-B

20mM Tris-HCl (pH 7.8 or 8); 1M NaCl; filter buffer trough a 0.22µm membrane and degas

MS-A

20mM MES-NaOH (pH 6); filter buffer through a 0.22µm membrane and degas

MS-B

20mM MES-NaOH (pH 6); 1M NaCl; filter buffer through a 0.22µm membrane and degas

40% Percoll

330mM sorbitol; 50mM HEPES-KOH (pH 7.6); 40% Percoll; store at -20°C

80% Percoll

330mM sorbitol; 50mM HEPES-KOH (pH 7.6); 80% Percoll; store at -20°C

P-ISO

330mM sorbitol; 20mM MOPS; 13mM Tris; 0.1% BSA; 3mM $MgCl_2$; store at 4°C (<1 week)

PS-A buffer

50mM sodium phosphate buffer (pH 7; prepared according to Lab FAQS from Roche Applied Sciences); filter buffer through a 0.22µm membrane and degas

PS-B buffer

PS-A buffer + 1.5M $(NH_4)_2SO_4$; filter buffer through a 0.22µm membrane and degas

PurB buffer

Buffer S200-A + 350mM NaCl; 0.5% Triton X-100

P-WASH I

330mM sorbitol; 50mM HEPES-KOH (pH 7.6); 3mM $MgCl_2$; store at -20°C

P-WASH II

330mM sorbitol; 50mM HEPES-KOH (pH 7.6); 0.5mM $CaCl_2$; store at -20°C

P-WASH III

330mM sorbitol; 50mM HEPES-KOH (pH 7.6); 5mM EDTA; store at -20°C

2x RB buffer

600mM sorbitol; 40mM Tricine-KOH (pH 7.6); 10mM $MgCl_2$; 5mM EDTA; store at 4°C (<1 week)

RNA extraction buffer

1% SDS; 10mM EDTA; 200mM $NaCH_3COO$; pH 5.2

S200-A buffer

50mM Tris-HCl (pH 7.8); 50mM NaCl; 10mM $MgCl_2$; filter buffer through a 0.22µm membrane and degas

SDS-running buffer

25mM Tris; 250mM glycine; 0.1% SDS

SEPP buffer

10mM Tricine-KOH (pH 8); 10mM $MgCl_2$; add 1mM DTT and 1 tablet Complete Mini EDTA-free (Roche Applied Science, Penzberg, Germany) prior to use

SRP buffer

50mM Tris-CH_3COOH (pH 7.5); 250mM KCH_3COO; 2.5mM $Mg(CH_3COO)_2$; 1mM DTT

50x TAE buffer

2M Tris; 50mM Na_2EDTA; 5.71% glacial acetic acid; adjust pH to 8 with HCl

TB buffer

10mM $CaCl_2$; 10mM Pipes; 15mM KCl; 55mM $MnCl_2$; adjust pH to 6.7 with HCl (to prevent the formation of water-insoluble MnO(OH), it is crucial to adjust pH prior to the addition of $MnCl_2$); sterile filtrate

TBS-T

50mM Tris-HCl (pH 7.4); 150mM NaCl; add 0.1% TWEEN 20 prior to use

<u>1x TE buffer</u>

10mM Tris; 1mM Na$_2$EDTA; adjust pH to 8 with HCl

<u>THY buffer</u>

25mM Tricine-KOH (pH 8); 10mM MgCl$_2$; 10mM isoascorbic acid; 2mM β-mercaptoethanol; store at -20°C

<u>5x THYSO buffer</u>

125mM Tricine-KOH (pH 8); 10mM MgCl$_2$

2.8. RNA methods

When working with RNA always DEPC treated ddH$_2$O and solutions prepared with DEPC treated ddH$_2$O were used in order to prevent degradation of RNA by RNases. Furthermore pipette tips and reaction tubes were stored in a separate, clean place and only used for RNA applications.

Total RNA isolation from Arabidopsis leaves

Leaves (~150mg) were harvested, transferred to a 1.5ml tube and immediately frozen in liquid nitrogen. Two glass beads were added and sample was homogenized for 2x 1min using a TissueLyser II (QIAGEN, Hilden, Germany). Subsequently 530µl RNA extraction buffer and 530µl phenol (pH 4; AppliChem, Darmstadt, Germany) were added followed by vortexing for 30s and centrifugation for 10min at 16100g. The supernatant was transferred to a new 1.5ml tube and extracted with 1 volume of PCI (25:24:1; ROTH, Karlsruhe, Germany). After vortexing and centrifugation as before, the supernatant was transferred to a new 1.5ml tube and extraction was repeated with 1 volume of CHCl$_3$. After centrifugation the supernatant was transferred to a new 1.5ml tube and mixed with $^1/_3$ volume of 10M LiCl. RNA was precipitated o/n at 4°C. The next day RNA was pelleted by centrifugation for 15min at 16100g and 4°C. The supernatant was decanted and the pellet was washed once with 500µl 2.5M LiCl and once with 500µl 70% ethanol (centrifugation as before). Finally RNA was dried for ~15min at 45°C, resuspended in 25µl ddH$_2$O and RNA concentration was determined:

- OD$_{260}$ of 2µl RNA in 500µl ddH$_2$0 was measured
- RNA concentration was calculated using following formula:

$$OD_{260} * 10 = \mu g/\mu l \; RNA$$

RNA was stored at -80°C.

Arabidopsis cDNA synthesis

First strand cDNA was synthesized from total RNA isolated from Arabidopsis leaves using the M-MLV reverse transcriptase from Promega (Madison, WI, USA). 2µg RNA were mixed with 1.5µl 100µM oligo(dT) primer, filled up to 14µl with ddH$_2$O and incubated for 5min at 70°C (in order to melt secondary structures within the RNA molecules which would interfere with reverse

transcription). After incubation on ice for 5min 1.25µl dNTP mix (10mM each), 5µl 5x buffer, 1µl M-MLV reverse transcriptase and 3.75µl ddH$_2$O were added and cDNA synthesis was carried out for 1h at 40°C. cDNA was stored at -20°C.

2.9. DNA methods

Polymerase chain reactions (PCR)

Amplification of DNA was done by common PCR. PCR mixes and programs are listed below (Tab.1+2). For cloning of DNA fragments or the introduction of mutations into genes following polymerases with proof-reading activity were used: Phusion, Vent$_R$ (both NEB, Ipswich, MA, USA) and PfuTurbo (Stratagene, La Jolla, CA, USA). Proof-reading ensured that DNA amplification occurred at a high fidelity.

In case of RT-PCR, verification of agrobacterial plasmids or genomic DNA genotyping, where only the presence or absence of specific DNA fragments had to be analyzed, the polymerase GoTaq (Promega, Madison, WI, USA) lacking proof-reading activity was used. After amplification all PCR products were analyzed by agarose gel electrophoresis. In case a DNA fragment of interest was needed for further applications, it was cut out of the gel and extracted (see "DNA extraction from agarose gels").

Tab. 1. PCR mixes for different applications. Optionally Mg^{2+} concentration was altered to increase PCR yield. After PCR with Phusion, Vent$_R$ or PfuTurbo polymerase 15µl DNA loading buffer were added and the whole sample was analyzed by agarose gel electrophoresis. For PCRs with GoTaq the Green GoTaq Reaction Buffer was used which already includes the loading dye and thus allowed direct loading of the sample onto an agarose gel. Usually 12µl were loaded. LL = Lacroute library (Minet et al., 1992).

components	cDNA (Phusion)	cDNA (Vent$_R$)	gen. DNA/ RT-PCR (GoTaq)	agrobacterial colony PCR (GoTaq)	PCR mutagenesis (PfuTurbo)
µl ddH$_2$0	x	x	15.875	18.375	31.6
µl buffer	10	5	5	5	4
µl primer 5' (10µM)	1	1	0.5	0.5	1
µl primer 3' (10µM)	1	1	0.5	0.5	1
µl dNTPs (10mM each; NEB)	1	1	0.5	0.5	1
µl DNA template	1 LL or 2.5 cDNA	1 LL or 2.5 cDNA	2.5 gen. DNA/cDNA	1 colony of agrobacteria	1 (MIDI-DNA 1:30)
µl polymerase	0.5	0.2	0.125	0.125	0.4
µl MgSO$_4$	-	2	-	-	-
µl total	**50**	**50**	**25**	**25**	**40**

Tab. 2. PCR programs for different applications. *standard annealing temperature; +/-2°C to optimize PCR. **elongation time depending on enzyme capacity: 1min/kb except for Phusion: 30s/kb.

gen. DNA/RT-PCR (GoTaq)	95°C	52°C*	72°C
1x	5min	2min	3min
35x	45s	1min	3min
1x	-	-	10min

PCR mut. (PfuTurbo)	95°C	53°C*	72°C
1x	2min	2min	10min
20x	45s	2min	10min
1x	-	-	15min

cDNA (Vent$_R$)	95°C	52°C*	72°C
1x	45s	-	-
30x	45s	1min	**
1x	-	-	10min

cDNA (Phusion)	98°C	54°C*	72°C
1x	30s	-	-
30x	10s	30s	**
1x	-	-	10min

agrob. PCR (GoTaq)	95°C	52°C*	72°
40x	1min	1min	3min

Comment:

After PCR-mutagenesis of an entire plasmid the PCR-product was digested with the restriction enzyme DpnI which recognizes only methylated sites. Since the template DNA was

isolated from *E.coli* it contained methylated nucleotides whereas the newly synthesized PCR product did not. Thus, DpnI (NEB, Ipswich, MA, USA) treatment resulted in a template free PCR product. 36µl of the PCR product were mixed with 8µl ddH$_2$O, 5µl buffer and 1µl DpnI. Digestion was carried out for 1.5h at 37°C followed by heat inactivation of the enzyme for 20min at 80°C.

DNA Quick-Preparation from *E.coli*

(All centrifugation steps were carried out at 16100g and RT)

For quick isolation of ~20-30µg plasmid DNA from an *E.coli* culture the alkaline lysis method according to the Molecular Cloning Laboratory Manual (Sambrook et al., 1989) was used. One colony of *E.coli* was inoculated in 5ml LB medium containing the appropriate antibiotic (according to the resistance marker of the plasmid) and incubated o/n at 37°C. The next day 2ml of the culture (in case of pBIN-Basta vector 2x 2ml due to low copy number) were transferred into a 2ml tube and centrifugated for 2min. The supernatant was discarded and 200µl of DNA Quick-Prep buffer 1 were added and cells resuspended. Subsequently 200µl of DNA Quick-Prep buffer 2 (necessary for lysis of cells) were added and tube was shaken gently. Then 200µl of DNA Quick-Prep buffer 3 (for neutralization) were added and again tube was shaken gently and left on ice for 20min (for digestion of RNA by RNase). After centrifugation for 4min the supernatant was transferred to a new 1.5ml tube and plasmid DNA was precipitated by addition of 0.7-fold volume of isopropanol (~420µl). After vigorously shaking DNA was pelleted by centrifugation for 10min and supernatant was discarded. DNA pellet was washed with 500µl of 70% ethanol by inverting tube and incubating for 5min at RT. After centrifugation for 5min the supernatant was decanted. After a last centrifugation step for 2min residual ethanol was removed with a pipette and the pellet was dried for ~15min at 40°C.   Finally the DNA was resuspended in 40µl 0.5x TE buffer (in case of pBIN-Basta: 30µl). DNA was stored at -20°C.

DNA MIDI-Preparation from *E.coli*

To isolate ~300µg plasmid DNA from an *E.coli* culture the JetStar 2.0 MIDI Prep Kit (GENOMED, Löhne, Germany) was used. A o/n culture of bacteria carrying the plasmid of choice was prepared in 200ml LB plus the appropriate antibiotic. The next day cells were harvested in a 50ml tube (centrifugation for 10min at 3166g and 4°C) and JetStar protocol was followed until washing of membrane with solution E5. After the second washing step the

plasmid DNA was eluted into a 12ml tube (KABE Labortechnik, Nümbrecht-Elsenroth, Germany) with 5ml solution E6. 5ml isopropanol were added and DNA was precipitated for 30min at -20°C. Subsequently the DNA was pelleted by centrifugation for 30min at 12000g and 4°C (using a SS34 rotor) and supernatant was discarded. Pellet was resuspended in 300µl ddH$_2$0 and transferred to a 1.5ml tube. A 2.5-fold volume of cold 100% ethanol (-20°C; ~750µl) was added, the tube was inverted and then centrifugated for 10min at 16100g and RT. The supernatant was discarded and 800µl cold 70% ethanol (-20°C) were added. The tube was inverted and centrifugated as before. The supernatant was discarded and after a last centrifugation step as before, residual ethanol was removed with a pipette. The pellet was dried for ~15min at 40°C and finally resuspended in 50µl ddH$_2$0. Subsequently, DNA concentration was determined and adjusted to1µg/µl:

- OD$_{260}$ of 2.5µl DNA in 500µl ddH$_2$0 was measured
- Concentration was adjusted to 1µg/µl using following formula:

$$OD_{260} * 475 - 47.5 = V_{add}$$

$$V_{add} \ldots.. \text{Volume of ddH}_2\text{0 to add to obtain } 1µg/µl$$

DNA was stored at -20°C.

Control plasmid DNA restriction

Plasmids obtained from DNA Quick-Preparation were verified by restriction fragment analysis. Suitable restriction enzymes that ideally cut once within the insert and once within the vector background were selected using the software Vector NTI (Invitrogen, Paisley, UK). All enzymes were obtained from NEB (Ipswich, MA, USA). Reaction mixes are listed in Tab.3. The DNA was digested for 1.5h at the appropriate temperature (see manufacturer's instruction), mixed with 5µl DNA loading buffer and subsequently analyzed by agarose gel electrophoresis (~12µl were loaded).

Tab. 3. Control restriction mixes. BSA was added when specified by manufacturer.

components	restriction mix (1 enzyme)	restriction mix (2 enzymes)
µl ddH$_2$O	x	x
µl buffer	2	2
µl enzyme 1	0.2	0.2
µl enzyme 2	-	0.2
(µl BSA)	(0.2)	(0.2)
µl plasmid	3	3
µl total	**20**	**20**

Preparative plasmid DNA restriction

For subcloning of DNA fragments preparative restriction mixes were prepared (Tab.4). Suitable restriction sites were selected using Vector NTI (Invitrogen, Paisley, UK) and all used enzymes were purchased from NEB (Ipswich, MA, USA).

5µl plasmid DNA from a Quick- or MIDI-Preparation were digested for 1.5h at the appropriate temperature (see manufacturer's instruction), mixed with 15µl DNA loading buffer and subsequently analyzed by agarose gel electrophoresis (reaction mix was completely loaded onto gel). Subsequently the fragment of interest was cut out of the gel and the DNA was extracted (see "DNA extraction from agarose gels").

Tab. 4. Preparative DNA restriction mixes. BSA was added when specified by manufacturer.	
µl ddH$_2$O	x
µl buffer	5
µl enzyme 1	0.5
µl enzyme 2	0.5
(µl BSA)	(0.5)
µl plasmid	5
µl total	50

Comment:

In case a restriction enzyme had to be used that is also present within a fragment of interest, a partial restriction digest was carried out. Therefore only 0.2µl of the respective enzyme were used and the digestion was carried out for only 1-10min.

DNA extraction from agarose gels

A fragment of interest was cut out from an agarose gel and extracted using the Wizard SV Gel and PCR Clean-Up System from Promega (Madison, WI, USA). The protocol was followed and the DNA finally eluted in 40µl ddH$_2$0. Successful extraction was controlled by agarose gel electrophoresis (3µl of eluted DNA + 3µl DNA loading buffer were loaded) and in case the DNA concentration was too low the DNA was concentrated by evaporation using a SpeedVac.

Ligation of DNA fragments

PCR fragments (Vent$_R$ and Phusion polymerase) were ligated into the carrier vector pCR-Blunt using the Zero Blunt PCR Cloning Kit (Invitrogen, Paisley, UK).

Ligations for subcloning of cut DNA fragments were done using the T4 ligase from NEB (Ipswich, MA, USA). First a vector background fragment and an insert were digested with the same restriction enzyme(s) to obtain compatible ends ("Preparative plasmid DNA restriction"). Subsequently the two fragments were ligated to obtain a single, functional

plasmid (see Tab.5 for ligation mix). The amount of insert and vector fragment used was estimated by agarose gel electrophoresis and usually a ~5-fold excess of insert was employed. The ligation was carried out for 20min at RT and then the reaction mix was directly used for transformation of competent *E.colis*.

Tab. 5. DNA ligation mix.	
µl vector	0.5-1
µl insert	x
µl buffer	1
µl ligase	0.2
µl ddH$_2$O	x
µl total	10

Comment:

In case two fragments with one compatible and one incompatible end had to be ligated, this was done in combination with a Klenow treatment using the DNA Polymerase I Klenow Fragment (NEB, Ipswich, MA, USA). Following a normal ligation, 0.1µl dNTPs (10mM each) and 0.2µl Klenow fragment were added to the reaction mix, which was then incubated for 55min at RT. This resulted in the formation of blunt ends from the incompatible sticky ends, which subsequently were ligated. Finally the reaction mix was transformed into competent *E.colis*.

Agarose gel electrophoresis

DNA samples were analyzed by agarose gel electrophoresis. According to the size of the analyzed DNA molecules gels varying between 0.8%-2% agarose in 1xTAE were used. Staining was done with ethidium bromide (5mg/ml; 5µl per 100ml gel) and the size marker GeneRuler 1kb Plus DNA Ladder (Fermentas, Burlington, ON, Canada) was used for all gels.

DNA sequencing

Sequencing reactions were done using the Economy Run Service of Microsynth (Balgach, Switzerland). Per sequencing reaction ~0.8µg of DNA in 10µl ddH$_2$0 were used. Sequencing primers M13 and M13r were used for genes in pCR-Blunt vector.

Cloning of genes

All genes of interest were amplified by PCR from cDNA. Primers specific for full-length coding sequences were used except for genes bigger than ~2200bp due to experimental handling constraints. In such cases N-terminal parts of the respective genes of usually ~500bp were amplified instead.

The 5'-primer always contained an ApaI site whereas the 3'-primer contained a NotI site. This allowed subcloning into different target vectors that previously were optimized for ApaI-NotI cloning. Furthermore the NotI site replaced the stop codon in order to allow subcloning in frame with C-terminal YFP which was already present in the vector pBIN-Basta. In pBIN-Basta the stop codon is localized immediately after the YFP coding sequence and the vectors pBAT and pGEX4T-3 contain a stop codon downstream of the cloning site.

Agarose gel-extracted PCR products were ligated into pCR-Blunt ("Ligation of DNA fragments"). After DNA Quick-Preparation and successful control restriction, the constructs were sent for DNA sequencing. Only if a sequence was completely correct or contained only minor mutations (ones that do not alter the encoded amino acid or do not change the chemical property: e.g. glycine to alanine), a gene of interest was cut out of pCRBlunt and subcloned ("Preparative plasmid DNA restriction" and "Ligation of DNA fragments"). All constructs were verified by control restriction analysis, subjected to DNA MIDI-Preparation, and stored at -20°C.

Genomic DNA isolation from Arabidopsis leaves

Leaves (~150mg) were harvested, transferred to a 1.5ml tube and immediately frozen in liquid nitrogen. Two glass beads were added and the sample was homogenized for 2x 1min using a TissueLyser II (QIAGEN, Hilden, Germany). After centrifugation for 5min at 16000g and RT the supernatant was transferred to a new reaction tube and mixed with 1vol of isopropanol. The tube was briefly vortexed, incubated for 10min at RT (to precipitate the DNA) and centrifugated for 5min at 16000g and RT. The supernatant was decanted and the pellet was washed with 300µl of 70% ethanol. After centrifugation as before the supernatant was completely removed with a pipette and the pellet was dried for ~10min at 42°C. Finally, the pellet was resuspended in 50µl of 0.5x TE and the tube was gently shaken at 65°C for 10min. The isolated genomic DNA was stored at -20°C. For PCR the DNA was thawed, spun down and only the supernatant was used.

2.10. Protein methods

Recombinant GST-fusion protein expression in and purification from *E.coli*

For recombinant protein expression the gene of interest was C-terminally fused to glutathione S-transferase (GST) by cloning into the vector pGEX4T-3. The plasmid then was transformed into the *E.coli* strain ER2566 which is optimal for protein expression.

Either 1 bacterial colony or an aliquot of *E.colis* carrying the plasmid of choice was inoculated in 30ml LB+AMP medium and incubated o/n at 37°C. The next day the cells were diluted to an OD_{600} of ~0.1 in a volume of 300ml LB+AMP medium and grown at 37°C to a OD_{600} of ~0.8. Subsequently, the culture was supplied with 1mM IPTG to induce protein expression, which was carried out for 4h at 30°C (prior to addition of IPTG 100µl uninduced and after 4h of protein expression 100µl induced sample were taken; after centrifugation for 2min at 16100g and removal of the supernatant, the cell pellets were resuspended in 30µl 2x Laemmli buffer). The cells were harvested in a GS-3 tube by centrifugation for 10min at 1519g and 4°C (from now on all steps were carried out at 4°C). The pellet was resuspended in 10ml GST buffer and transferred to a 50ml tube. After centrifugation as before the cells were resuspended in another 10ml of GST buffer and broken by sonification (4x 30s with 30s break in between; 100%-continous intensity). Immediately after disruption 1% Triton-X 100 was added and the lysate was transferred to a 12ml tube. Cell debris was pelleted by centrifugation for 30min at 12000g and 4°C using a SS-34 rotor and the supernatant was transferred to a 15ml Greiner tube.

In the meantime Glutathione Sepharose^{TM} 4B (GE Healthcare, Chalfont St. Giles, England) was prepared (always cut tips were used): 100µl of resuspended sepharose slurry was transferred to a 1.5ml tube. After centrifugation (all centrifugation steps were carried out for 5min at 200g and 4°C) and removal of the supernatant, the sepharose was washed with 750µl GST buffer. Centrifugation was repeated, the supernatant discarded and the sepharose resuspended in 75µl GST buffer.

The bacterial lysate was mixed with the prepared sepharose and incubated for 1h at 4°C on a spinning wheel. After centrifugation and removal of supernatant the sepharose was washed twice with 5ml GST buffer. The sepharose was resuspended in 300µl GE buffer and incubated for 1h at 4°C on a spinning wheel to elute bound proteins. After centrifugation the eluate was transferred to a new 1.5ml tube and elution was repeated twice (10µl sample of each eluate were taken and mixed with 5µl 4x Laemmli buffer). Purified recombinant proteins were stored at -20°C.

All samples (uninduced, induced and eluates) were analyzed by SDS-PAGE. After heating for 5min at 95°C and centrifugation for 5min at 16100g, the samples were analyzed by SDS-PAGE (5µl of the uninduced as well as the induced sample and all of the eluate samples were loaded onto the gel).

SDS-PAGE (polyacrylamide gel electrophoresis)

Protein molecular weight determination was done by gel electrophoresis using a high-molarity Tris buffer system without urea (Fling and Gregerson, 1986). The Mini-PROTEAN II system from Bio-Rad (Hercules, CA, USA) was used to cast and run gels.

For the analysis of protein samples gels consisting of a stacking gel and a 12% separating (resolving) gel were used. They were run at 20mA/gel until samples migrated into separating gel, then the gels were further run at 40mA/gel. In all cases the molecular weight marker PageRuler[TM] Plus Prestained Protein Ladder (Fermentas, Burlington, ON, Canada) was used. Proteins were detected by Coomassie staining (gels were soaked in Coomassie staining/destaining solution while gentle shaking). Finally gels were dried using a Slab Gel Dryer.

In vitro protein kinase assay

Protein kinase activity of samples was determined by incubation with radioactively labeled ATP-γ-^{32}P (reaction mixes are listed in Tab.6+7). The kinase reactions were carried out for

Tab. 6. *In vitro* protein kinase assay reaction mixes. *Substrates refers to kinase assays of samples from crude stroma in the presence of general protein kinase substrates. The substrates used were casein, histone III-S, MBP and phosvitin (1-2µg/µl each). **CaCl$_2$/MgCl$_2$ concentrations varied between 0.5-250mM. ***The general protein kinase inhibitors Purvalanol B (PurB), sorafenib, staurosporine and sunitinib were used at concentrations of 8µM-8mM (in DMSO).

components	S200/S75/ATP/PurB/ FSBA/solubilization	Ca^{2+}/Mg^{2+}/ EGTA	Inhibitors	cKin3	NDPK2/ GST	Substrates*
µl 5x kinase buffer	4	4	4	4	4	4
µl sample	15.9	15.3	13.3	1	15.9/1	14.9
µl ATP-γ-^{32}P	0.1	0.1	0.2	0.1	0.1	0.1
µl 500mM EGTA	-	0.2	-	-	-	-
µl CaCl$_2$/MgCl$_2$**	-	0.4	-	-	-	-
µl inhibitor***	-	-	2.5	2.5	-	-
µl substrate	-	-	-	1	-	1
µl ddH$_2$O	-	-	-	11.4	0/14.9	-
µl total	**20**	**20**	**20**	**20**	**20**	**20**

20min at RT. Subsequently 8µl 4x Laemmli buffer were added to each reaction mix and 8µl were directly loaded onto a 12% SDS gel. The gel was run and after Coomassie staining/destaining and drying of the gel, the incorporation of γ-^{32}P into proteins was analyzed using a Storage Phosphor Screen (dried gel was put on screen o/n) and a Typhoon Trio Imager (GE Healthcare, Chalfont St. Giles, England).

Tab. 7. *In vitro* protein kinase assays for cKin18. Chloroplast stroma and thylakoids were already rebuffered to 1x kinase buffer prior to assays.

components	stroma	thylakoids
µl 5x kinase buffer	2	1
µl thylakoids	-	15
µl stroma	10	-
µl ATP-γ-^{32}P	0.1	0.1
µl cKin18	0/3	0/1/2.5/4
µl ddH$_2$O	x	x
µl total	20	20

Comment on FSBA kinase assays:

Prior to the kinase reaction a sample was incubated with FSBA (in DMSO; end concentration 1mM or 2mM) for 2h30min at 37°C. Subsequently 15.9µl of the reaction mix were used for the *in vitro* protein kinase assay (see table above). DTT was omitted from the kinase buffer because it would have reacted with FSBA.

Protein precipitation by trichloroacetic acid (TCA)

A protein containing sample was mixed with ¼ volume of 50% TCA and vortexed for 10s. Precipitation was carried out on ice for 20min. Subsequently the sample was centrifugated for 10min at 16100g and RT. The supernatant was decanted and 600µl 100% acetone was added. After vortexing for 10s the sample was centrifugated as before. The supernatant was discarded and after another centrifugation step for 2min at 16100g and RT, residual acetone was removed with a pipette and the pellet was dried for ~15min at 40°C. Finally the pellet was resuspended in 30µl 2x Laemmli buffer and in case of yellow coloring (due to residual TCA in sample) 0.1µl 5M NaOH was added. After heating for 5min at 95°C and centrifugation for 5min at 16100g and RT, the sample was analyzed by SDS-PAGE.

ATP/Purvalanol B affinity chromatography

C$_{10}$-linked Aminophenyl-ATP-Sepharose was purchased from Jena Bioscience (Jena, Germany). Preparation of Purvalanol B (PurB) affinity Sepharose was done according to Daub (Wissing et al., 2007). Affinity sepharoses were poured into disposable polystyrene columns (Thermo Scientific, Waltham, MA, USA). Columns were run by gravity flow at RT.

PurB column: Column (500µl of slurry) was equilibrated with 10 column volumes (CV) of PurB buffer. Pooled S200 eluate from pea (~1.5mg protein) was adjusted to PurB buffer and then the sample was applied to the column. Subsequently column was washed with 20CV of PurB buffer and bound proteins were eluted with 6CV of 0.5% SDS.

ATP column: Column (500µl of slurry) was equilibrated with 10CV of ATP buffer. Pooled S200 eluate from pea or Arabidopsis (1.5mg or 0.8mg protein, respectively) was adjusted to ATP buffer and then the sample was applied to the column. Subsequently the column was washed with 20CV ATP buffer and bound proteins were eluted with 6CV 0.5% SDS.

All fractions were collected and subsequently precipitated with TCA. All samples were analyzed by 12% SDS-PAGE. Proteins were visualized by Coomassie staining, bands were excised and subjected to mass spectrometry.

Protein sample preparation for mass spectrometry (MS)

(all working steps were done on a clean sterile bench; all used equipment had to be as clean as possible)

Coomassie or silver stained SDS-PAGE gel bands were used for nano-electrospray liquid chromatography (LC)-MS/MS investigations (silver staining was done according to Blum (Blum et al., 1987) but using formaldehyd instead of glutaraldehyd). A gel band was chopped, transferred to a 600µl tube (genXpress, Wiener Neudorf, Austria) and washed 3x 10min with 200µl ddH$_2$O (incubated on a thermomixer at 800rpm). In case of a Coomassie stained band it was destained with a mixture of 160µl acetonitrile (Chromasolv®; Sigma-Aldrich, St. Louis, MO, USA) and 200µl freshly prepared 50mM NH$_4$HCO$_3$ (Sigma-Aldrich, St. Louis, MO, USA) by briefly vortexing followed by incubation on a thermomixer for 15min at 800rpm. If gel pieces were not completely destained the last step was repeated with fresh solutions. Subsequently the supernatant was discarded and 160µl acetonitrile were added. After incubation on a thermomixer for 5min at 800rpm the supernatant was removed and gel pieces were air-dried. The proteins then were reduced by addition of 200µl freshly prepared 10mM DTT (Roche Applied Science, Penzberg, Germany) in 50mM NH$_4$HCO$_3$. Incubation was carried out on a thermomixer for 30min at 800rpm and 56°C. Then the supernatant was removed followed by addition of 160µl acetonitrile and incubation on thermomixer for 5min. Acetonitrile was discarded and 100µl freshly prepared 10mg/ml iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) in 50mM NH$_4$HCO$_3$ were added. Alkylation was carried out for 20min in the dark. Subsequently iodoacetamide was removed and gel pieces were washed 3x for 10min with 200µl 50mM NH$_4$HCO$_3$ (incubation on thermomixer). Then ammonium

bicarbonate was discarded, 200µl acetonitrile were added and after incubation on thermomixer for 5min the gel pieces were air-dried again. Trypsin (proteomics grade; Roche Applied Science, Penzberg, Germany) was used as protease (1µg was resuspended in 4µl 1mM HCl and stored at -80°C). Prior to use 196µl 50mM NH_4HCO_3 were added. Dried gel pieces were soaked in trypsin solution (40µl/sample) for 10min at 4°C. Finally the solution was removed, the gel pieces were covered with 50mM NH_4HCO_3 and digestion was carried out o/n at 37°C. The next day the protease reaction was stopped by addition of 4µl 10% HCOOH. The peptides were extracted by mixing in ultrasonic bath (cooled with ice) for 10min. After brief centrifugation the supernatant was transferred to a 200µl tube and extraction was repeated twice with 20µl 5% HCOOH. The supernatants were pooled and after centrifugation for 90s at 20000g transferred to a new 200µl tube. Subsequently peptides were subjected to LC-MS/MS analysis.

Liquid chromatography (LC)-MS/MS analysis of protein samples

The HPLC used was an UltiMate™ system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a PepMap C18 purification column (300µm x 5mm) and a 75µm x 150mm analytical column of the same material. 0.1% TFA (Thermo Scientific, Waltham, MA, USA) was used on the Switchos module for the binding of the peptides and a linear gradient of acetonitrile (Chromasolv®; Sigma-Aldrich, St. Louis, MO, USA) and 0.1% formic acid in water was used for the elution. LC-MS/MS analysis was carried out with the UltiMate™ system interfaced to an LTQ (Thermo Scientific, Waltham, MA, USA) linear ion trap mass spectrometer. The nanospray source of Proxeon (Odense, Denmark) was used with the distal coated silica capillaries of New Objective (Woburn, MA, USA). The electrospray voltage was set to 1500V. Peptide spectra were recorded over the mass range of m/z 450 to 1600, MS/MS spectra were recorded in information dependent data acquisition and the default charge state was set to 3. The mass range for MS/MS measurements was calculated according to the masses of the parent ions. One full spectrum was recorded followed by four MS/MS spectra for the most intense ions, automatic gain control was applied and the collision energy was set to the arbitrary value of 35. Helium was used as collision gas. The instrument was operated in data dependent modus. Fragmented ions were set onto an exclusion list for 20 seconds. Raw spectra were interpreted by Mascot 2.2.04 (Matrix Science Ltd., London, England) using Mascot Daemon 2.2.2. Peptide tolerance was set to +/- 2Da, MS/MS tolerance was set to +/- 0.8Da. Carbamidomethylcysteine was set as static modification, oxidation of methionine

residues was set as variable modification. Trypsin was selected as protease and 2 missed cleavages were allowed.

Mascot results were loaded into Scaffold (Ver. 2.01.01.1; Proteome Software Inc., Portland, OR, USA) for a X! Tandem Search. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99% probability as assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Additionally at least two identified peptides were required. In the case of Arabidopsis the full genome sequence from TAIR was used for search and in the case of pea a recently created EST database was used (Brautigam et al., 2008).

In vitro protein translation/myristoylation assay

Proteins were translated from the vector pBAT in the presence of radioactively labeled methionine or myristate using the TNT[®] T3 Coupled Wheat Germ Extract System (Promega, Madison, WI, USA). Reaction mixes are listed below (Tab.8).

1µg of plasmid DNA per reaction was dried up in a 600µl tube using a SpeedVac. For a myristoylation assay additionally 5µl of myristate-^{3}H were dried up in a separate 600µl tube. The reaction mix was prepared in a different tube, transferred to the DNA containing tube and resuspended. In case of a myristoylation assay the reaction mix was completely transferred from the DNA containing tube to the myristate containing tube and resuspended again. All reaction mixes were incubated for 1h at 30°C.

Tab. 8. *In vitro* protein translation/myristoylation mixes. Proteins to translate were encoded on a plasmid which was dried up prior to the addition of the reaction mix. Myristate was also dried up in a separate tube.

components	translation mix	myristoylation mix
µl wheat germ extract	6.25	6.25
µl reaction buffer	0.5	0.5
µl T3 RNA polymerase	0.25	0.25
µl amino acids – methionine (1mM each)	0.25	0.25
µl 1mM methionine	-	0.25
µl methionine-[35]S	0.75	-
µl RNasin	0.25	0.25
µl ddH$_2$O	4.25	4.75
µl total	**12.5**	**12.5**

Subsequently 2µl of a translation sample were mixed with 10µl 2x Laemmli buffer and analyzed by SDS-PAGE. After Coomassie staining/destaining of the gel, the incorporation of

methionine-^{35}S into proteins was analyzed using a Storage Phosphor Screen (dried gel was put on screen o/n) and a Typhoon Trio Imager (GE Healthcare, Chalfont St. Giles, England).

In case of a myristoylation assay 3µl sample were mixed with 12µl 2x Laemmli buffer and analyzed by SDS-PAGE. After Coomassie staining/destaining the gel was soaked for 30min in Amplify solution (GE Healthcare, Chalfont St. Giles, England) while gentle shaking. Subsequently, the gel was dried and the incorporation of myristate-^{3}H into proteins was analyzed by exposure to an X-ray film for ~10 days at -80°C. The film was developed using a CURIX 60 processor (AGFA, Mortsel, Belgium).

In vitro signal recognition particle (SRP) assay

Proteins were translated from the vectors pBAT and pGEM3 in the presence or absence of SRP using the TNT$^{®}$ T3/SP6 Coupled Wheat Germ Extract System (Promega, Madison, WI, USA; T3 polymerase for pBAT; SP6 polymerase for pGEM3). Therefore 0.5µg of each plasmid DNA were dried up in a 600µl tube using a SpeedVac. The reaction mixes (Tab.9) were prepared in a different tube, transferred to the DNA containing tubes and resuspended. Translation was carried out for 20min at 30°C. After addition of 4µl 4x Laemmli buffer to each reaction mix 5µl were loaded onto a 12% SDS gel. After Coomassie staining/destaining of the gel, the incorporation of methionine-^{35}S into proteins was analyzed using a Storage Phosphor Screen (dried gel was put on screen o/n) and a Typhoon Trio Imager (GE Healthcare, Chalfont St. Giles, England).

Tab. 9. SRP assay reaction mix. SRP was obtained from Bernhard Dobberstein (ZMBH, University of Heidelberg, Germany). *****SRP was used either crude (1µl or 2µl) or 1:10 diluted in SRP buffer (1µl 1:10 = 0.1µl; 2µl 1:10 = 0.2µl). ******Amount of SRP buffer was dependent on the amount of SRP (in total 2µl).

components	SRP mix
µl wheat germ extract	3
µl reaction buffer	0.24
µl T3/SP6 RNA polymerase	0.12
µl amino acids – methionine (1mM each)	0.12
µl methionine-^{35}S	0.4
µl RNasin	0.12
µl SRP*	0-2
µl SRP buffer**	0-2
µl total	**6**

Gel filtration – Superdex 200 (S200)

Proteins extracted from chloroplast stroma ("Chloroplast stroma extraction") were buffer exchanged to buffer S200-A using PD-10 Desalting columns (GE Healthcare, Chalfont St. Giles, England) according to manufacturer's instructions. Afterwards the protein extract was concentrated^{3} to ~500µl using a Centriprep Centrifugal Filter Unit (NMWL: 10kDa; Millipore,

Billerica, MA, USA). After clarification by centrifugation for 10min at 16100g and 4°C the supernatant was applied to a S200 gel filtration column (GE Healthcare, Chalfont St. Giles, England). Size exclusion chromatography was performed on a FPLC system (GE Healthcare, Chalfont St. Giles, England) at a flow rate of 0.8ml/min (using running buffer S200-A). The eluate was fractionated and the fractions (1.44ml each) were analyzed for their protein content by SDS-PAGE after TCA precipitation and for protein kinase activity by *in vitro* protein kinase assays. All protein samples were stored at 4°C until further treatment.

Gel filtration – Superdex 75 (S75)

The fractions of interest from a S200 gel filtration run were pooled and concentrated to ~500µl using a Centriprep Centrifugal Filter Unit (NMWL: 10kDa; Millipore, Billerica, MA, USA). After clarification by centrifugation for 3min at 16100g and 4°C the supernatant was applied to a S75 gel filtration column (GE Healthcare, Chalfont St. Giles, England). Size exclusion chromatography was performed on a FPLC system (GE Healthcare, Chalfont St. Giles, England) at a flow rate of 0.4ml/min (using running buffer S200-A). The eluate was fractionated and the fractions (0.5ml each) were analyzed for their protein content by SDS-PAGE after TCA precipitation and for protein kinase activity by *in vitro* protein kinase assays. All protein samples were stored at 4°C until further treatment.

Ion exchange chromatography – MonoQ/MonoS

Root culture protein extracts (~10mg protein/sample) were buffer exchanged to MS-A (for MonoS) or MQ-A (for MonoQ) buffer using PD-10 Desalting columns (GE Healthcare, Chalfont St. Giles, England) according to manufacturer's instructions. Protein samples obtained from size exclusion chromatography were kept in buffer S-200A.

All samples were concentrated to ~500µl using a Centriprep Centrifugal Filter Unit (NMWL: 10kDa; Millipore, Billerica, MA, USA). After clarification by centrifugation for 10min at 16100g and 4°C, the supernatants of the samples were applied to a MonoS (cation exchange) or MonoQ (anion exchange) column (GE Healthcare, Chalfont St. Giles, England). Ion exchange chromatography was performed on a FPLC system (GE Healthcare, Chalfont St. Giles, England) at a flow rate of 2ml/min. Proteins were eluted by applying a gradient of MQ-A and MQ-B or MS-A and MS-B buffer for the MonoQ or MonoS column, respectively. The eluate was fractionated and the fractions (0.5ml or 1ml each) were analyzed for their protein content by SDS-PAGE after TCA precipitation and in case of MonoQ runs also for protein

kinase activity by *in vitro* protein kinase assays. All protein samples were stored at 4°C until further treatment.

Hydrophobic interaction chromatography – Phenyl-Superose

A root culture protein extract was buffer exchanged to PS-B buffer using PD-10 Desalting columns (GE Healthcare, Chalfont St. Giles, England) according to manufacturer's instructions. Afterwards the sample was concentrated to ~500µl using a Centriprep Centrifugal Filter Unit (NMWL: 10kDa; Millipore, Billerica, MA, USA). After clarification by centrifugation for 10min at 16100g and 4°C the supernatant was applied to a Phenyl-Superose column (GE Healthcare, Chalfont St. Giles, England). Hydrophobic interaction chromatography was performed on a FPLC system (GE Healthcare, Chalfont St. Giles, England) at a flow rate of 2ml/min. A gradient of PS-B and PS-A buffer was applied. The eluate was fractionated and fractions (1ml each) were analyzed for their protein content by SDS-PAGE after TCA precipitation.

FSBA immunoprecipitation

25mg dry Protein A Sepharose CL-4B beads (GE Healthcare, Chalfont St. Giles, England) were soaked in 1ml cold IP buffer on ice for 10 min and then centrifugated for 30s at 4000g and RT. The supernatant was removed and the sepharose taken up in 1.1ml cold IP buffer + 6µl α-FSBA antibody (0.2µg/µl; ~2µg antibody/100µg protein). Coupling was performed on a spinning wheel for 2.5h at 4°C. Subsequently the bead-antibody complexes were pelleted by centrifugation for 1min at 4000g and 4°C. The supernatant was decanted and the beads resuspended in 500µl IP buffer (total volume ~650µl).

During antibody coupling to the beads, a protein sample was treated with FSBA. 760µl IP buffer were mixed with 20µl BSA (1µg/µl), 5µl recombinant cKin3 (~1µg/µl), and 40µl FSBA (in DMSO; end concentration 10µM or 100µM) and incubated for 2h30min at 37°C.

After FSBA treatment the protein sample was incubated with 200µl of prepared sepharose for 1h at 4°C on a spinning wheel. Subsequently the bead-antibody-protein complexes were pelleted by centrifugation for 1min at 4000g and 4°C. The supernatant was transferred to a 1.5ml tube and the beads were washed twice with 1ml IP buffer and twice with 1ml IP-W buffer (each time incubation for 10min at 4°C on a spinning wheel followed by centrifugation as before). The beads were resuspended in 30µl 2x Laemmli buffer, boiled for 5min at 95°C and after centrifugation the supernatant was analyzed by SDS-PAGE.

2.11. Bacteria methods

Preparation of competent *E.coli* bacteria (DH5α and ER2566)

A small amount of *E.coli* cells (kept in stock at -80°C) was plated on a LB plate and incubated o/n at 37°C. The next day one colony was inoculated in 20ml LB medium + 20mM $MgSO_4$ and incubated o/n at 23°C. 2ml of the preculture were inoculated in 300ml LB medium + 20mM $MgSO_4$ and grown o/n at 23°C to an OD_{600} of ~0.4-0.6. The cells were transferred to 50ml tubes and incubated on ice for 10min (all further steps were carried out on ice). Cells were harvested by centrifugation for 10min at 755g and 4°C. The supernatant was discarded and the pellets resuspended in a total volume of 64ml TB buffer. Subsequently bacteria were incubated on ice for 30min and then centrifuged for 10min at 425g and 4°C. The supernatant was removed and the cells were resuspended in a total volume of 10ml TB buffer + 7% DMSO. After incubation on ice for 1h the competent *E.colis* were aliquoted in 1.5ml tubes and immediately frozen on dry ice. Aliquots were stored at –80°C.

Preparation of electro-competent agrobacteria (AGL1)

A small aliquot of agrobacteria (kept in stock at -80°C) was inoculated in 2ml LB medium + rifampicin (25µg/ml) and incubated o/n at 30°C. The next day the cells were diluted 1:100 in LB+RIF medium and again were grown o/n at 30°C to an OD_{600} of ~1-1.5. The culture was cooled on ice, transferred to a sterile 250ml centrifuge tube and centrifugated for 6min at 4066g and 4°C. The cell pellet was resuspended in 50ml sterile 1mM Hepes (pH 7), transferred to a 50ml tube and centrifugated for 12min at 3166g and 4°C. The supernatant was decanted and the washing step repeated twice with 50ml 1mM Hepes and once with sterile 10% glycerol. Finally the competent agrobacteria were resuspended in 4ml 10% glycerol, aliquoted in 1.5ml tubes and immediately frozen in liquid nitrogen. Aliquots were stored at -80°C.

Transformation of *E.colis* by heat-shock

50µl of competent *E.colis* were thawed on ice, mixed with 10µl ligation mix or ~0.1µg of isolated plasmid and incubated on ice for 20min. After a heat-shock for 1min at 42°C the cells were recovered by addition of 800µl LB medium and shaking for 1h at 37°C. Subsequently the cells were pelleted by centrifugation for 90s at 16100g and the supernatant was discarded.

The pellet was resuspended in ~70µl LB medium and plated on a LB plate containing the appropriate antibiotic using glass beads. The plate was incubated o/n at 37°C.

Transformation of agrobacteria by electroporation

50µl of electro-competent agrobacteria were thawed on ice and mixed either with 2.5µl DNA from a Quick-Preparation or with 0.5µl DNA from a MIDI-Preparation in an electroporation cuvette. Agrobacteria were electroporated at 2kV using a Gene Pulser (Bio-Rad, Hercules, CA, USA). Immediately afterwards 500µl LB medium were added, the content of the cuvette was transferred into a 1.5ml tube and agrobacteria were recovered by shaking for 30min at 30°C. Subsequently the cells were pelleted by centrifugation for 90s at 16100g and the supernatant was discarded. The pellet was resuspended in ~70µl LB medium and plated on a LB plate containing the appropriate antibiotic using glass beads. The plate was incubated for 2-3 days at 30°C.

Preparation of *E.coli* and agrobacteria stocks

Bacteria were mixed with sterile 50% glycerol in a 1:1 ratio and stored at -80°C.

2.12. Plant methods

Agrobacterium-mediated transfection of tobacco leaf epidermal cells

A gene of interest was N-terminally fused to YFP by cloning into the vector pBIN-Basta. Subsequently the construct was transformed into agrobacteria. Then one colony was inoculated in 5ml LB+KAN medium and incubated o/n at 30°C. The next day 1ml of the preculture was inoculated in 50ml LB+KAN medium and further grown for 4h at 30°C. Cells were harvested in a 50ml tube by centrifugation for 12min at 3023gand RT. The pellet was resuspended in 40ml LB medium without antibiotic and supplemented with 150µM acetosyringone. Agrobacteria were grown for another 2h at 30°C and again harvested by centrifugation for 12min at 3023g and RT. Finally cells were taken up in ~10ml (according to the size of the pellet) 5% sucrose and injected into punctured leaves of ~6 week old tobacco plants grown under short day conditions (8h light/16h dark photoperiod at $100 – 150µmol\ m^{-2}\ s^{-1}$; 22°C +/- 5; humidity 60% +/- 20%) using a syringe without needle. Plants were put into a plastic bag o/n and the next day transferred into a greenhouse. Two days after infiltration the

subcellular localization of the YFP-fusion protein was analyzed by confocal laser scanning microscopy (Axioplan microscope; Zeiss, Oberkochen, Germany).

Comment:

For co-localization analyses a plant organelle marker (e.g. ER-mCherry) was transformed into agrobacteria and a culture was raised as mentioned before. This culture was mixed with a culture containing a YFP-fusion protein construct in a 1:1 ratio and infiltrated into tobacco leaves. Localization analysis was done by confocal laser scanning microscopy using different filters for mCherry and YFP.

Isolation of *Pisum sativum* chloroplasts

(all steps were carried out at 4°C)

Chloroplast isolation was adapted from Schleiff (Schleiff et al., 2003). Pea seedlings were grown for 8 to 9 days under long day conditions (16h light/8h dark photoperiod at ~70µmol m^{-2} s^{-1}; 21°C +/- 5; humidity 70 to 90%). Leaves were cut and homogenized in P-ISO buffer using a Waring blender (3 x 3s pulses: low – high – low). The homogenate was filtered through four layers of Miracloth (Merck, Darmstadt, Germany) into four 50ml tubes and centrifugated for 2 min at 2800g. The pellets were resuspended in 1 ml P-WASH I buffer each and loaded on top of 2 preformed Percoll gradients (12 ml 40% Percoll; 7 ml 80% Percoll). After centrifugation for 5 min at 8000g (brake "off") using a HB-4 swing-out rotor intact chloroplasts were recovered from the 40%-80% interphase. They were transferred into two 50ml tubes, washed with ~30ml P-WASH I buffer (centrifugation for 2min at 2800g) and the supernatant was decanted. In case the chloroplasts were used for import assays the washing step was repeated. The pellets were resuspended in ~500µl P-WASH I buffer, pooled and either directly used for import assays or immediately frozen in liquid nitrogen and stored at -80°C.

Isolation of *Arabidopsis thaliana* chloroplasts

(all steps were carried out at 4°C)

Chloroplast isolation was adapted from Kunst (Kunst, 1998). Arabidopsis plants were grown for ~8 weeks under short day conditions (8h light/16h dark photoperiod at 100 – 150µmol m^{-2} s^{-1}; 22°C +/- 5; humidity 60% +/- 20%). Prior to harvesting of leaves four Percoll gradients were prepared: Per gradient 15ml Percoll (GE Healthcare, Chalfont St. Giles, England) were

mixed with 15ml 2x RB buffer in SW28 centrifugation tubes and ultracentrifugated for 30min at ~53000g in a SW28 rotor using an Optima L-80 ultracentrifuge (Beckman Coulter, Brea, CA, USA; brake "slow") to form a continuous gradient. In the meanwhile 90g of leaves were harvested and homogenized in 500ml of HOMO buffer using a Waring blender (3 pulses: low – low – high; 2-3s each). The homogenate was filtered through four layers of Miracloth (Merck, Darmstadt, Germany) into one GS3 tube and centrifugated for 5 min at 1519g. The pellet was carefully resuspended in 20ml 1xRB buffer (using a fine paintbrush) and loaded on top of the four preformed Percoll gradients, 5ml on each. After centrifugation for 6min at ~10700g (brake "slow") the intact chloroplasts (lower green band of each gradient) were recovered and transferred into two 50ml tubes. The chloroplasts were washed with 1x RB buffer (centrifugation for 3min at 1700g). The pellets were resuspended in ~300µl 1x RB buffer, pooled and immediately frozen in liquid nitrogen and stored at -80°C.

Determination of the chlorophyll content of isolated chloroplasts

Chlorophyll measurement was done according to Arnon (Arnon, 1949). 5µl sample were mixed with 5ml 80% acetone and centrifugated for 2min at 3166g. The OD_{645}/OD_{663} of the supernatant was measured (using quartz cuvettes) and the chlorophyll concentration was determined using following formula:

$$(OD_{645}*20.2 + OD_{663}*8.02)*1000 = µg/ml \text{ chlorophyll in sample}$$
$$(1000…..\text{dilution factor})$$

In vitro **chloroplast import assay**

(when handling chloroplasts always cut pipette tips were used)

A protein translated from pBAT in the presence of methionine-^{35}S was incubated with isolated pea chloroplasts for 20min at RT in the dark (see Tab.10 for reaction mix). Subsequently intact chloroplasts were re-isolated through a 300µl 40% Percoll cushion by centrifugation for 5min at 4500g and RT. The Percoll was discarded and the pellet washed in 200µl P-WASH I buffer (centrifugation for 1min at 1150g and 4°C). The supernatant was removed, the chloroplast pellet resuspended in 200µl P-WASH I buffer and split to two 1.5ml tubes. After centrifugation for 1min at 1150g and 4°C the supernatants were removed and one pellet was resuspended in 10µl 4x Laemmli buffer. The other pellet was resuspended in 200µl P-WASH II buffer + 3µl thermolysin (3µg/µl) and protease treatment was carried out on ice

for 20min. The reaction was stopped by addition of 0.5µl 0.5M EDTA and chloroplasts were pelleted by centrifugation for 1min at 1150g and 4°C. The supernatant was removed and the chloroplasts washed with 200µl P-WASH III buffer (centrifugation as before). The supernatant was discarded and finally the pellet was resuspended in 10µl 4x Laemmli buffer. All samples were heated for 3min at 95°C and subsequently loaded onto a 12% SDS gel. As a control 0.2µl of the translated protein were mixed with 10µl 4x Laemmli buffer and also loaded onto the gel. After Coomassie staining/destaining the gel was analyzed using a Storage Phosphor Screen (dried gel was put on screen o/n) and a Typhoon Trio Imager (GE Healthcare, Chalfont St. Giles, England).

Tab. 10. Premix and import reaction mix. For each import reaction 24µl premix were prepared. ATP for premix was always freshly prepared. All other solutions were stored at -20°C. *Chloroplasts corresponding to 20µg chlorophyll were used. **(100µl – µl of added chloroplasts)/10 = µl of 10x HMS buffer to add; because chloroplasts were already resuspended in the correct buffer.

components	premix		components	import mix
µl 250mM methionine	4		µl premix	24
µl 250mM cysteine	4		µl ddH$_2$O	x
µl 1M NaHCO$_3$	1		µl chloroplasts*	x
µl 1M K-gluconate	2		µl 10x HMS buffer**	x
µl 20%BSA	1		µl translated protein	2-3
µl 100mM ATP	3		µl total	100
µl ddH$_2$O	9			
µl total	24			

Chloroplast stroma extraction

Chloroplasts (~20mg of chlorophyll) were incubated in ~1.5vol of SEPP buffer on ice for 5 min. After centrifugation for 6min at 12000g and 4°C the supernatant was transferred to a 50ml tube and the extraction was repeated once with ~5ml SEPP buffer. Stromal extracts were pooled and kept on ice until further treatment. The thylakoid pellet was resuspended in ~5ml THY buffer and stored at -80°C.

Thylakoid solubilization

Thylakoids (120µg chlorophyll; isolated during stromal extraction of chloroplasts) were mixed with a detergent (0.5-1.5% end concentration), 5x THYSO buffer and ddH$_2$O in a total volume of 160µl (the volume of 5x THYSO buffer was dependent on the volume of thylakoids because they were already resuspended in the correct buffer; e.g. if the volume of thylakoids was 60µl then 20µl of 5x THYSO buffer had to be added). After incubation for

30min at 4°C on a spinning wheel, the reaction mix was centrifugated for 1h at 18000g and 4°C. The supernatant containing solubilized proteins was transferred into a new reaction tube and analyzed for protein kinase activity by *in vitro* protein kinase assays as well as for protein content by SDS-PAGE after TCA precipitation. The non-solubilized pellet was mixed with 4x Laemmli buffer and analyzed by SDS-PAGE.

2.13. Bioinformatic methods

Proteomic approach - Data validation

Identified proteins (always referring to AGI codes) were imported into excel for further analyses. Redundant protein identifications were removed using Excel's Advanced filter. Proteins were searched against PPDB (Sun et al., 2008), plprot database (Kleffmann et al., 2006), AMPDB (Heazlewood et al., 2004) and Araperox (Reumann et al., 2004). All proteins not found in any of the above-mentioned databases were manually inspected regarding experimental verification of subcellular localization by searching in publications found in TAIR AGI entry (www.arabidopsis.org) or ENTREZ search engine (http://www.ncbi.nlm.nih.gov/sites/gquery). Furthermore identified proteins were individually analyzed for nucleotide binding features using TAIR annotation (www.arabidopsis.org), Expasy – Prosite (Hulo et al., 2008) and Expasy – Enzyme (Bairoch, 2000). Subcellular targeting prediction was done using TargetP (Emanuelsson et al., 2000), ChloroP (Emanuelsson et al., 1999), Aramemnon consensus prediction (Schwacke et al., 2007) and MultiP (Lee et al., 2008). In case of multiple splicing forms always the one with the lowest number was used.

Arabidopsis whole proteome and kinome myristoylation prediction

The AGI codes as well as the sequences of all Arabidopsis proteins were obtained from the TAIR homepage (TAIR8 release; www.arabidopsis.org). The AGI codes of all Arabidopsis protein kinases were obtained from the PlantsP database (Gribskov et al., 2001). Myristoylation prediction was conducted using the PlantsP Myrist Predictor but only N-terminal myristoylation sites were evaluated (Podell and Gribskov, 2004).

3. Results

3.1. Protein kinases and protein phosphorylation in chloroplasts

In eukaryotic genomes it is estimated that 1 to 3% of all genes encode for protein kinases (Stone and Walker, 1995). For example the human kinome consists of 518 protein kinases out of ~22200 proteins in total (2.3%)(Manning et al., 2002; Orchard et al., 2005). 2.3% of the Arabidopsis proteome (27379 unique proteins; TAIR9 release) correspond to 630 protein kinases. But the PlantsP database which is dedicated to plant phosphorylation lists a total number of 965 protein kinases (~3.5%)(Gribskov et al., 2001). This higher number is a result of multiple gene duplications that are generally found in plant's genomes (Chevalier and Walker, 2005). Given that ~2100 proteins are estimated to be imported into the chloroplasts at least 73 protein kinases are expected (3.5% of 2100). But so far only a handful chloroplast-localized protein kinases are thoroughly described in the chloroplast: the "state transition" kinases STN7 and STN8, the plastid transcription kinase CKII, and the chloroplast sensor kinase CSK (Ogrzewalla et al., 2002; Bonardi et al., 2005; Salinas et al., 2006; Puthiyaveetil et al., 2008). Furthermore, MSK4 from *Medicago sativa*, NtDSK1 from tobacco and TAK1-3, CIPK13, and AT1G51170 from Arabidopsis have been reported to be chloroplast-localized (Snyders and Kohorn, 1999; Cho et al., 2001; Kempa et al., 2007; Schliebner et al., 2008). Obviously there is a gap between the expected and observed number of protein kinases inside the chloroplast. Since the chloroplast harbors many different processes that are crucial for plant's survival and these processes clearly need to be regulated, more protein kinases are expected to be present in the chloroplast than identified so far.

Protein phosphorylation in the thylakoids and in the chloroplast stroma has already been extensively studied since the 1970s and 1980s, respectively (Bennett, 1977; Laing and Christeller, 1984). By incubation of chloroplast protein extracts with radioactively labeled ATP and by probing with a phosphothreonine antibody, the phosphorylation of various substrates has already been demonstrated (Foyer, 1985; Rintamaki et al., 1997).

Since calcium is known to be an important secondary messenger and is also known to be involved in the regulation of protein kinases, I performed a Ca^{2+}-dependent protein kinase assay on stromal proteins extracted from isolated chloroplasts (Fig.6). To this end, the proteins were incubated with radioactively labeled ATP and the incorporation of γ-P_i into substrates, which can only be catalyzed by protein kinases, was analyzed by autoradiography. Ca^{2+} was either added to or depleted from the reaction mix by addition of the chelator EGTA.

Increasing calcium levels did not change the phosphorylation pattern at all, whereas addition of EGTA inhibited phosphorylation of two proteins at a size of ~50kDa. Phosphorylation could be rescued by addition of calcium but not magnesium, indicating that besides extensive phosphorylation of various substrates, there is also Ca^{2+}-dependent phosphorylation occurring in the stroma of chloroplasts.
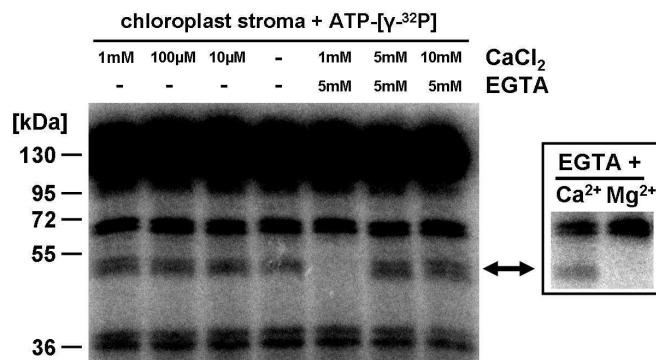

Fig. 6. Ca^{2+}-dependent phosphorylation of stromal proteins. Extracted stromal proteins were incubated in the absence or presence of $CaCl_2$ and/or EGTA. Insert shows protein kinase assay in the presence of 5mM EGTA and 5mM $CaCl_2$ or $MgCl_2$.

3.2. Identification of novel chloroplast protein kinases – candidate approach

Already for my diploma thesis I set out to identify new chloroplast-localized protein kinases in Arabidopsis via a candidate approach. Since no protein kinases are encoded in the chloroplast genome nuclear-encoded protein kinases were investigated. The four protein kinases, cKin1-4 (<u>c</u>hloroplast <u>kin</u>ase), were selected based on the presence of a TargetP predicted chloroplast transit peptide (cTP) and their localization was investigated by yellow fluorescent protein (YFP)-fusion analysis. To this end, all candidate genes were N-terminally fused to YFP and in an agrobacterium-mediated process they were transfected into *Nicotiana tabacum* epidermal leaf cells. Subsequently, the subcellular localization of the YFP-fusion proteins was analyzed by confocal laser scanning microscopy. Pictures were recorded using filters for YFP and chlorophyll autofluorescence.

Summarized, there was evidence for chloroplast localization of cKin1 and cKin3, for cKin2 no pictures could be obtained due to technical problems and the localization of cKin4 remained ambiguous. Thus, I repeated the YFP localization experiments and furthermore performed chloroplast import assays.

cKin1-4 – revised localization

Repetition of the YFP analyses showed that cKin1-4 all were clearly localized at the plasma membrane and except for cKin2 also in the nucleus (Fig.7A). To confirm these results I performed *in vitro* import assays (Fig.7C). Radioactively labeled, recombinant cKin proteins

were incubated with isolated chloroplasts and subsequently treated with the protease thermolysin. Finally the proteins were analyzed by SDS-PAGE and autoradiography. Successful chloroplast import, as it was shown for the positive control ferredoxin-NADP$^+$ reductase (FNR), included both a processing of the precursor protein to its mature form by SPP and inaccessibility of the mature protein to thermolysin. In contrast, cKin3 and cKin4,

A

B

C
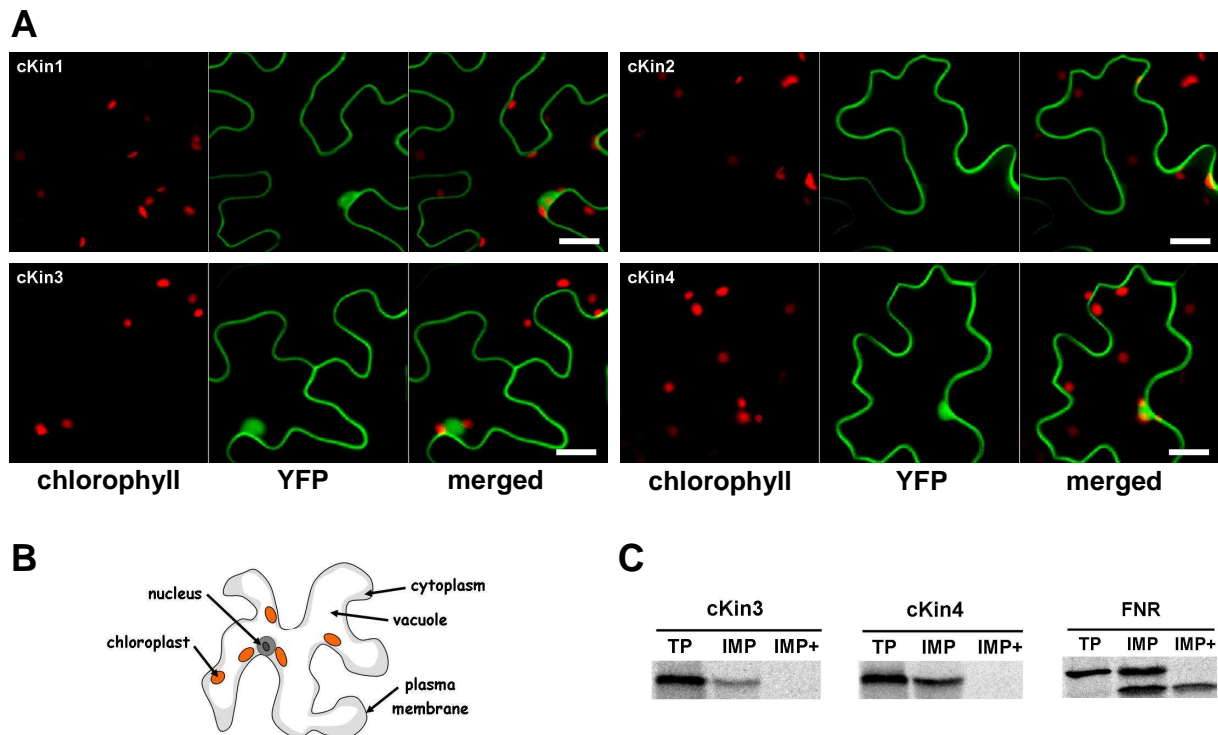
Fig. 7. Localization analysis of cKin1-4. A, Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel is a merged image. Bar = 20µm. **B,** Scheme of a tobacco leaf epidermal cell. **C,** Chloroplast import assays of cKin3, cKin4 and the positive control FNR are shown. TP = translation product. IMP = import reaction: protein incubated with isolated chloroplasts. IMP+ = thermolysin added after import reaction.

representative for cKin1 and cKin2, were not imported into the chloroplast.

Concluding, the selected candidate proteins cKin1-4 were not localized inside the chloroplast. Hence, I extended the candidate approach to more proteins.

New candidate protein kinases

New candidate genes were selected based on sequence homology to cKin1-4, as analyzed by BLAST search (www.arabidopsis.org), and chloroplast prediction by TargetP (Emanuelsson et al., 2000). In total seven proteins were selected, cKin6-12 (Tab.11), but cKin8 and cKin12 could not be amplified by PCR. The already described chloroplast protein kinase CKII, designated cKin5, served as a positive control.

Haphazardly, the protein kinases cKin13 and cKin14 were selected due to the fact that the amino acid sequence of many chloroplast imported proteins starts with MAS. But both genes could not be amplified neither from leaf nor seedling cDNA because much likely they are only expressed in mature pollen according to the eFP browser (Winter et al., 2007). Furthermore, I searched available organellar protein databases

Tab. 11. cKin6-12. Chloroplast prediction score and homology to cKin1-4 are listed.

name	AGI	TargetP (cTP)	homology
cKin6	At4g35600	0.932	cKin1 (e-103)
cKin7	At1g26970	0.911	cKin1 (e-147)
cKin8	At1g76360	0.932	cKin3 (e-103)
cKin9	At1g72540	0.921	cKin1 (3e-86)
cKin10	At1g69790	0.968	cKin4 (e-142)
cKin11	At1g71530	0.769	cKin2 (e-150)
cKin12	At4g34440	0.967	cKin1 (9e-64)

and the literature for evidences of chloroplast protein kinases. The candidates cKin15 and cKin16 were identified in a chloroplast mass spectrometric study (Zybailov et al., 2008) but according to Prosite (http://www.expasy.org/prosite) cKin15 is lacking its kinase active site and therefore it was eliminated from the test set. The plastidiar plprot database contained three protein kinases, plpKin1-3, whereas plpKin3 had already been confirmed to be localized in the nucleus and the cytoplasm and plpKin1 was removed from the database in a more recent update. Thus, only plpKin2 was analyzed. In the database PPDB besides the already known chloroplast protein kinases STN7, STN8 and CKII, only four members of the ABC1 family of proteins kinases are listed. Representatively, ABC1 and ABC2 were selected which were identified in proteomic studies of plastoglobules (Vidi et al., 2006; Ytterberg et al., 2006). Additionally, Andreas Weber a collaboration partner from Düsseldorf, Germany, who is working on chloroplast proteomics, reported the identification of five protein kinases, WKin1-5. Since WKin4 and WKin5 are lacking the protein kinase active site according to Prosite and WKin1 could not be obtained by PCR, I only analyzed the localization of WKin2 and WKin3. Finally, I tried to confirm the localization of already published chloroplast protein kinases. I selected MSK4 and not only analyzed the original protein from *Medicago sativa*, MsMSK4, but also the closest Arabidopsis homolog AtMSK4. I also added AtDSK1, the closest homolog of NtDSK1 from tobacco, to the test set. YFP alone was included as a negative control. The subcellular localization of all candidate genes fused to YFP was analyzed by confocal laser scanning microscopy after transfection of tobacco epidermal cells.

Among all analyzed candidates chloroplast localization, indicated by overlapping YFP and chlorophyll autofluorescence signals, could only be confirmed for the positive control cKin5 and the two ABC1 family protein kinases (Fig.8). YFP alone was restricted to the nucleus and the cytoplasm. cKin6, cKin16, WKin3 and plpKin2 were localized at the plasma membrane.
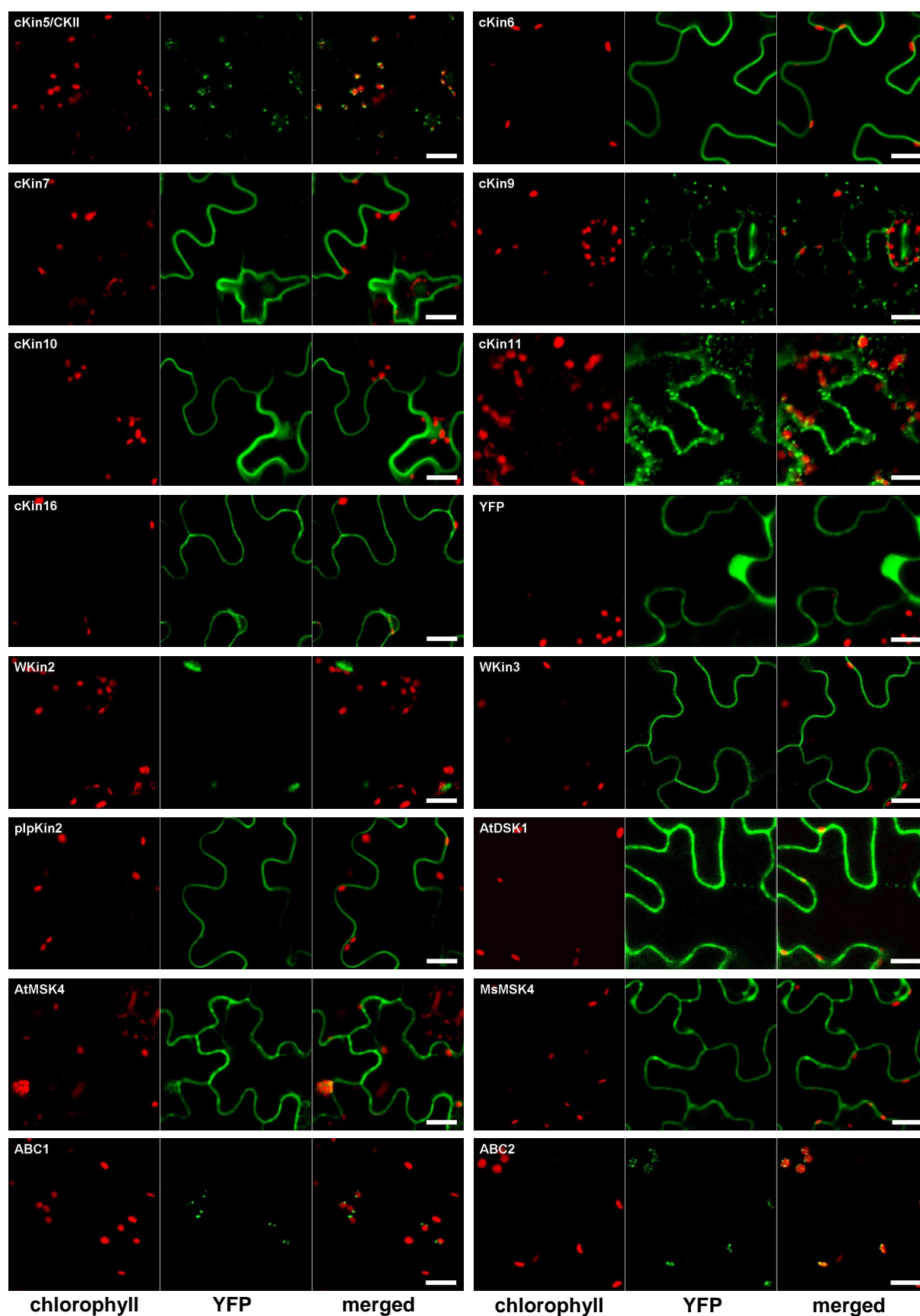
Fig. 8. Subcellular localization of selected candidate protein kinases and YFP. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm.

In contrast, AtDSK1, AtMSK4 and MsMSK4 seemed to be localized rather in the cytoplasm than at the plasma membrane, because the YFP-signal at the border of the cells was discontinuous and also cytoplasmic strands were visible. Besides the membrane localization of cKin7 and cKin10, a slight signal was originating from the nucleus and WKin2 was present exclusively in the nucleus. cKin9 and cKin11 were probably localized in mitochondria indicated by the small vesicles visible throughout the cytoplasm, whereas cKin11 was also present in the nucleus.

Obviously, the candidate strategy proved to be not successful in the identification of novel chloroplast protein kinases. Alternatively, a phylogenetic and a proteomic strategy was developed which will be explained in the next chapters.

3.3. Identification of novel chloroplast protein kinases – phylogenetic approach

It is widely accepted that chloroplasts are derived from endosymbiotic cyanobacteria. In contrast to eukaryotes, where serine/threonine-specific protein kinases (STKs) are the key players in signal transduction, it was assumed for a long time that in cyanobacteria this part is fulfilled by two-component systems consisting of sensor histidine kinases (HK) and response regulators (RR)(Parkinson, 1993). HK and RR domains are sometimes even found within one protein, which is then called a hybrid kinase (HY).

In *Anabaena variabilis* and Nostoc, for example, the number of annotated putative two-component genes is 202 and 260, respectively. Anabaena contains 73 HKs, 74 RRs and 55 HYs whereas Nostoc contains 95 HKs, 94 RRs and 71 HYs (Ashby and Houmard, 2006). In contrast, Arabidopsis contains 54 proteins with two-component signatures (Puthiyaveetil et al., 2008). But beginning with the first identification of a STK in a cyanobacterium and supported by the sequencing of cyanobacterial genomes many STKs have already been identified. *Anabaena variabilis*, for example, contains 53 predicted STKs and *Nostoc punctiforme* 56 (Zhang et al., 2007).

It is possible that after completion of the endosymbiotic process STKs were maintained in the chloroplast. So I set out to identify evolutionary conserved protein kinases between cyanobacteria and Arabidopsis. A similar approach has already been published and has been shown to be successful in the identification of chloroplast proteins in general (Sato et al., 2005).

Localization of evolutionary conserved chloroplast protein kinases

First attempts to identify homologous protein kinases in Arabidopsis and cyanobacteria by BLAST searches (Altschul et al., 1990) turned out to be unrewarding, because protein kinases already share high sequence homologies due to their highly conserved catalytic domain, regardless of their origin. Thus, a strategy had to be developed that predominantly evaluates sequences other than the protein kinase domain, which contain more information on evolutionary conservation. Therefore I engaged in a collaboration with bioinformaticians from the Center of Integrative Bioinformatics (Vienna, Austria). They not only analyzed protein kinases but extended their approach to the whole Arabidopsis proteome. Applying their strategy and highly stringent criteria out of the ~27000 Arabidopsis proteins 465 were identified as evolutionary conserved between Arabidopsis and cyanobacteria. Unexpectedly, only five protein kinases were identified: cKin21, cKin22, ABC2, ABC4 and ABC5. Using relaxed criteria further two protein kinases showed up: PIDPK and PHOT1 (AT3G45780). ABC2 was already shown to be chloroplast-localized in the candidate approach, ABC5 is already curated to be chloroplast-localized according to PPDB and PHOT1 is a well-known plasma membrane localized phototropin (Sakamoto and Briggs, 2002). Hence, only cKin21, cKin22, PIDPK and ABC4 were subjected to YFP localization analysis (Fig.9). In the case of cKin21 and cKin22 only the N-termini were fused to YFP. Using the full-length sequence of a protein is not important here, as it is known that for analysis of chloroplast localization the N-terminus harboring the cTP is sufficient to mediate chloroplast import (Jarvis, 2008; Lee et al., 2008).
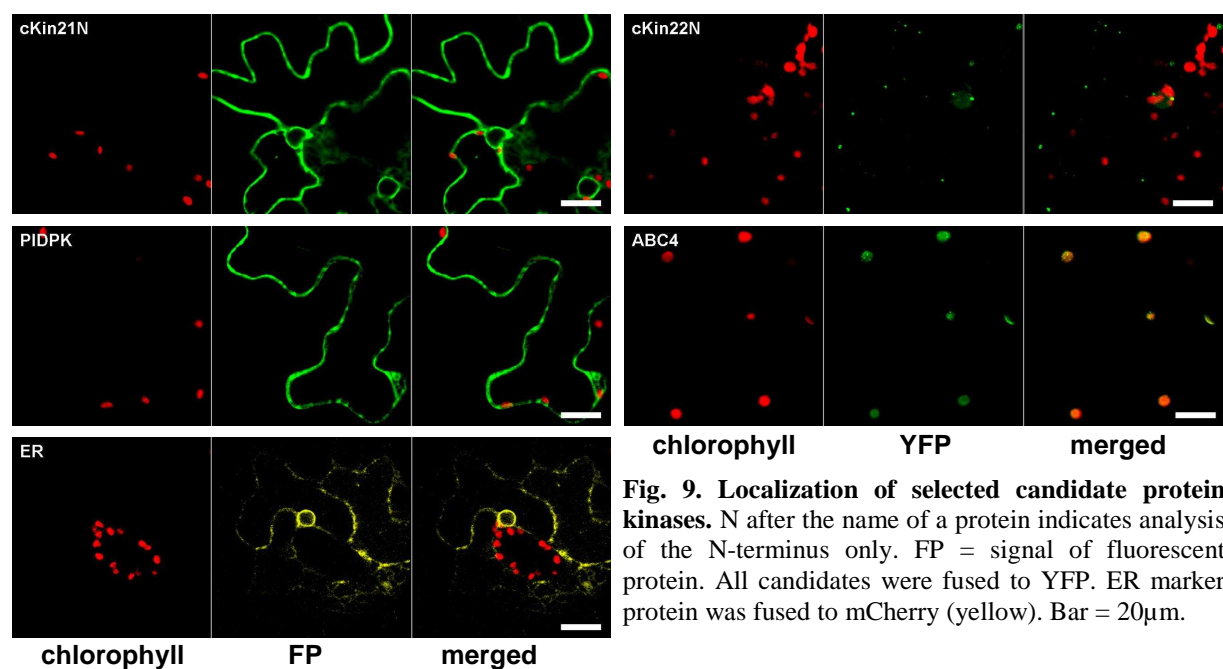

Fig. 9. Localization of selected candidate protein kinases. N after the name of a protein indicates analysis of the N-terminus only. FP = signal of fluorescent protein. All candidates were fused to YFP. ER marker protein was fused to mCherry (yellow). Bar = 20µm.

cKin21 localized to the plasma membrane and to a ring-like structure visible around the nucleus. This ring-like structure together with a speckled distribution pattern within the cytoplasm is typical for ER localization, as it was shown by an ER-marker protein fused to the fluorescent protein mCherry. Thus, it is possible that cKin21 localized to the ER. cKin22 was probably present in mitochondria and PIDPK showed plasma membrane association. Only ABC4 exhibited clear chloroplast localization. But it turned out that ABC4 had already been identified in a chloroplast proteomic study and in a proteomic study on plastoglobules (Ytterberg et al., 2006; Zybailov et al., 2008). Thus, also the phylogenetic approach did not lead to the discovery of novel chloroplast-localized protein kinases.

Nevertheless, together with my colleague Simon Stael I extended the investigations on the data set of evolutionary conserved proteins. We examined the localization fate of proteins derived from the cyanobacterial ancestor, after their genes were transferred from the cyanobacterial genome into the nucleus of the new host cell.

Proof of concept of the phylogenetic strategy

In order to proof that the phylogenetic strategy led to the identification of chloroplast-localized proteins a homoserine kinase (HSK), a fructosamine kinase (FAK), an aspartate/glutamate/uridylate kinase (AGUK), and a NAD kinase (NADK1) were selected and subjected to YFP analysis.

AGUK, HSK and FAK exhibited clear chloroplast localization, whereas FAK also seemed to be localized to mitochondria (Fig.10). Only NADK1 was localized outside of the chloroplast.

Fig. 10. YFP localization of selected candidate proteins. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm.

This result was in good accordance with the finding that out of all 465 conserved proteins 62.4% have already been curated to be localized inside the chloroplast according to PPDB. Compared to 14.9% chloroplast predicted proteins of the whole Arabidopsis proteome (TargetP analysis of TAIR9 proteome release), this showed that conservation of proteins between Arabidopsis and cyanobacteria is a strong indicator for chloroplast localization.

Chloroplast prediction based on sequence conservation

Furthermore, we assessed whether evolutionary conservation comprises targeting prediction potential especially for proteins with non-canonical targeting peptides. To this end, we selected six evolutionary conserved proteins predicted not to be localized to the chloroplast by the consensus prediction feature of Aramemnon, which integrates the algorithms of 16 independent prediction programs (Schwacke et al., 2003).
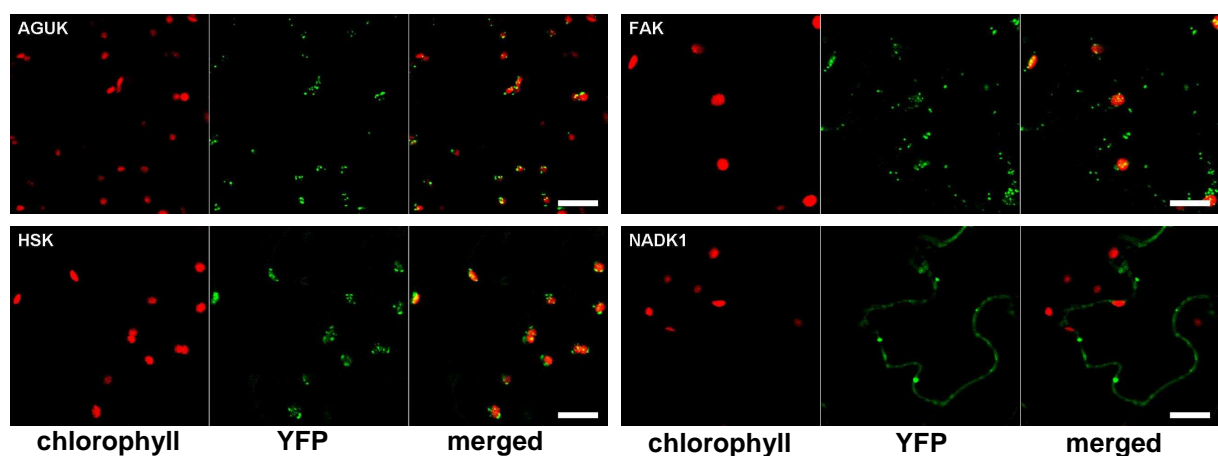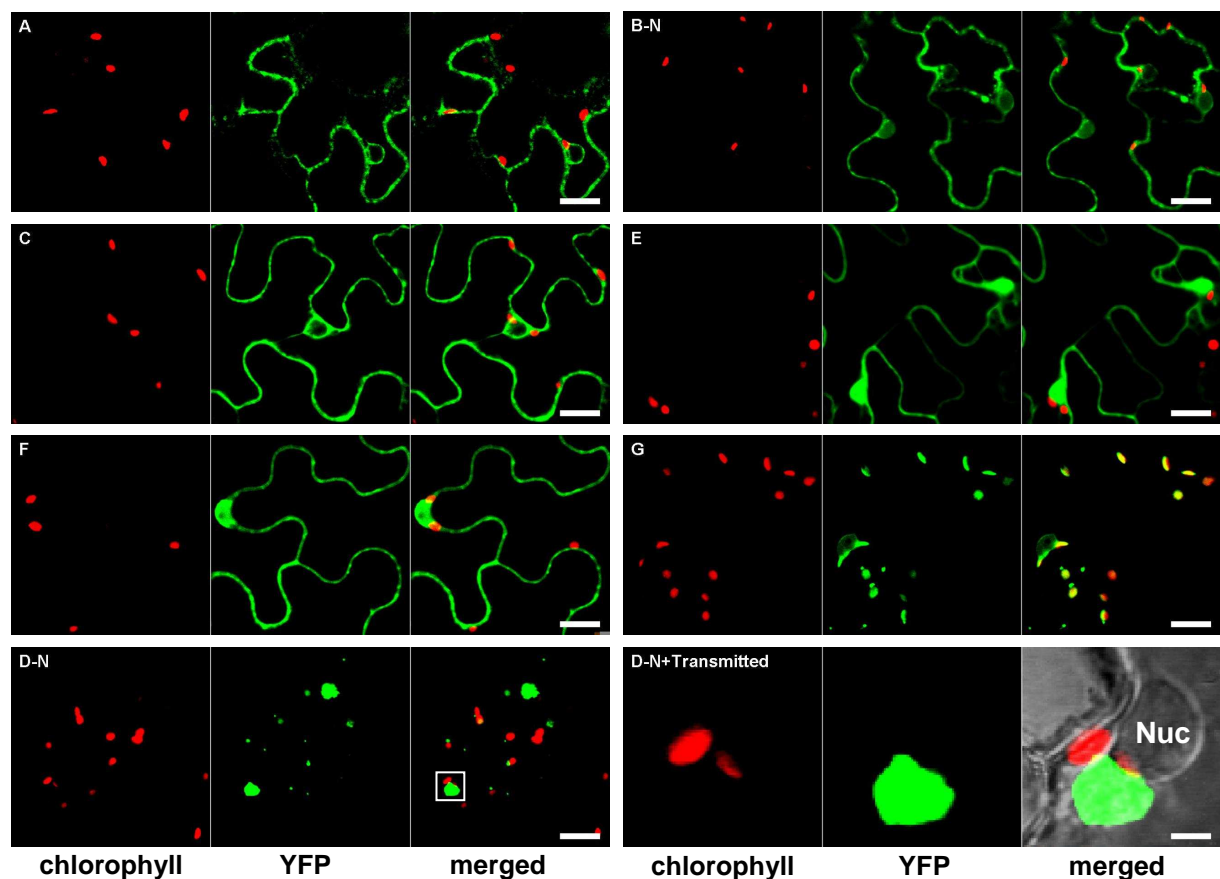

Fig. 11. YFP localization of selected candidate proteins. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image; only in the case of D-N+Transmitted, which is an enlargement of the section marked with a white square in D-N, the transmitted light was included in the merged image. N after the name of a protein indicates that only the N-terminus was analyzed. Nuc = nucleus. Bar = 20µm; only in the case of D-N+Transmitted: Bar = 4µm.

The selected proteins included an alpha-amylase (designated A), a glutamate-ammonia ligase (B), the EMB1075 carboxy-lyase (C), a pyridoxal-5'-phosphate-dependent putative cysteine synthase (D), a putative alpha subunit of tryptophan synthase (E) and a homoserine dehydrogenase. Lactoylglutathione lyase (G) served as a positive control, as it has already been identified in a chloroplast proteomic study (Zybailov et al., 2008). All proteins were subjected to YFP-fusion protein analysis. In the case of D only the N-terminus of the protein was used for localization studies, because two splicing forms differing in the C-terminus were reported.

Interestingly, none of the selected candidate proteins except for the positive control G were localized in chloroplasts (Fig.11). Besides chloroplasts, G was also targeted to small vesicles, probably mitochondria. In contrast, A and B were much likely targeted to the ER. C, E and F and seemed to be present in the cytoplasm, whereas E and F also exhibited nuclear localization. The putative cysteine synthase D showed by far the most interesting distribution pattern. Besides presence in small vesicles, D also localized to big nuclear-sized structures. A close-up of the image including transmitted light revealed that these structures were located next to the nuclei. Co-infiltration experiments with mitochondrial and peroxisomal markers demonstrated that D exclusively localized to mitochondria (Fig.12).
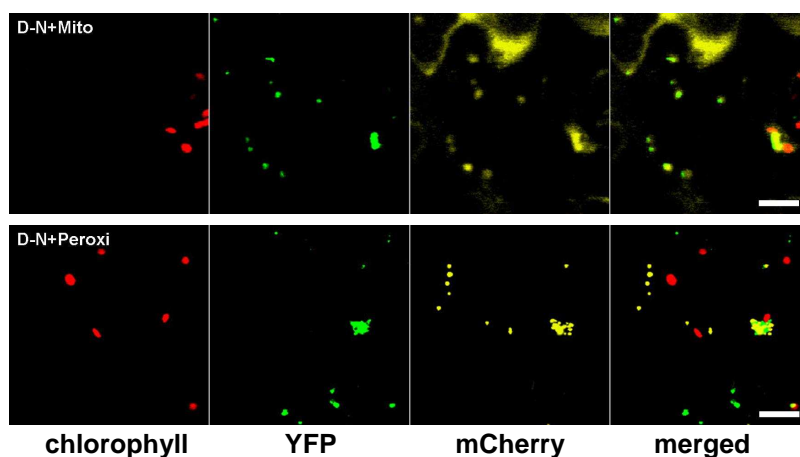
Fig. 12. Co-infiltration studies of D. Tobacco leaves were infiltrated with D-YFP and a mitochondrial or peroxisomal marker protein fused to mCherry. Localization was analyzed by confocal laser scanning microscopy two days after infiltration. The first channel shows chlorophyll autofluorescence (red). The YFP (green) and mCherry (yellow) signal are shown in the second and third channel, respectively. The fourth channel is the merged image. Bar: D-N+Mito = 10µm; D-N+Peroxi = 20µm.

3.4. Identification of novel chloroplast protein kinases - proteomic approach

Since the sequence-based identification of chloroplast-localized protein kinases by targeting prediction or evolutionary conservation turned out not to be successful, a proteomic approach was developed. This approach aimed at the direct identification of protein kinases in protein extracts of isolated chloroplasts by mass spectrometry.

Since most preceding chloroplast proteomics studies focused on the exploration of the thylakoid protein complement, I decided to focus on the stromal proteome, because of the

higher potential to discover new proteins. Furthermore, the stroma, as the aqueous compartment of the chloroplast, per se contains mainly soluble proteins. They are easily accessible by standard chromatographic separation techniques, in contrast to hydrophobic proteins originating from thylakoid preparations.

Traditionally, highly pure and intact chloroplasts used for functional studies were obtained from *Spinacia oleracea* or pea. Since their genomes have not been sequenced so far, chloroplast isolation protocols were adapted for Arabidopsis. But it is known that during isolation Arabidopsis chloroplasts tend to break, lose their stromal content and reseal again (Fig.13)(Halliwell, 1978). Therefore in addition to Arabidopsis chloroplasts, pea chloroplasts were used in combination with a recently created pea expressed sequence tag (EST) database that already proved to be useful in proteomic studies of the chloroplast envelope (Brautigam et al., 2008).

Currently, mass spectrometric approaches are limited by the detection of low-abundant proteins in complex protein samples.

Fig. 13. Isolated chloroplasts. Ps = *Pisum sativum*. At = *Arabidopsis thaliana*. Stroma and thylakoid proteins extracted from isolated chloroplasts were analyzed by SDS-PAGE. The amount of the large subunit of Rubisco (52kDa) is a measure of chloroplast intactness.

Protein kinases, as regulatory proteins, are usually present in minute amounts. Therefore I had to enrich them from the complex chloroplast stroma with the most abundant protein Rubisco prior to mass spectrometry. This could be achieved by gel filtration, which is a technique to separate proteins according to their size.

Gel filtration of stromal extracts – Superdex 200

After extraction of stromal proteins from isolated chloroplasts of Arabidopsis and pea, size exclusion chromatography was performed on a S200 column. This resulted in the separation of the multimeric Rubisco protein complex with a size of ~540kDa and ribosomes from all other proteins that are of smaller size (Fig.14A). I fractionated the eluate and analyzed all fractions for their protein content by Coomassie staining and tested for protein kinase activity by kinase assays (Fig.14B). Based on the estimated size of protein kinases (~30-100kDa), kinase activity accumulated as expected in the lower-size fractions eluting after the prominent Rubisco peak. The fractions with highest protein kinase activity were pooled and either directly subjected to protein identification by mass spectrometry or to downstream purification approaches to further reduce sample complexity. All experimental strategies will be described in the following chapters.

A

B
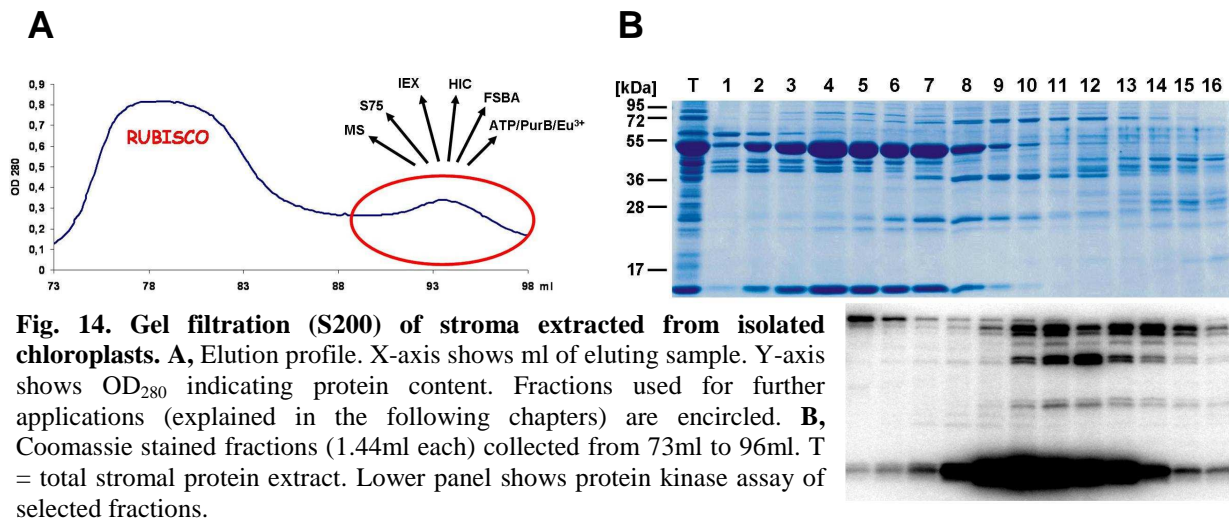
Fig. 14. Gel filtration (S200) of stroma extracted from isolated chloroplasts. A, Elution profile. X-axis shows ml of eluting sample. Y-axis shows OD_{280} indicating protein content. Fractions used for further applications (explained in the following chapters) are encircled. **B,** Coomassie stained fractions (1.44ml each) collected from 73ml to 96ml. T = total stromal protein extract. Lower panel shows protein kinase assay of selected fractions.

Direct mass spectrometry of gel-filtrated stroma

An aliquot of the pooled Arabidopsis stromal proteins after S200 gel filtration was analyzed by SDS-PAGE. After electrophoresis and Coomassie staining all visible bands were cut and subjected to mass spectrometry.

In total only the three protein kinases cKin23-25 could be identified. All were subjected to YFP analysis but none of them could be confirmed to be localized within the chloroplast
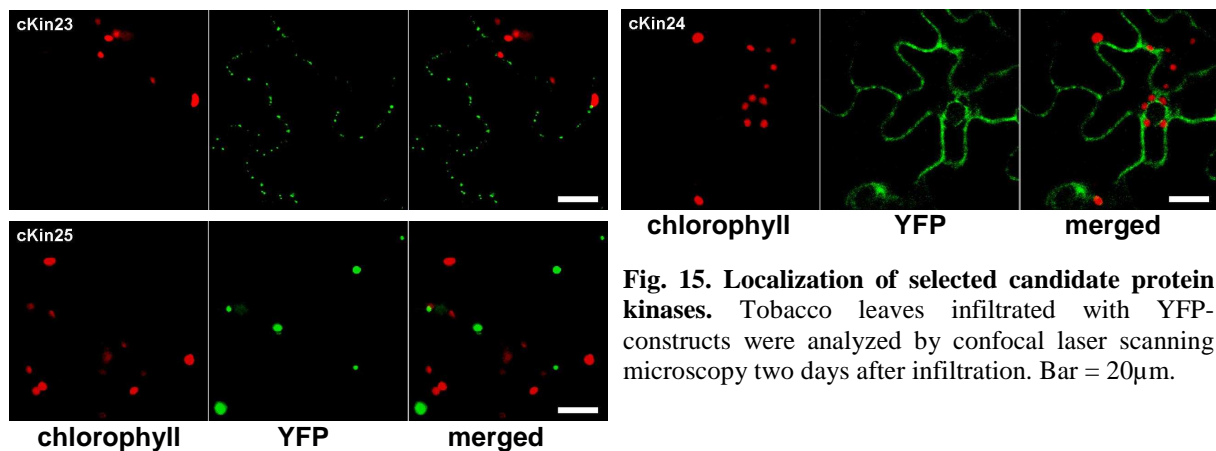
Fig. 15. Localization of selected candidate protein kinases. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Bar = 20µm.

(Fig.15). cKin23 localized to many small vesicles within the cytoplasm which first were assumed to be mitochondria. But co-localization studies argued against mitochondrial as well as peroxisomal localization (Fig.16A). Thus, cKin23 was probably localized to oleosomes (oil bodies) which show a mitochondrial-like subcellular distribution as visualized by GFP (Fig.16B). cKin24 probably is an ER-resident protein because it exhibited the ER-typical speckled pattern within the cytoplasm and a ring-like structure around the nucleus. Although cKin25 seemed to localize to peroxisomes, this could not be verified by co-localization analysis (Fig.16A).

A

chlorophyll   YFP   mCherry   merged

B

Fig. 16. A, Co-infiltration studies of cKin23 and cKin25. Tobacco leaves were infiltrated with cKin23- or cKin25-YFP (green) and a mitochondrial or peroxisomal marker protein fused to mCherry (yellow). Localization was analyzed by confocal laser scanning microscopy two days after infiltration. Bar = 20µm. **B, Localization of Oleosin.** Oleosin-GFP expressed in a tobacco leaf cell (Wahlroos et al., 2003). Bar = 10µm.

Furthermore, I was able to identify the protein band at ~20kDa corresponding to the strongest signal in the protein kinase assay of the gel-filtrated fractions. The band was cut and subjected to protein identification by mass spectrometry. In total 100 different proteins were identified within this sample, but according to spectral counts (the number of identified peptides) the major protein was identified as NDPK2. This protein is a chloroplast-localized nucleoside diphosphate kinase that has been shown to undergo autophosphorylation on a histidine residue (Shen et al., 2006). By performing a kinase assay with recombinant NDPK2 purified via an N-terminal GST-tag, the autophosphorylation ability of this protein could be confirmed (Fig.17). GST alone, which was included as a control, did not show any autophosphorylation.
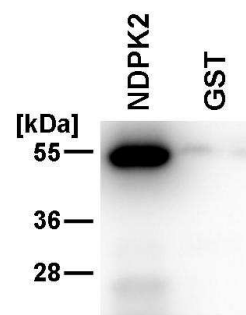
Fig. 17. NDPK2 kinase assay. GST-NDPK2 has a calculated molecular weight of ~53kDa and GST alone of ~27kDa.

Gel filtration - Superdex 75

The pooled fractions after S200 gel filtration were also applied to a second gel filtration column, S75, which has an optimum separation range for proteins of 3-70kDa in contrast to the 10-600kDa separation range of the S200 column. According to the elution profile, using S75 resulted in a further separation of proteins indicated by two major and one smaller peak (Fig.18A). Again the eluate was fractionated (from 8 to 16ml) and analyzed for protein kinase

activity (Fig.18B). Although the separation of the protein mixture could be enhanced, protein kinase activity was strongly reduced. Thus, I decided not to continue with this approach.

A

B
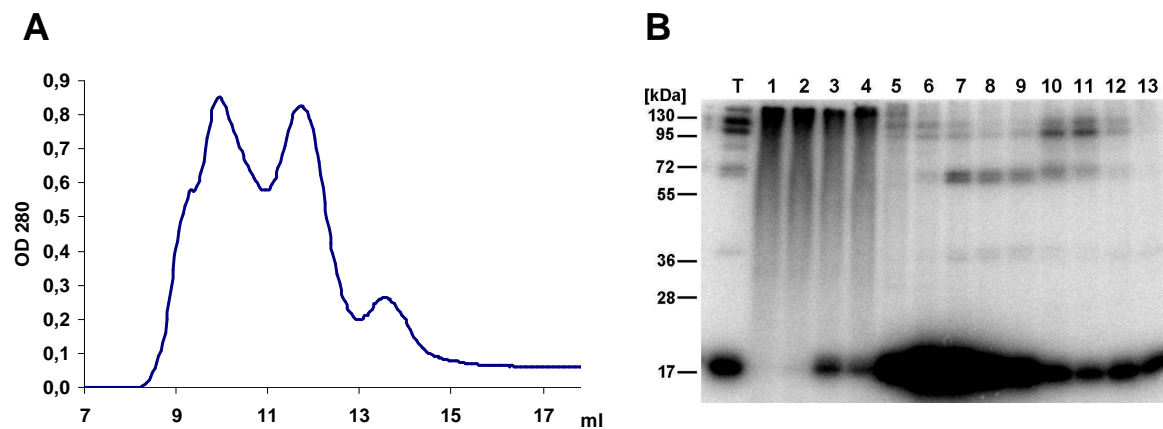
Fig. 18. Gel filtration (S75) of proteins collected after first gel filtration (S200). A, Elution profile. X-axis shows ml of eluting sample. Y-axis shows OD_{280} indicating protein content. **B,** Protein kinase assay of S75-fractions (0.5ml each) collected from 8.5ml to 15ml. T = total sample before S75 run.

Ion exchange chromatography - MonoQ/MonoS

Ion exchange chromatography separates proteins according to their net charge. Proteins are bound to an either positively or negatively charged matrix. Subsequently, the proteins are usually eluted by increasing the ionic strength in the running buffer although changing the pH is also a possibility.

In the lab the cation-exchange column MonoS (containing methyl sulfonate groups) and the anion-exchange column MonoQ (containing quaternary ammonium groups) were available. First, test runs with root cell culture protein extracts were carried out. The cation exchange column MonoS was not functional anymore because the proteins were not able to bind to the column but eluted immediately after sample injection (Fig.19A). The MonoQ proved to be

MonoS   **MonoQ**
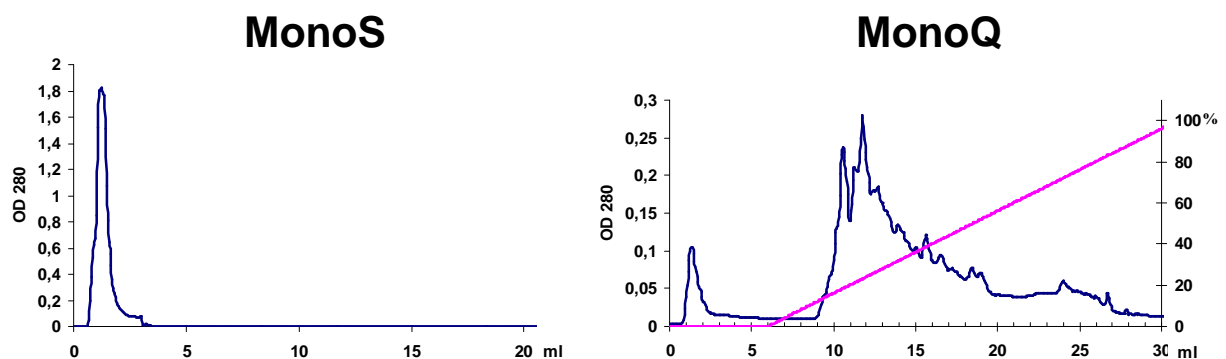
Fig. 19. Ion exchange chromatography – test runs with root cell culture extracts. Elution profiles of MonoS and MonoQ columns are shown. X-axis shows ml of eluting sample. Y-axis shows OD_{280} indicating protein content. In case of MonoQ right Y-axis shows NaCl concentration (in %) of running buffer. Salt gradient is indicated by pink line in the elution profile.

functional as could be inferred from the elution pattern along the applied salt gradient (Fig.19B).

Consequently, I used the MonoQ column for the purification of S200 gel-filtrated stromal extracts of pea. I fractionated the eluate and analyzed the fractions for protein content and protein kinase activity (Fig.20). Purification of stromal proteins via the MonoQ column resulted in a very efficient separation of proteins indicated by the different band patterns of the Coomassie-stained fractions. Most protein kinase activity accumulated in fractions 6-8,

A

B

C

Fig. 20. MonoQ chromatography of stromal proteins.
A, Elution profile. X-axis shows ml of eluting sample. Left Y-axis shows OD_{280} indicating protein content. Right Y-axis shows NaCl concentration (in %) of running buffer. Salt gradient is indicated by pink line in the elution profile. **B,** Coomassie stained fractions (0.5ml each) collected from 16ml to 21ml; only these fractions exhibited significant protein kinase activity. T = total sample before MonoQ. **C,** Protein kinase assay of MonoQ-fractions.

whereas fraction 8 exhibited lowest sample complexity. Thus, visible bands in the size range of expected protein kinases (~30-100kDa) were cut from fraction 8 and subjected to mass spectrometry for protein identification. Not a single protein kinase could be detected and therefore I decided not to continue with this approach.

Hydrophobic interaction chromatography – Phenyl-Superose

Hydrophobic interaction chromatography is based on the reversible interaction of hydrophobic parts of proteins with a matrix containing hydrophobic residues. Proteins are bound in the presence of high salt concentrations and eluted by decreasing ionic strength.

I tested a Phenyl-Superose column (containing hydrophobic phenyl residues) with a root cell culture protein extract. According to the elution profile the column was attested to be

functional (Fig.21). But hydrophobic interaction chromatography has not been repeated with gel-filtrated stromal extracts so far but seems to be promising for the enrichment of protein kinases.
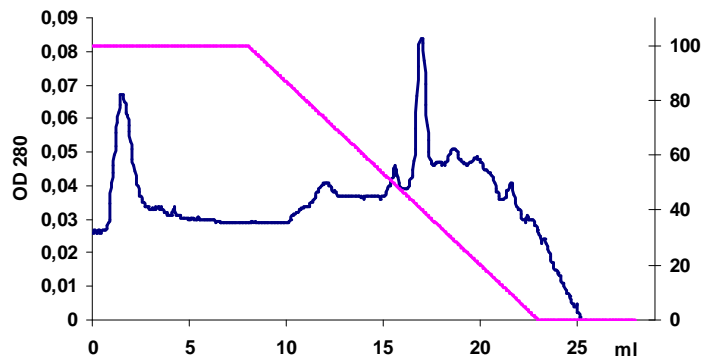
Fig. 21. Phenyl-Superose chromatography – test run with root cell culture extract. Elution profile. X-axis shows ml of eluting sample. Left Y-axis shows OD_{280} indicating protein content. Right Y-axis shows $(NH_4)_2SO_4$ concentration (in %) of running buffer. Salt gradient is indicated by pink line in the elution profile.

Affinity chromatography – ATP/PurB/Eu^{3+}

In an alternative approach to further reduce the sample complexity of gel-filtrated stroma from pea, my colleague Simon Stael and I performed affinity chromatography, which is based on the specific and reversible interaction of a ligand with its target protein. This functional aspect is the major advantage over other separation strategies such as MudPIT, which at the moment probably represents the most powerful separation technique in connection with MS (Wolters et al., 2001). While MudPIT integrates cation-exchange and reversed-phase chromatography to separate complex mixtures of peptides, affinity chromatography is applied to whole functional proteins. Many different ligands are routinely used for the affinity purification of diverse target proteins. Classical examples are antigens for the purification of antibodies, lectins for glycoproteins or hormones for receptors (Jones, 1991). The selection of ligands used for this study was based on our interest in understanding cellular signaling. Key players in cellular signaling are protein kinases, which are involved in the regulation of most cellular processes and calcium-binding proteins that decode calcium signals that are elicited under various conditions (Baginsky and Gruissem, 2009; Bussemer et al., 2009). We focused not only on protein kinases but extended our approach to ATP-binding proteins in general. Therefore we used ATP and the ATP-site directed protein kinase inhibitor PurB as ligands in independent chromatographic runs. PurB was originally developed to inhibit activity of the human CDK2-cyclin A complex but has already been successfully employed in the identification of protein kinases from total human cell extracts by affinity chromatography coupled to MS (Gray et al., 1998; Wissing et al., 2007). Additionally, we used Eu^{3+} as a ligand in order to purify calcium-binding proteins. We could not use Ca^{2+}, because it easily

67

gets desorbed from the affinity matrix in a process called metal ion transfer whereas Eu^{3+} was demonstrated to be stably attached and to selectively adsorb calcium-binding proteins (Chaga et al., 1996).

After gel filtration of stroma extracted from pea chloroplasts, the Rubisco depleted fractions were applied to all three different affinity ligands. In an alternative approach to deplete Rubisco, isolated pea chloroplasts were heated and soluble proteins recovered after centrifugation (Fig.22). This step was established primarily to enrich for heat-stable calmodulins, which are calcium-binding proteins expected to be present inside chloroplasts (Huo et al., 2004; Bussemer et al., 2009). But empiric results in our lab showed that this procedure also leads to an almost complete separation of Rubisco and to an enrichment of a sub-pool of heat-resistant proteins. The sample after chloroplast heating was only applied to the Eu^{3+}-column. Subsequently, all samples were analyzed by SDS-PAGE (Fig.22C). The different band pattern of the eluting fractions compared to the original sample indicated a specific enrichment of proteins and thereby attesting functionality of the columns. Visible bands in all eluting fractions were cut and subjected to identification by MS using the pea EST database. As described by Bräutigam et al. each identified protein was queried against the Arabidopsis genome database and the corresponding Arabidopsis gene identifier (AGI) of the closest homolog was determined.
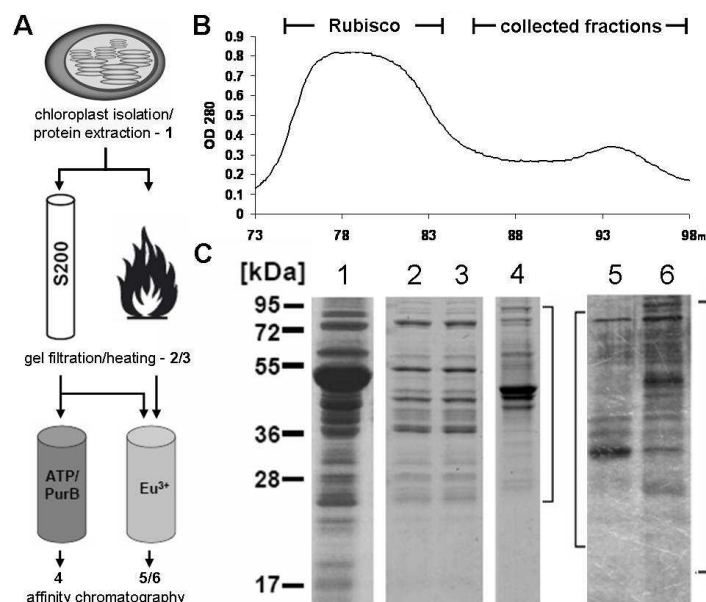
Fig. 22. Experimental strategy and procedure. A, Flow scheme. **B,** Elution profile of gel filtration. X-axis shows ml of eluting sample. Y-axis shows absorbance at 280 nm indicating relative protein content. **C,** Affinity chromatography. 1-6, protein samples analyzed by SDS-PAGE: 1, crude chloroplast protein extract. 2, sample after gel filtration prior to affinity chromatography. 3, flow-through of ATP-affinity column. 4, elution of ATP-affinity column. 5, Citrate elution of Eu^{3+}-affinity column. 6, EDTA strip of Eu^{3+}-affinity column. In lanes 4-6 the region, where protein bands were cut, is indicated.

Number of identified proteins

In total 448 unique proteins were identified with high confidence (Fig.23). Using the ligands ATP and PurB 175 proteins were identified exclusively, 129 were found only with the Eu^{3+}-column, and 144 were identified with both affinity strategies. This is the result of the analysis of more than three biological replicates and several technical replicates. Based on all obtained results saturation curves were calculated referring to identified proteins (Fig.23D). For each affinity strategy three biological samples were analyzed and the percentage of all new identified proteins per sample was plotted. Using the ATP-affinity strategy in total 319 proteins were identified. 82% of all proteins were already identified in the first biological sample, the second and third biological replicates led only to the detection of further 4% and 14% of all proteins, respectively. Using the Eu^{3+}-column 273 proteins were identified. While 54% were discovered with the first biological sample, the second and the third biological sample gave rise to 1% and 45% of all identified proteins, respectively. It is important to note that the experimental procedure had been modified in the third experiment.

Localization of identified proteins

In order to get an idea about the enrichment of chloroplast-localized proteins in the data set the number of predicted chloroplast proteins was analyzed using TargetP (Fig.23B). Out of the 448 identified proteins 84.3% are predicted to contain a cTP compared to 14.9% proteins of the whole Arabidopsis proteome (TAIR9 release). Furthermore to assess the quality of the data set regarding the amount of already experimentally verified chloroplast proteins and non-chloroplast contaminants the available databases PPDB, plprot, AMPDB, SUBA and AraPerox were queried. The localization of all remaining proteins that were not found in any

TargetP	% of all 448	% of 49 new	% of TAIR9
M	7,95	14,29	11,38
C	84,32	61,22	14,85
S	0,91	2,04	19,83
-	6,82	22,45	53,94
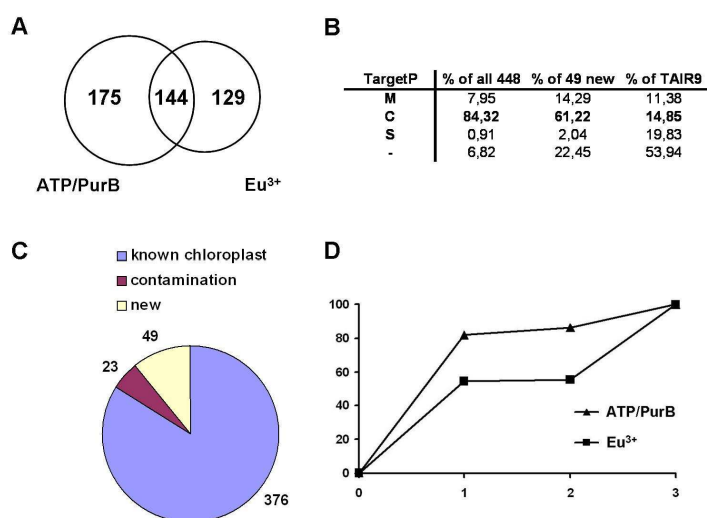
Fig. 23. Analysis of 448 identified proteins and saturation curves. A, Comparison of ATP/PurB and Eu^{3+} affinity strategy regarding identified proteins. B, Targeting prediction by TargetP. Organelle encoded proteins were excluded. C=chloroplast; M=mitochondrion; S=secretory system; - =other localization. C, Experimentally verified localization of identified proteins. Contamination = other localization than chloroplast. New = putative new to the chloroplast. D, Saturation curves of ATP/PurB and Eu^{3+} runs. X-axis shows the number of biological replicates. Y-axis shows identified proteins in percentage of all proteins identified with the respective affinity strategy. The number of uniquely identified proteins with consecutive biological samples was added up.

of the above-mentioned databases was manually curated by evaluating the literature. Only if no information about the subcellular localization could be found neither in databases nor in the literature, a protein was considered to be a putative novel chloroplast-localized protein.

All in all 376 proteins were already experimentally identified within the chloroplast (Fig.23C). In contrast only 23 proteins were non-chloroplast contaminants. Most of the non-chloroplast contaminants were of mitochondrial origin with 15 proteins out of 23. The remaining 49 proteins were considered as putative novel chloroplast proteins (Tab.12). All of these 49 proteins that have been reported to be chloroplast-localized during preparation of this thesis are indicated in Tab.12.

Among all 448 identified proteins not a single protein kinase and only two proteins with putative calcium-binding features could be identified. A P-type ATPase cation transporter that has already been identified in a previous chloroplast proteomic study (Zybailov et al., 2008) and a cation efflux family protein with so far unknown localization.

Tab. 12. All 49 putative novel chloroplast proteins. AGI codes of all proteins together with functional annotation from TAIR9, classification in MAPMAN BINs from PPDB and TargetP prediction are shown. C=chloroplast; M=mitochondrion; S=secretory system; --- =other localization. Whether or not a protein was identified with the ATP/PurB and/or Eu^{3+} strategy is depicted by + or -, respectively. Proteins selected for YFP confirmation are written in bold. Proteins already reported to be chloroplast-localized during preparation of this thesis are labeled by superscript lowercase letters, which are explained at the bottom of the table.

AGI code	functional annotation (TAIR9)	TargetP	ATP/PurB	Eu^{3+}	PPDB BIN
AT1G06510	unknown protein	C	-	+	35
AT1G06900	**metalloendopeptidase/zinc ion binding**	**---**	**-**	**+**	**29**
AT1G15730[a,b]	**PRL1-interacting factor L, putative**	**C**	**+**	**-**	**31**
AT1G19920[a]	APS2; ASA1; sulfate adenylyltransferase (ATP)	C	+	-	14
AT1G21500[a]	unknown protein	C	-	+	35
AT1G22410	2-dehydro-3-deoxyphosphoheptonate aldolase	C	+	+	13
AT1G23800	ALDH2B7; 3-chloroallyl aldehyde dehydrogenase (NAD)	M	+	-	5
AT1G30510[a]	ATRFNR2; root FNR 2)	C	+	-	7
AT1G36280[a,b]	adenylosuccinate lyase	C	+	-	23
AT1G42430	unknown protein	---	+	+	35
AT1G54310	RNA binding	M	+	-	35
AT1G60000[a,b]	29 kDa ribonucleoprotein	C	+	+	27
AT1G66530	arginyl-tRNA synthetase, putative	---	+	-	29
AT1G71720[b]	S1 RNA-binding domain-containing protein	C	-	+	29
AT1G71920[a]	histidinol-phosphate aminotransferase, putative	C	+	-	13
AT1G74920	ALDH10A8; 3-chloroallyl aldehyde dehydrogenase	---	+	-	16
AT1G76690	OPR2; 12-oxophytodienoate reductase	---	+	-	17
AT1G77122	unknown protein	C	+	+	35
AT1G77670[a]	**aminotransferase class I and II family protein**	**M**	**+**	**-**	**16**
AT1G77930	DNAJ heat shock N-terminal domain-containing protein	C	-	+	27
AT1G79530[c]	GAPCP-1; glyceraldehyde-3-phosphate dehydrogenase	C	+	-	4
AT1G79870[a]	oxidoreductase family protein	---	+	-	26
AT2G04620	**cation efflux family protein**	**---**	**-**	**+**	**34**
AT2G17240	unknown protein	C	-	+	35
AT2G17340	pantothenate kinase-related	---	+	-	18

AT2G21350	RNA binding	C	-	+	35
AT2G23390	Acyl-CoA N-acyltransferase	M	+	-	35
AT2G25870	**haloacid dehalogenase-like family protein**	**M**	**+**	**-**	**35**
AT2G31890[b]	ATRAP; putative RNA binding domain	C	+	-	35
AT2G44760	unknown protein	C	+	-	35
AT2G47590	**photolyase/blue-light receptor 2**	**---**	**+**	**-**	**30**
AT3G02900[a]	unknown protein	C	-	+	35
AT3G04650	oxidoreductase	C	+	-	35
AT3G25110	AtFaTA; Arabidopsis FatA acyl-ACP thioesterase	C	+	-	11
AT3G29185[a]	unknown protein	C	+	+	35
AT3G49140[a]	**pentatricopeptide repeat-containing protein**	**M**	**+**	**+**	**26**
AT3G54470	**uridine 5'-monophosphate synthase/UMP synthase**	**-**	**+**	**-**	**23**
AT3G55870	anthranilate synthase, alpha subunit, putative	S	+	-	13
AT3G57810	**OTU-like cysteine protease family protein**	**M**	**-**	**+**	**29**
AT3G59040	pentatricopeptide (PPR) repeat-containing protein	C	+	-	26
AT4G27070[a]	TSB2; tryptophan synthase beta subunit 2	C	+	+	13
AT4G33670	**L-galactose dehydrogenase**	**-**	**+**	**-**	**3**
AT5G02590	tetratricopeptide (TPR) repeat-containing protein	C	-	+	35
AT5G14460	pseudouridine synthase/transporter	C	+	-	23
AT5G15390	tRNA/rRNA methyltransferase (SpoU) family protein	C	+	-	27
AT5G22620[a,b]	**phosphoglycerate mutase family protein**	**C**	**+**	**-**	**35**
AT5G52010	zinc finger (C2H2 type) family protein	C	+	-	27
AT5G62990	embryo defective 1692 (ubiquitin thiolesterase)	C	+	+	35
AT5G64840[b]	ATGCN5; *A. thaliana* general control non-repressible 5	C	+	-	34

[a]Protein is present in the recently launched AT_CHLORO database. [b]Protein is chloroplast-localized according to recent PPDB update. [c]Chloroplast-localization already published (Munoz-Bertomeu et al., 2009).

Functional classification

To get insight into the functional distribution within the data set each identified protein was assigned to one of the 35 MAPMAN BIN categories (Thimm et al., 2004). The modified BIN classifications provided by PPDB were used. Most of all proteins with assigned functions are related to protein synthesis (BINs 29.1 and 29.2; 14%) followed by "amino acid metabolism" (11%), "protein folding, proteolysis and sorting" (BINs 29.3-29.8; 9%), "photosynthesis" (8%), "RNA" (5%), "nucleotide metabolism" (4%) and "lipid metabolism" (4%). Examples for categories with only a few identified proteins are "metal handling" (0.2%), "stress" (0.5%) and "hormone metabolism" (0.5%).

Verification of the subcellular localization of selected proteins

As mass spectrometric detection of proteins in organellar preparations alone is not a convincing proof of localization due to the risk of detecting contaminants, 13 candidates were selected for further experimental investigation by YFP fusion analysis (Tab.13).

The cation efflux family protein CAT, the two proteases MPP and OTL, the protein HAC, which belongs to the superfamily of haloacid dehalogenases, and the aminotransferase ATF

were chosen to be analyzed. Further, the pentatricopeptide repeat-containing protein PPR, the uridine 5'-monophosphate synthase UMP, and the protein PIF, which was shown to interact with the WD40 domain of the nuclear factor PRL1, were selected. The photolyase PHR2, the L-galactose dehydrogenase GDH, and the phosphoglycerate mutase PGL were also included in the test set. Finally the P-type ATPase PAP, which is besides CAT one of only two identified putative calcium-binding proteins, was added. Although PAP has already been assigned previously to the chloroplast by MS, the confirmation of its localization was carried out as a proof of concept for the affinity purification strategy.

For most candidate proteins the full-length coding sequences were cloned. In the case of MPP, CAT and PPR only N-terminal parts were cloned, because MPP and CAT are simply too long for easy experimental handling with 3075 bp and 2397 bp, respectively, and for PPR two splicing forms are described that differ in the C-terminus. The subcellular localization of all candidate proteins was analyzed by confocal laser scanning microscopy (Fig.24).

Tab. 13. The 13 candidate proteins selected for YFP localization. AGI codes of selected proteins, arbitrary name and functional annotation from TAIR9 are shown. YFP indicates the experimentally determined subcellular localization. Results of targeting prediction by TargetP, ChloroP, MultiP and Aramemnon (Aram.) are included as well. C=chloroplast; M=mitochondrion; S=secretory system; --- =other localization. Whether or not a protein was identified with the ATP/PurB and/or Eu^{3+} strategy is depicted by + or -, respectively.

AGI code	name	functional annotation (TAIR9)	YFP	TargetP	ChloroP	MultiP	Aram.	ATP/ PurB	Eu^{3+}
AT1G06190	PAP	P-type ATPase, cation-transport	C	C	C	C	C	-	+
AT1G06900	MPP	metalloendopeptidase/zinc ion binding	---	---	---	---	---	-	+
AT1G15730	PIF	PRL1-interacting factor L, putative	C/M	C	C	C	C	+	-
AT1G77670	ATF	aminotransferase class I and II family protein	C/---	M	C	---	---	+	-
AT2G04620	CAT	cation efflux family protein	M	---	C	---	---	-	+
AT2G25870	HAC	haloacid dehalogenase-like family protein	C	M	C	----	M	+	-
AT2G47590	PHR2	photolyase/blue-light receptor 2	S	---	---	---	---	+	-
AT3G49140	PPR	pentatricopeptide repeat-containing protein	M	M	---	---	---	+	+
AT3G54470	UMP	uridine 5'-monophosphate synthase	---	---	---	---	---	+	-
AT3G57810	OTL	OTU-like cysteine protease family protein	C	M	C	C	C	-	+
AT4G33670	GDH	L-galactose dehydrogenase	---	---	---	---	---	+	-
AT5G22620	PGM	phosphoglycerate mutase family protein	C	C	C	C	C	+	-

Out of the 13 candidate genes seven showed chloroplast localization. HAC, OTL, PAP and PGL showed exclusive chloroplast localization, whereas ATF seemed to be dually localized to chloroplasts and the cytoplasm, and PIF to chloroplasts and probably also to mitochondria. In the case of PIF stromules were visible as well. For the remaining six proteins chloroplast localization could not be confirmed. However, it is important to note here that they have been selected for confirmation despite their negative prediction for chloroplast localization based on their function, adding potentially unexpected pathways to the chloroplast. PPR and CAT were probably localized to mitochondria, whereas CAT showed also nuclear localization.

GDH and MPP were localized in the nucleus and cytoplasm and UMP exclusively in the cytoplasm. The vesicle-like distribution of PHR2 within the cytoplasm and the slightly visible ring-like structure around the nucleus pointed towards localization in the endoplasmatic reticulum (ER).

chlorophyll   YFP   merged

Fig. 24. YFP localization of selected candidate proteins. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel is a merged image of the previous two plus transmitted light. N after the name of a protein indicates that only the N-terminus was analyzed. Bar = 20µm.

Pea vs. Arabidopsis chloroplasts

To assess the identification potential of the pea EST database compared to the complete genome database of Arabidopsis, the ATP-affinity approach was repeated with chloroplasts isolated from mature Arabidopsis plants. The procedure was exactly the same as for pea.

By using Arabidopsis 365 unique proteins were identified in total compared to 234 with pea.

The overlap between both organisms accounted for 160 proteins. Out of the 365 Arabidopsis proteins 94% were already known to be localized in the chloroplast compared to 86.3% with the pea approach (Fig.25). The number of non-chloroplast contaminants slightly decreased from 4.7% with pea to 3.6% with Arabidopsis. The biggest difference between both data sets was obviously the number of novel chloroplast-localized proteins, which was with pea (9%) almost four times higher than with Arabidopsis (2.5%).
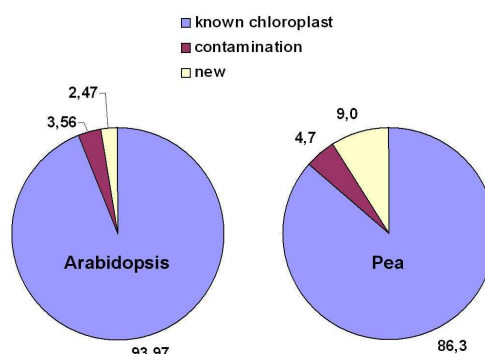
Fig. 25. Pea vs. Arabidopsis ATP affinity run. Proteins belonging to each localization group are calculated in percentage of all proteins identified with the respective organism. New = putative new to the chloroplast.

Localization of protein kinases identified with the proteomic approach

Since not a single protein kinase was identified with the very stringent criteria the raw data were re-evaluated using relaxed identification parameters. By this means, four protein kinases, cKin17-20, could be identified and all were subjected to YFP analysis (Fig.26).
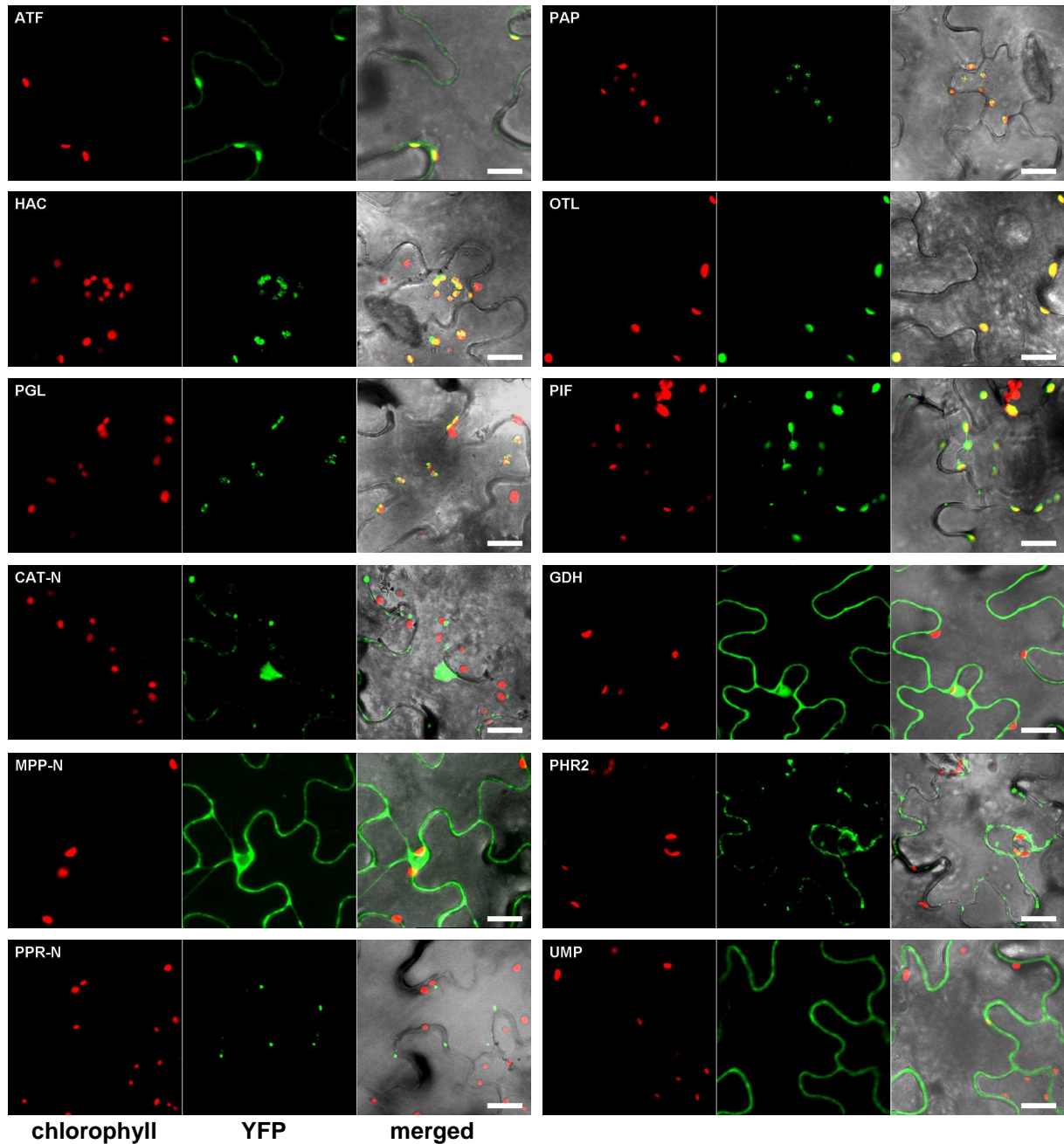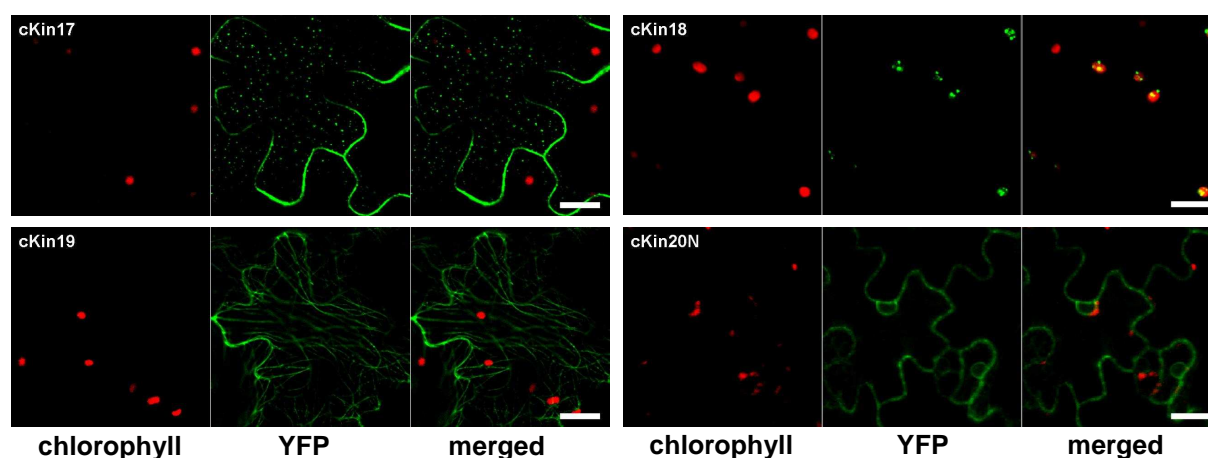

Fig. 26. YFP localization of identified protein kinases. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm

cKin17 localized to small vesicles throughout the cytoplasm but mitochondrial or peroxisomal localization could be ruled out due to the regular distribution of the vesicles. cKin18 was clearly localized to chloroplasts, cKin19 was associated with the cytoskeleton and cKin20 with the ER.

cKin18

cKin18 was the only novel chloroplast-localized, putative protein kinase identified within all experiments done. But according to Prosite it is lacking a conserved aspartic acid residue within its catalytic domain, which is predicted to be important for protein kinase activity. Thus, cKin18 was recombinantly expressed and incubated with stroma and thylakoid protein extracts in the presence of radioactively labeled ATP, in order to investigate its protein kinase activity (Fig.27A). Unexpectedly, cKin18 was able to phosphorylate a substrate of ~38kDa in the chloroplast stroma. In contrast, no phosphorylation of thylakoid substrates could be observed but, interestingly, phosphorylation of thylakoid proteins of the size of ~95kDa was inhibited upon addition of cKin18.

Furthermore, to investigate the physiological function of cKin18 T-DNA insertion were searched. Since neither a line with insertion in an exon nor an intron was available, the line SALK_047737 carrying the T-DNA insertion within the promoter region of cKin18 was ordered. Unfortunately, the promoter region is shared with the neighboring gene AT5G16820, the putative transcription factor HSF3 (Fig.27B).
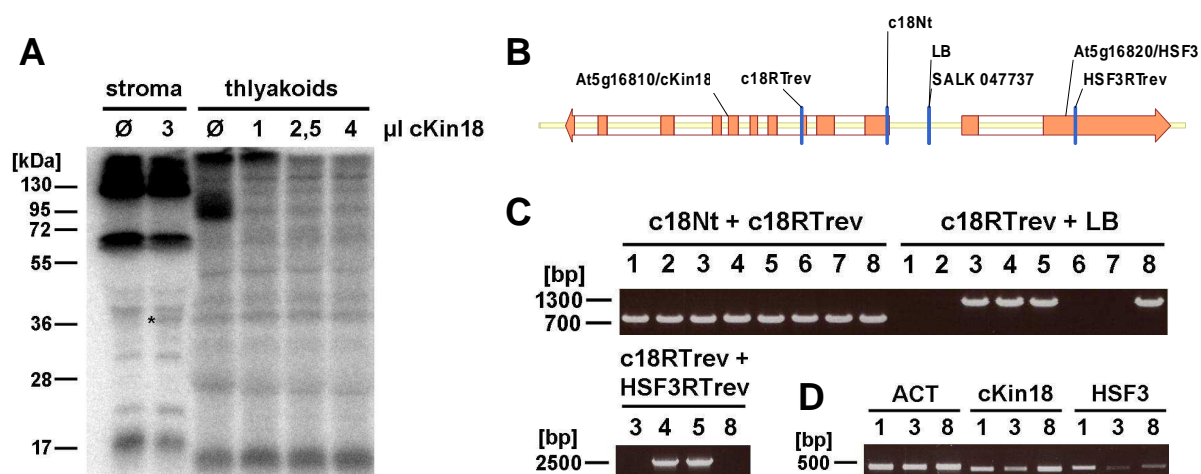
Fig. 27. cKin18 protein kinase assay and T-DNA insertion line analysis. A, cKin18 was incubated with stromal and thylakoid protein extracts and radioactively labeled ATP. Asterisk labels substrate specifically phosphorylated by cKin18. **B,** Scheme of genomic locus of cKin18 and neighboring HSF3. Exons are orange colored. SALK_047737 indicates position of T-DNA insertion of this specific line. c18Rtrev, c18Nt, LB, and HSF3RTrev are the primers used for PCR genotyping and RT-PCR. **C,** SALK_047737 insertion line PCR genotyping. **D,** RT-PCR analyses of line SALK_047737. In the case of ACT the primers ACT3-5' + ACT3-3', in the case of cKin18 the primers c18Nt + c18RTrev, and in the case of HSF3 the primers HSF3RTfw + HSF3RTrev were used.

The insertion line was analyzed by PCR genotyping using primers specific for the T-DNA insertion and cKin18 (Fig.27C). Of the eight lines analyzed, lines 3, 4, 5, and 8 carried the insertion as indicated by the obtained PCR-product using the primers c18RTrev and LB. But only line 3 and 8 were homozygous regarding the insertion, because when using the primers c18RTrev and HSF3RTrev no PCR-product could be obtained. This was due to the presence of the T-DNA insertion, with a size of ~4500bp too big for amplification, on both chromosomes.

The effect of the insertion on the expression of cKin18 as well as HSF3 was investigated by RT-PCR (Fig.27D). Actin 3 (ACT) was used as control. Transcript levels of cKin18 were reduced in the lines 3 and 8 compared to line 1, which exhibited a wild-type genotype. Unfortunately, expression of HSF3 was even more reduced. Therefore this T-DNA insertion line could not be used for functional studies, because the influence of the impaired expression of the transcription factor HSF3 could not be ruled out.

Purification of protein kinases by fluorosulfonylbenzoyladenosine

Since classical chromatographic approaches were not successful in the identification of novel chloroplast protein kinases, I searched the literature for alternative strategies.

I found reports about fluorosulfonylbenzoyladenosine (FSBA), which is an ATP analogue that has already been successfully used for the selective labeling of protein kinases. It is able to react with
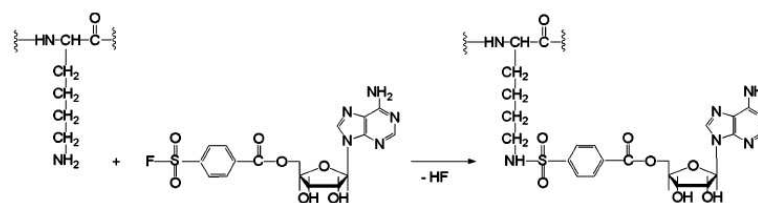
Fig. 28. Scheme of FSBA enzymatic activity. FSBA is able to bind to the ATP binding pocket of proteins and to covalently modify lysine residues there (Renzone et al., 2006).

primary amino groups such as the invariant lysine that is present within the ATP-binding pocket of protein kinases (Fig.28). In combination with FSBA-specific antibodies protein kinases can be purified by immunoprecipitation (Parker, 1993; Moore et al., 2004).

I performed a protein kinase assay of gel-filtrated stroma after incubation with 1 or 2mM FSBA for 2h30min in order to assess the reactivity of FSBA on chloroplast protein kinases (Fig.29A). Besides a control reaction without FSBA, an additional control reaction including DMSO was prepared, since FSBA was dissolved in DMSO. It turned out that already DMSO alone reduced kinase activity to a small extent. But in the presence of FSBA, regardless of whether 1 or 2mM, a further significant decrease in kinase activity could be observed. This especially affected the incorporation of γ-P_i into proteins of ~130kDa. Thus, FSBA-treatment

in combination with immunoprecipitation using an FSBA-directed antibody seemed to be a promising strategy for the purification of chloroplast protein kinases.

The functionality of a purchased α-FSBA antibody was tested in a preliminary experiment using recombinant cKin3 (a conventional STK) and BSA, a protein without ATP-binding cleft, which therefore should not be targeted by FSBA. BSA and cKin3 were treated with FSBA

Fig. 29. FSBA kinase assay and immunoprecipitation. A, Stromal protein extracts were incubated for 2h30min at 37°C in the absence or presence of FSBA prior to kinase assay. Ø = control. D = control with DMSO. 1 and 2 = 1mM and 2mM FSBA, respectively. B, Coomassie stained poteins after immunoprecipitation of cKin3 and BSA using a α-FSBA antibody.

and subsequently incubated with the antibody, which had been coupled to sepharose beads before. After washing the immunoprecipitated proteins were analyzed by SDS-PAGE (Fig.2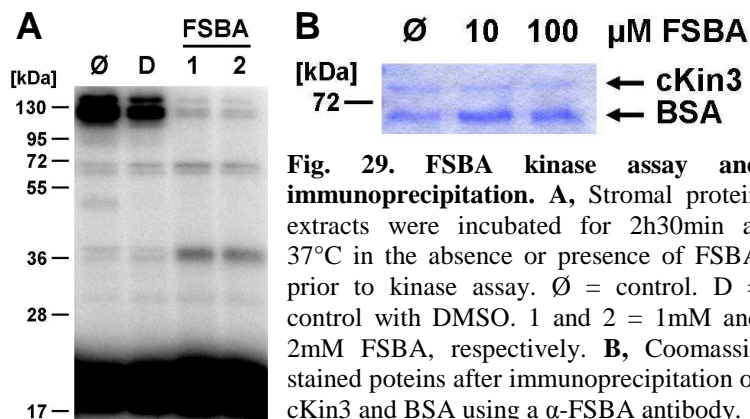9B). Unfortunately, the antibody recognized BSA as well as cKin3 even when FSBA was excluded from the reaction mix. Thus, the antibody could not be used for the purification of protein kinases from stromal extracts. Alternatively, after FSBA treatment of stromal proteins FSBA-labeled protein kinases could be identified by mass spectrometry, as it has already been successfully conducted (Renzone et al., 2006).

Interaction of chloroplast protein kinases with general kinase inhibitors and substrates

Abnormal protein phosphorylation is linked with various human diseases, above all cancer. Therefore protein kinases have become one of the most important groups of drug targets nowadys (Cohen, 2002). In clinical research tremendous efforts have been made to identify and characterize novel protein kinase inhibitors. In contrast to inhibitors for specific protein kinases that make use of individual protein characteristics, broad-range inhibitors are targeting the ATP-binding site that is common to all protein kinases. In a recent study 38 inhibitors were investigated for their specificity to more than 300 human protein kinases (Karaman et al., 2008). It was shown that the inhibitor staurosporine bound and inactivated by far the most protein kinases followed by the inhibitor sunitinib.

Since protein kinases are highly conserved throughout all organisms, human protein kinase inhibitors can be applied to plant protein kinases as well. I used Purvalanol B, staurosporine, sunitinib and sorafenib in protein kinase assays of crude or gel-filtrated stroma (Fig.30A). Unexpectedly, sorafenib and sunitinib did not influence kinase activity at all. Staurosporine and Purvalanol B could inhibit phosphorylation of a protein of ~50kDa and Purvalanol B

additionally reduced phosphorylation of two proteins of ~65kDa. But both inhibitors acted at a concentration of 100µM. In contrast, staurosporine has been shown to exhibit half maximal inhibition of a serine/threonine- and a tyrosine- specific protein kinase already at a concentration of 3nM and 2nM, respectively (Meggio et al., 1995). Also, phosphorylation of the general protein kinase substrate MBP by recombinant cKin3, which is a conventional STK, was already abolished completely at an inhibitor (PurB) concentration of 1µM (Fig.30C).

In addition to the unexpected poor effect of protein kinase inhibitors, chloroplast-localized protein kinases phosphorylated only weakly the general substrate histone (Fig.30B). Furthermore, they completely failed to phosphorylate the substrates casein, phosvitin, and MBP, which together with histone all have already been successfully used to study the activity of various protein kinases (Yang et al., 1987; Schinkmann and Blenis, 1997; Trojanek et al., 2004). In contrast MBP was heavily phosphorylated by cKin3 (Fig.30C).

These results strongly suggest that most chloroplast-localized protein kinases are unusual compared to conventional eukaryotic protein kinases.
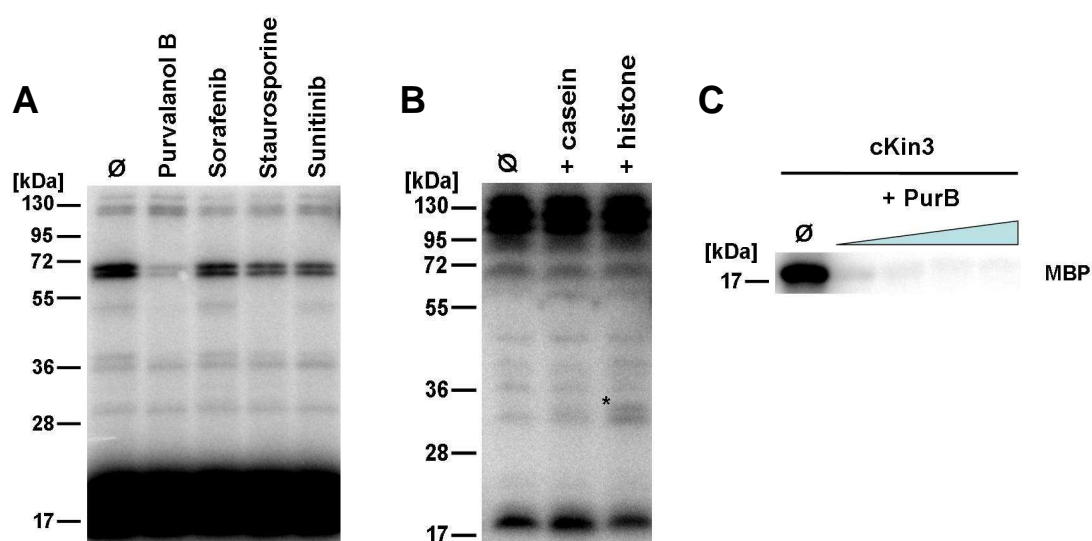
Fig. 30. Protein kinase assays with inhibitors and substrates. A, Protein kinase assay of gel-filtrated stromal protein extracts in the presence of protein kinase inhibitors (100µM). Ø = control reaction without inhibitor. **B,** Kinase assay of crude stromal extracts in the presence of general protein kinase substrates. Casein is representative for MBP and phosvitin. Asterisk indicates minor phosphorylation of histone. **C,** Phosphorylation of MBP by cKin3 in the presence of PurB. Ø = control. Triangle incidactes increasing PurB concentration: 1, 10, 100, 1000µM.

3.5. The effect of acylation on the subcellular localization of proteins

It is well-known that acylation, the N-terminal attachment of myristic and/or palmitic acid, has an influence on the subcellular localization of proteins. In order to asses the effect of acylation on the localization of protein kinases, I set out to analyze the extent of myristoylation, which in most cases is a prerequisite for palmitoylation, within the Arabidopsis kinome compared to the whole proteome.

According to PlantsP out of all 27235 Arabidopsis proteins (TAIR8 release) 965 are protein kinases and 26270 have another annotated function (Gribskov et al., 2001). Strikingly, using the Myrist predictor program (Podell and Gribskov, 2004) 7% of all protein kinases but only 1% of all other proteins were predicted to be myristoylated. Thus, it seemed that myristoylation plays an important role especially for protein kinases.

Interestingly, cKin1, cKin3, cKin4, and cKin6 contain a myristoylation consensus motif, which requires a glycine residue that has to be present at amino acid position 2. If this glycine is removed or exchanged with a different amino acid, then myritoylation is inhibited. Hence, to investigate the impact of acylation on the subcellular localization of cKin1, cKin3, cKin4, and cKin6, I generated G2A mutants of these protein kinases that carry an alanine residue instead of the glycine on position 2. YFP studies showed that in all cases membrane localization was drastically reduced and proteins accumulated in the nucleus compared to the wild-type proteins (Fig.31). These results confirmed previous findings that myristoylation is crucial for membrane attachment of proteins (Benetka et al., 2008).

Recently, a much more striking effect of myristoylation on the subcellular localization of a
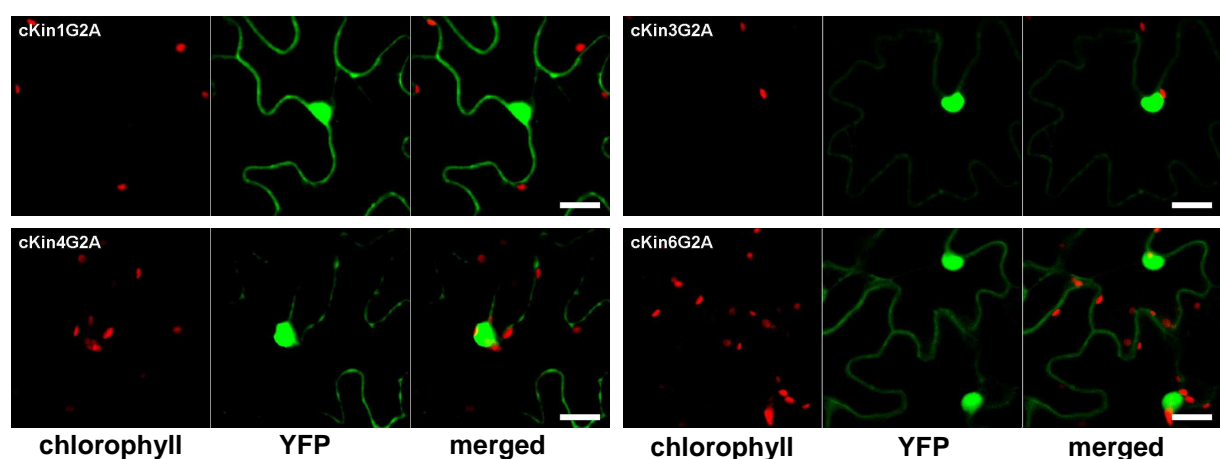
Fig. 31. YFP localization of G2A mutants of selected cKins. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm.

protein kinase has been described. The Ca^{2+}-dependent protein kinase CPK16, which is localized predominantly at the plasma membrane, is relocated to chloroplasts when the

glycine on position 2 is exchanged for an alanine (Fig.33)(Mehlmer, 2009). This observation implied that myristoylation interferes with chloroplast import. This is in accordance with the fact, that out of the ~1100 experimentally confirmed chloroplast proteins in PPDB (organelle encoded proteins were excluded) only 0.2% (2 proteins) are predicted to be myristoylated in contrast to 1.2% (320 proteins) of the whole Arabidopsis proteome. The two already experimentally identified chloroplast proteins that are predicted to be myristoylated are a protein phosphatase (AT4G03415) and a tRNA synthetase (AT2G25840) that has been shown to be dually targeted to chloroplasts and mitochondria (Duchene et al., 2005). However, it is still unclear whether these two proteins are really myristoylated *in vivo*.

Subsequently, the inhibition of chloroplast import by N-myristoylation was experimentally investigated. The influence of palmitoylation, which is often accompanied with myristoylation, on chloroplast import was also considered and analyzed.

The influence of protein acylation on chloroplast import

CPK16 harbors a cysteine residue on position 4 in addition to the glycine on position 2. This cysteine residue represents a possible site for palmitoylation. To assess the effect of myristoylation and palmitoylation on chloroplast targeting, the CPK16 mutants C4S and G2AC4S were created in addition to the G2A mutant. They have the cysteine on position 4 exchanged for serine and thus cannot be palmitoylated anymore. CPK16 and all mutants were subjected to myristoylation and YFP analyses. As expected, only wild-type CPK16 and CPK16C4S could be myristoylated as shown by the incorporation of radioactively labeled myristic acid into *in vitro* translated proteins (Fig.32). In control reactions with radioactively labeled methionine all mutants were successfully translated.
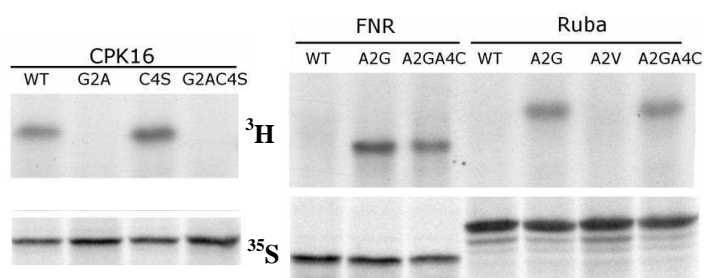
Fig. 32. Myristoylation assays and control translations of CPK16, FNR and Ruba and their mutants. [3]H and ^{35}S indicate incorporation of radioactively labeled myristic acid and methionine, respectively, into translated proteins.

YFP studies revealed that the C4S mutant, which can be myristoylated but not palmitoylated, was not targeted to chloroplasts but showed a localization pattern similar to wild-type CPK16 (Fig.33). In contrast, the G2AC4S mutant was localized to chloroplasts suggesting that myristoylation alone inhibits chloroplast import in the case of CPK16.

Based on these results my colleague Simon Stael and I started a project to investigate, whether it is possible to prevent import of canonical chloroplast proteins by the artificial introduction of myristoylation. Therefore we selected the two chloroplast proteins ferredoxin-NADP$^+$ reductase (FNR) and Rubisco activase (Ruba), which are lacking a glycine on
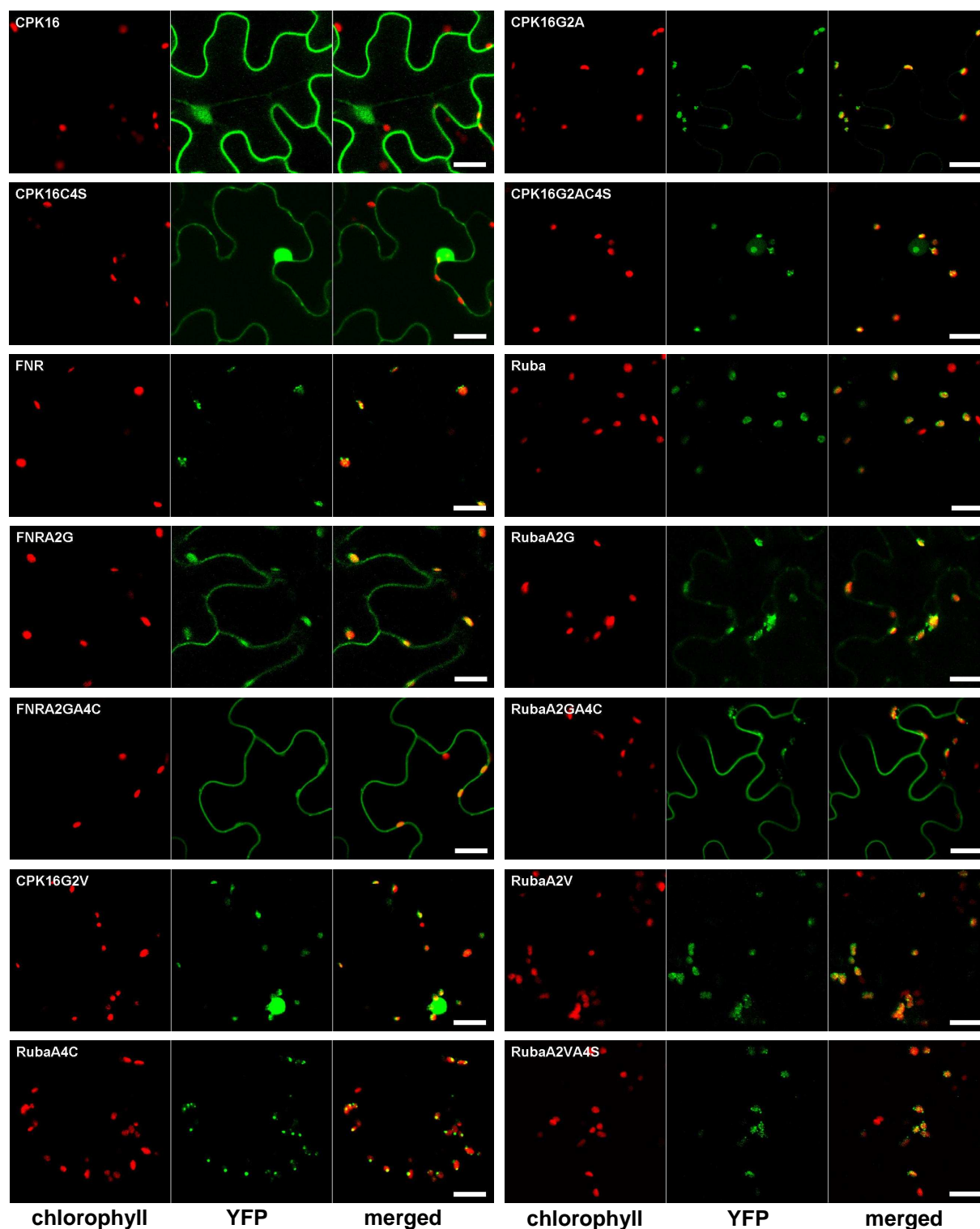
Fig. 33. YFP localization of CPK16, FNR, and Ruba and their mutants. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm.

position 2. But according to Myrist predictor the exchange of alanine on position 2 for a glycine results in the introduction of a strong myristoylation consensus motif in both proteins. Consequently, both FNRA2G and RubaA2G could be myristoylated *in vitro* (Fig.32).

Interestingly, YFP-fusion proteins of FNRA2G and RubaA2G still localized to chloroplasts but they were also present in the cytoplasm to a small extent, in contrast to the wild-type proteins (Fig.33). Considering a possible effect of palmitoylation on localization, A2GA4C mutants of FNR and Ruba containing a putative palmitoylation site in addition to the myristoylation site were created. Both mutants were still myristoylated *in vitro* indicating that introduction of the cysteine did not eliminate the myristoylation consensus motif (Fig.32). YFP analyses of FNRA2GA4C and RubaA2GA4C revealed a strong membrane attachment of both mutants and only residual chloroplast localization indicating that introduction of myristoylation and palmitoylation impedes chloroplast import (Fig.33).

To rule out the possibility that the exchange of amino acids led to chloroplast import inhibition due to perturbation of the cTP, control mutations were generated. In the case of CPK16 a G2V version was created, because it is known that alanine is frequently occurring on position 2 of chloroplast proteins and that it is a possible chloroplast targeting determinant (Pujol et al., 2007; Zybailov et al., 2008). Nevertheless, CPK16G2V still localized to chloroplasts (Fig.33). In the case of Ruba, representative for FNR, the control mutants A4C, A2V, and A2VA4S were created and all of them exhibited wild-type chloroplast localization (Fig.33). These results clearly indicated that inhibition of chloroplast import of CPK16, FNR, and Ruba was established by N-terminal acylation.

Additionally, for CPK16, FNR, Ruba, and their mutants *in vitro* chloroplast import assays were carried out, in order to analyze the effect of acylation on chloroplast targeting in an independent experiment (Fig.34). Surprisingly, for all analyzed proteins no significant differences in the efficiency of chloroplast import could be detected. This was in contradiction to the observed acylation-dependent differences in the subcellular localization of the YFP-fusion proteins.
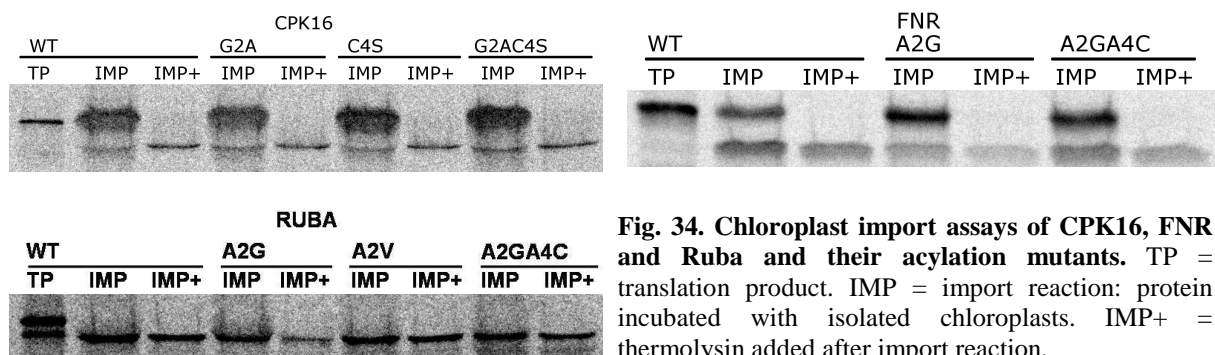
Fig. 34. Chloroplast import assays of CPK16, FNR and Ruba and their acylation mutants. TP = translation product. IMP = import reaction: protein incubated with isolated chloroplasts. IMP+ = thermolysin added after import reaction.

Other examples for the interference of myristoylation with chloroplast import?

In order to identify additional proteins that, similarly to CPK16, are relocated to the chloroplast when N-terminal myristoylation is inhibited, the whole Arabidopsis proteome was analyzed for proteins that are predicted to be both myristoylated and chloroplast-localized.

In total 22 proteins were identified including CPK16, CPK24 and two PP2C-type phosphatases (AT4G03415, AT3G02750). CPK24 has already been analyzed for its localization also as G2A version, but did not localize to chloroplasts at all (data not shown). Interestingly, the localization of both phosphatases has recently been analyzed in a study about chloroplast-predicted protein kinases and phosphatases (Schliebner et al., 2008). AT4G03415 was shown to be localized to the chloroplast and is one of the only two above-mentioned experimentally identified chloroplast proteins that are predicted to be myristoylated. The second protein phosphatase, PP1 (AT3G02750), seemed to be localized to the plasma membrane and therefore represented the most promising candidate. However, PP1G2A was localized in the nucleus and the cytoplasm (Fig.35).

Furthermore, I analyzed the localization of the closest homologs of CPK16: CPK18 and CPK28. CPK28 had already been investigated before and its G2A mutant exhibited exclusive nuclear localization (Mehlmer, 2009). The G2A mutant of CPK18, which is identical to CPK16 in the first seven amino acids (MGLCFSS), also did not exhibit chloroplast localization but similarly to PP1G2A was present in the nucleus and cytoplasm (Fig.35).

chlorophyll   **YFP**   **merged**   **chlorophyll**   **YFP**   **merged**

Fig. 35. YFP localization of the G2A mutants of PP1 and CPK18. Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm.

ER involvement in myristoylation-dependent targeting of CPK16?

In the case of CPK16 myristoylation was shown to be responsible for inhibition of chloroplast import but the molecular mechanism behind this phenomenon was still unclear. Assuming that CPK16 is targeted via the ER to its final destination, it is possible that myristoylation has an impact on the co-translational import into the ER via SRP. If myristoylation is the determinant for CPK16 to be co-translationally recognized by SRP, then the protein would not be available for the components of the post-translationally acting chloroplast import

machinery anymore. In contrast, CPK16G2A, which is lacking myristoylation, would not be recognized by SRP and therefore would be available for the chloroplast import machinery.

To test this hypothesis I analyzed the localization of CPK16 and CPK16G2A, which both were N-terminally fused to a YFP-mutant carrying the ER retention signal KDEL at the very C-terminus. A similar mutant has already been successfully used to demonstrate that the carbonic anhydrase CAH1 is transported to the chloroplast via the secretory pathway. In contrast to CAH1-GFP, which localized to chloroplasts, CAH1-GFP-KDEL was retained in the ER (Villarejo et al., 2005). In the case of CPK16 neither the wild-type protein nor the G2A mutant fused to YFP-KDEL exhibited ER retention (Fig.36), but both proteins rather showed the same subcellular distribution as when fused to the wild-type YFP version.
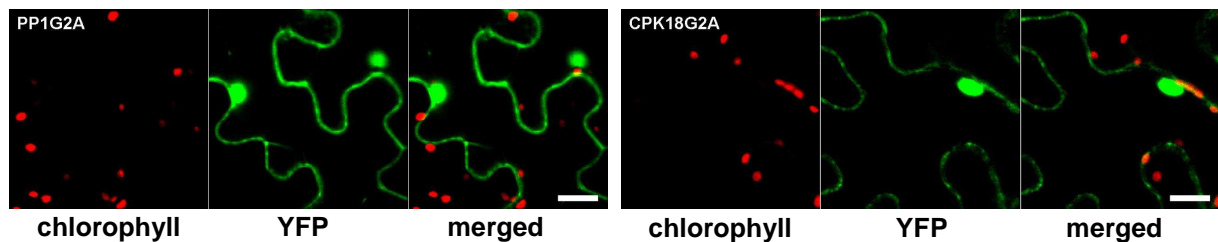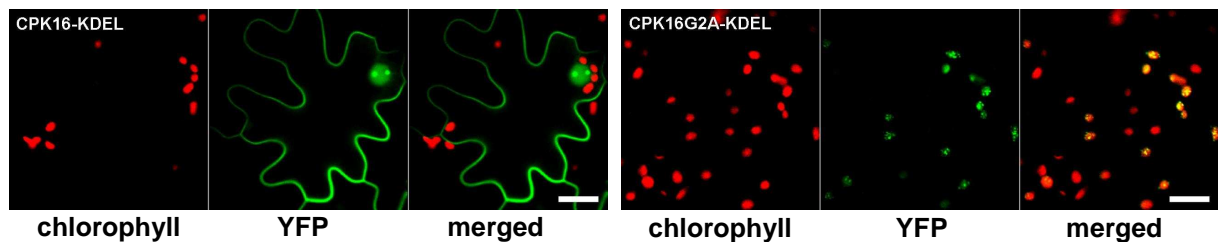

Fig. 36. YFP localization of CPK16- and CPK16G2A-KDEL. A, Tobacco leaves infiltrated with YFP-constructs were analyzed by confocal laser scanning microscopy two days after infiltration. Chlorophyll autofluorescence (red) is shown in the first channel and the YFP signal (green) in the second channel. The third channel shows the merged image. Bar = 20µm.

Since KDEL is triggering retrograde transport of proteins from the Golgi to the ER (Hadlington and Denecke, 2000), it could be excluded that CPK16 was targeted via the ER-Golgi pathway. But import into the ER still could not be ruled out. Therefore direct interaction assays with SRP were carried out.

Interaction of CPK16 and CPK16G2A with SRP

In vitro translation of proteins carrying an ER signal peptide (SP) is known to be arrested upon addition of SRP in the absence of microsomal membranes (Walter and Blobel, 1983). CPK16 and CPK16G2A were translated *in vitro* in the absence or presence of SRP using a wheat germ extract system as previously described for the mammalian NADH-cytochrome b(5) reductase (b5R)(Colombo et al., 2005). Incorporation of radioactively labeled methionine into the full-length protein served as a measure for translation. Interestingly, the translation of the CPK16 wild-type as well as the G2A mutant protein was inhibited with increasing SRP concentrations, indicating myristoylation-independent recognition of both proteins by SRP (Fig.37).

Nevertheless, a crucial control was missing: a protein that was not affected by SRP addition. Therefore, the SRP assay was repeated with the proteins b5Rwt and b5Rsol (obtained from Sara Colombo), which were used in the above-mentioned study (Fig.37). Unexpectedly, translation of b5Rwt was only weakly affected by SRP, whereas translation of b5Rsol was almost completely inhibited by the addition of 1µl SRP. This was in contradiction to the published results and therefore the SRP assay appeared to be error-prone. For this reason no conclusions on the interaction of CPK16 and CPK16G2A with SRP were drawn.
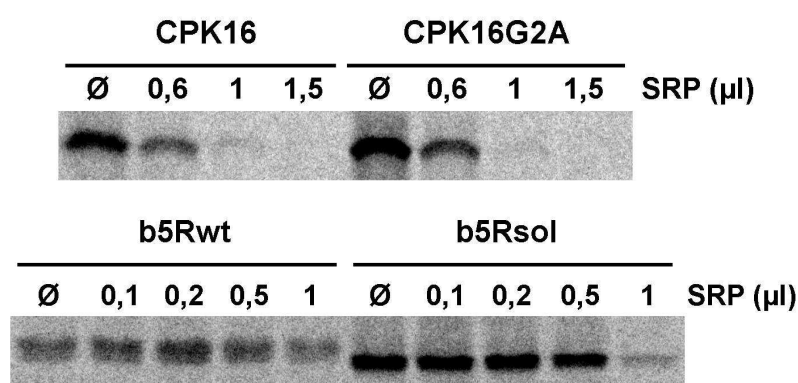
Fig. 37. SRP assay of CPK16, CPK16G2A, b5Rwt and b5Rsol.
Proteins were translated in the presence of indicated amount of SRP using a wheat germ extract system. Ø = control reaction without SRP.

3.6. Thylakoid-localized protein kinases – solubilization by detergents

As already mentioned protein phosphorylation is not restricted to the soluble compartment of the chloroplast. Historically, the first reports of protein phosphorylation within chloroplast thylakoids date back to the 1970s when the phosphorylation of light-harvesting complex (LHC) proteins was demonstrated (Bennett, 1977). The first thylakoid protein kinases that have been identified were the "state transition" kinases STN7 and STN8. Furthermore, the three protein kinases TAK1-3 have been shown to be able to phosphorylate LHC proteins *in vitro* (Snyders and Kohorn, 2001).

Thylakoid-localized protein kinases are hydrophobic as they are localized in a membranous environment and therefore they are hardly accessible for standard chromatographic separation techniques. Thus, it is necessary to solubilize thylakoid protein kinases prior to further purification procedures. This can be done by incubation of thylakoids with detergents, which are amphiphilic molecules that are routinely used for the purification of membrane proteins (Arnold and Linke, 2008). Depending on the biochemical properties of different detergents and their concentrations not only the amount but also the activity of solubilized proteins is

influenced. Therefore, a preliminary experiment was carried out to determine the optimal conditions for protein kinase solubilization from thylakoids.

Thylakoid membranes were separated from isolated chloroplasts and incubated with the detergents CTAB (cationic), dodecyl maltoside, Triton X-100, Tween 20, NP-40, Digitonin (all non-ionic), SDS, N-laurylsarcosine, deoxycholic acid (all anionic), and CHAPS (zwitterionic) at a final concentration of 0.5%. Subsequently solubilized proteins were analyzed by
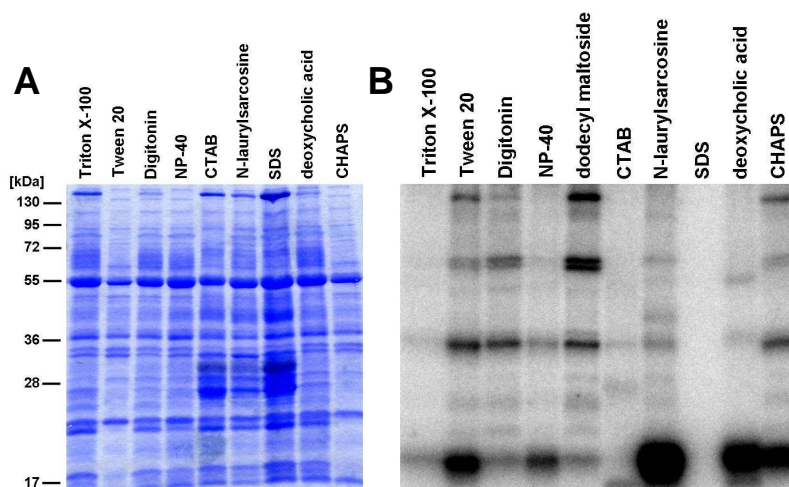
Fig. 38. Solubilization of thylakoid proteins using different detergents. A, Coomassie stain. **B,** Protein kinase assay.

SDS-PAGE and Coomassie staining and tested for protein kinase activity (Fig.38).

According to the amount of solubilized proteins the strongest detergents were SDS, N-laurysarcosine and CTAB. But as expected, these detergents also disturbed protein structure to an extent that protein kinase activity was drastically reduced and in the case of SDS even completely lost. Clearly the best performance regarding preservation of protein kinase activity showed dodecyl maltoside, although one protein kinase responsible for the phosphorylation of a ~18kDa substrate could not be solubilized (unfortunately, Coomassie stain of dodecyl maltoside sample is missing).

To determine the optimal detergent concentration the experiment was repeated with 1% and 1.5% of dodecyl maltoside (Fig.39). Indeed, increasing detergent concentration improved the solubilization of proteins but did not affect protein kinase activity, which completely was solubilized already at a concentration of 0.5%. Thus, for protein kinase solubilization from thylakoid membranes 0.5% dodecyl maltoside were found to be optimal. Subsequent experiments including mass
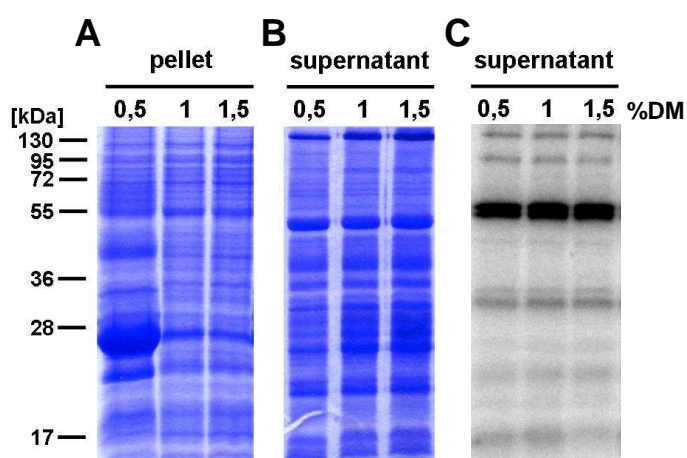
Fig. 39. Solubilization of thylakoid proteins using different concentrations of dodecyl maltoside. %DM = percentage of dodecyl maltoside used for solubilization. **A+B,** Coomassie stain of pellets and supernatants. **C,** Protein kinase assay of supernatants.

spectrometric identification of solubilized proteins or further purification procedures have not been conducted so far but seem to be promising regarding the identification of novel thylakoid-localized protein kinases.

4. Discussion

4.1. Protein kinase activity in chloroplasts

It has already been shown that various substrates in the chloroplast stroma and thylakoids are phosphorylated by chloroplast-localized protein kinases. Besides the extensive phosphorylation of stromal proteins, which was confirmed in my experiments, I could also demonstrate Ca^{2+}-dependent phosphorylation in the stroma.

The drawback of the described *in vitro* protein kinase assay is that only the incorporation of radioactively labeled γ-P_i into proteins is measured. Hence, it cannot be distinguished between phosphorylation of substrates or autophosphorylation of protein kinases, which is a common feature (Smith et al., 1993). But based on experimental experiences, autophosphorylation usually can only be observed when protein kinases are present at very high levels, for example after recombinant expression and purification. Thus, it is more likely that the signals observed in the protein kinase assays are derived from substrate phosphorylation.

Furthermore, based on the number of phosphorylated bands it is not possible to deduce how many protein kinases are present in the chloroplast stroma, because it is feasible that one protein kinase is able to phosphorylate different substrates. Moreover, if the substrate of a stromal protein kinase is only localized within the thylakoids, then its activity cannot be measured by incubation of stromal protein extracts with radioactively labeled ATP.

4.2. Candidate approach

In total I analyzed 15 different protein kinases that either were predicted to be targeted to chloroplasts by TargetP, that shared homology to predicted chloroplast-localized protein kinases or that were identified in chloroplast proteomic studies. Surprisingly, only ABC1 and ABC2, which both belong to the ABC1 family of protein kinases and which were already identified in proteomic studies of plastoglobules, could be verified to be chloroplast-localized. Especially the failure of TargetP to predict chloroplast protein kinases was unexpected, since this program was calculated to correctly predict chloroplast localization for 45% of analyzed proteins (Richly and Leister, 2004).
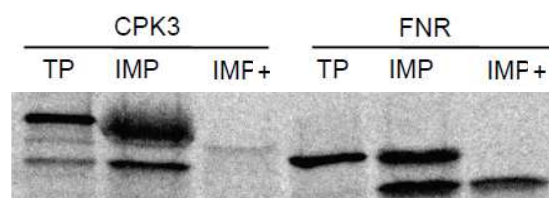
Fig. 40. Chloroplast import assay of CPK3. FNR was included as positive control. TP = translation product. IMP = import reaction: protein incubated with isolated chloroplasts. IMP+ = thermolysin added after import reaction.

Notably, quite a number of different protein kinases are predicted to be localized in chloroplasts but systematic analysis of their localization revealed that most of them are not targeted to chloroplasts *in vivo* (Schliebner et al., 2008). For example, the Ca^{2+}-dependent protein kinase CPK3 has a firm prediction for chloroplast targeting, but could not be imported into the chloroplast (Fig.40) and eventually turned out to be localized in the nucleus and at different cellular membranes (Mehlmer et al., 2010).

4.3. Phylogenetic approach

In a different approach I set out to identify Arabidopsis chloroplast-localized protein kinases based on sequence homology to cyanobacteria. In collaboration with bioinformaticians 465 Arabidopsis could be identified as inherited from the cyanobacterial ancestor using very stringent selection criteria. As a proof of concept a homoserine kinase (HSK), a fructosamine kinase (FAK), an aspartate/glutamate/uridylate kinase (AGUK), and a NAD kinase (NADK1) were subjected to YFP analyses whereas three of them could be confirmed to be chloroplast-localized.

Within the set of 465 proteins only five protein kinases were contained: cKin21, cKin22, ABC2, ABC4, and ABC5. Using relaxed criteria gave rise to further two protein kinases: PIDPK and PHOT1. Out of these seven proteins only the three ABC kinases were shown to be true chloroplast proteins.

Interestingly, already four members of the ABC1 family of protein kinases (ABC1, ABC2, ABC4, and ABC5) could be confirmed in the chloroplast. Intriguingly, three of them have initially been identified in proteomic studies of plastoglobules, which are thought be storage compartments and were shown to contain vitamin E, lipids and quinones (Vidi et al., 2006; Ytterberg et al., 2006).

The presence of three protein kinases in plastoglobules is very surprising regarding the fact that protein kinases are already appearing to be underrepresented in the chloroplast. But an alignment of the protein sequences of ABC2, ABC4 and a conventional STK (AT3G02810) revealed that despite an overall high conservation of the protein kinase domains, two crucial glycine residues within the ATP-binding site are missing (Fig.41). Additionally, compared to the ATP-binding region signature from Prosite, an unexpected leucine residue is present. Therefore it has to be considered that ABC1 family protein kinases possibly are not able to bind ATP and as a consequence are not able to phosphorylate substrates. This could be elucidated by investigating the enzymatic activity of recombinant ABC1 family proteins.

A

[LIV] - G - (P) - G - (P) - [FYWMGSTNH] -

B

```
                                              10        20
                                    ....|....|....|....|..
ABC2-ATPbinding                     IAAASLGQVYKAQLRYSGQVVA
ABC4-ATPbinding                     IAAASLGQVYRATLRATGEDVA
ProteinKinase-ATPbinding            LGEGGFGRVYKGTLKSTGQVVA
Consensus                           * * * G VY    L    G  VA
```

Fig. 41. ATP-binding site of ABC2 and ABC4. A, A part of the ATP-binding signature of protein kinases according to Prosite. **B,** Alignment of the putative ATP-binding sites of ABC2, ABC4, and a conventional STK (AT3G02810). Consensus sequence is also shown. Asterisks indicate positions of the missing glycine and the unexpected leucine residues.

4.4. Proteomic approach

Size exclusion chromatography proved to be a valuable tool in order to deplete stromal protein extracts of Arabidopsis and pea from the most abundant protein Rubisco and to enrich for low-abundant proteins such as protein kinases. Subsequently, the protein kinase-enriched fractions were subjected to direct protein identification by mass spectrometry, to a second gel filtration step using a S75 column, to anion-exchange chromatography using a MonoQ column and to affinity chromatography. In total I was able to identify seven protein kinases, cKin17-20 and cKin23-25, but only cKin18 could be confirmed to be localized in the chloroplast by YFP analysis.

In the case of MonoQ chromatography only one fraction exhibiting the strongest protein kinase activity was subjected to protein identification by MS while other fractions showed different phosphorylation patterns indicating the presence of different protein kinases. It would be interesting to analyze these fractions by MS as well. Furthermore, the pooled fractions after gel filtration on the S200 column could also be applied to hydrophobic interaction chromatography using the Phenyl-Superose column, which exhibited a very efficient separation of root extract proteins in a test run.

Disappointingly, the proteomic approach resulted in the identification of only a single chloroplast-localized, putative protein kinase. Nevertheless, the affinity strategy coupled to gel filtration led to interesting discoveries which will be discussed now.

4.5. Proteomic approach - ATP/PurB/Eu^{3+} affinity chromatography

A strategy to enrich for low-abundant proteins from chloroplasts of the non-model organism pea was developed and led to the identification of 448 proteins. First, all available data on the

localization of these proteins were extracted from databases and the literature in order to get an overview on what is already known. However, assigning protein localization is error-prone when data originating from different experiments in different laboratories are combined. Also, it has to be considered that proteins can be dually targeted (Karniely and Pines, 2005). Furthermore proteins are sometimes assigned to the wrong location, especially when identified in MS-based studies. For example some identified proteins are present in the mitochondrial AMPDB although they are already curated to be exclusively localized in the chloroplast according to PPDB (e.g. AT1G14810, AT2G21170 or AT4G34200).

In order to assign putative novel chloroplast proteins, all identified proteins were divided into three classes: contamination, already known chloroplast and putative novel chloroplast. In case of contradicting evidences for different localizations a protein was assigned to the chloroplast when at least one experiment pointed towards chloroplast localization. Only if no experimental evidence on the localization of a protein had been presented so far, it was considered as a putative novel chloroplast protein. It may well be that putative novel chloroplast proteins were overlooked, because dually targeted proteins or chloroplast proteins identified in other non-chloroplast proteomic studies were assigned as contamination. The example of cKin18, which was clearly localized to the chloroplast but only showed up when using relaxed identification criteria, further suggests that the affinity approach has the potential to identify even more putative novel chloroplast proteins. But it has to be considered that lowering the identification criteria would certainly increase the false-positive rate.

All in all, the localization analysis of the 448 identified proteins showed a good overall quality of the chloroplast isolations as reflected by the high rate of known chloroplast proteins being 84% and the low contamination rate of 5%. In total 11% or 49 proteins were classified as putative new to the chloroplast.

Chloroplast targeting prediction

Using a test set of proteins with known localization the sensitivity of TargetP was calculated with 72% (Richly and Leister, 2004). But when applied to the experimentally identified data set the program performed much better. Out of the 368 already known chloroplast proteins that are not encoded within the chloroplast, 337 were predicted to contain a cTP (92%). Interestingly, chloroplast prediction decreased from 84% for all 448 proteins to 61% for all 49 putative novel chloroplast proteins. This means that either non-chloroplast contaminants are enriched or that several proteins with non-canonical targeting peptides were identified (Bhattacharya et al., 2007).

Out of the 13 selected candidate proteins seven were confirmed to be chloroplast-localized by YFP-fusion analysis. By a combination of the four prediction programs TargetP, ChloroP, MultiP and Aramemnon all experimentally verified chloroplast proteins were at least once correctly predicted. The best individual performance showed ChloroP, which predicted six out of the seven chloroplast-localized proteins correctly.

Saturation of protein identifications

Most of the peptide measurements were performed on a LTQ XL mass spectrometer. It can be argued that using an instrument with higher sensitivity could increase the amount of protein identifications. A LTQ Velos for example was able to identify almost twice as many proteins compared to a LTQ XL instrument when analyzing 1µg of *Caenorhabditis elegans* digest (Second et al., 2009).

In the case here, analyzing the calculated saturation curves for both affinity strategies showed that already with the second biological sample there was almost no increase in new protein identifications (Fig.23D). A significant increase only occurred with the third biological sample but this was due to changes in the experimental setup. In the case of the ATP binding strategy the ligand of the affinity column was changed from PurB to ATP and in the case of the Eu^{3+} column gel filtrated stroma was used instead of heated chloroplasts. This, together with the fact that the sample complexity already was highly reduced prior to MS measurements, suggests that the experimental setup was the limiting factor for protein identifications rather than the type of instrument used. Reanalyzing the samples with an instrument such as LTQ Velos would allow verification of this hypothesis.

Identified proteins - ATP/PurB vs. Eu^{3+}

Affinity chromatography was performed using the ligands ATP, PurB and Eu^{3+}. With each affinity ligand a specific subset of proteins could be identified (Fig.42). As expected, the overlap between ATP and PurB was bigger (75 proteins) than between ATP and Eu^{3+} (14 proteins) or PurB and Eu^{3+} (39 proteins). This reflects the different nature of the ligand's binding affinities. Nevertheless, even with PurB and ATP several unique proteins could be identified, indicating a
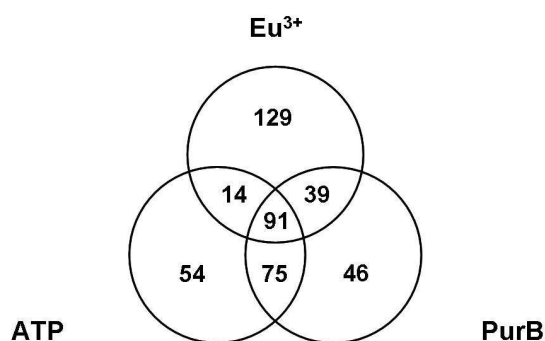

Fig. 42. Comparison of proteins identified in ATP, PurB, and Eu^{3+} affinity runs. Numbers and overlaps of identified proteins using the three different affinity ligands are shown in this Venn diagram.

slightly different mode of action on ATP-binding proteins. Unexpectedly, 91 proteins were identified with all three ligands which may be due to unspecific binding of proteins to the column matrices. On the other hand, since chromatography was carried out under native conditions and thus protein complexes were maintained, it is possible that one protein of a multimeric complex bound ATP while another protein interacted with metal ions. Also, it is possible that a single protein bound to two different ligands.

Selected candidate proteins

In total 13 candidate proteins, 11 from the 49 putative novel chloroplast proteins, were selected for verification by YFP-fusion analysis. Only seven proteins could be confirmed to be chloroplast-localized. The overrepresentation of non-chloroplast contaminants is probably due to the fact that in some cases candidate selection was directed to proteins with a function unexpected in chloroplasts. The L-galactose dehydrogenase GDH for example is involved in the biosynthetic pathway of L-ascorbic acid which is assumed to be carried out in the cytoplasm except for the last step which is localized in mitochondria (Smirnoff, 2000). YFP analysis showed that GDH is localized in the cytoplasm which confirms the classical pathway. A further example is PHR2 a protein with sequence similarity to photolyases, which are enzymes that repair UVB-induced DNA damages. Arabidopsis chloroplasts were reported to lack photolyase activity which is supported by the putative ER localization of PHR2 (Chen et al., 1996). All confirmed chloroplast-localized proteins will now be discussed in detail.

OTL (OTU-like cysteine protease)

The protein OTL belongs to the family of proteases and clearly showed chloroplast localization. Chloroplast proteases have a very important function in processes such as cTP removal and degradation of misfolded and damaged proteins or proteins lacking their cofactors (Nair and Ramaswamy, 2004). OTL belongs to the OTU-like superfamily of predicted cysteine proteases. Homologous proteins are conserved throughout all kingdoms of life (Makarova et al., 2000). In plants the importance of cysteine proteases for chloroplast function was investigated by overexpressing the cysteine protease inhibitor cystatin in tobacco leaves. Cysteine proteases were shown to be involved in the turnover of Rubisco as well as Rubisco activase and to regulate chloroplast protein composition (Prins et al., 2008). In Arabidopsis no cysteine protease inside the chloroplast has been identified so far. Thus, it would be interesting to test whether cysteine protease activity of OTL can be confirmed *in vivo*.

Furthermore, OTL harbors the canonical PTS1 peroxisomal targeting signal SKL at the very C-terminus (Subramani, 1996). Since YFP localization studies were carried out using a C-terminal YFP-fusion, the PTS1 was masked. Repeating localization studies using an N-terminal YFP-fusion would allow analysis of peroxisomal targeting of OTL.

ATF (aminotransferase)

ATF is annotated as aminotransferase. Its closest homolog in Arabidopsis is the aspartate aminotransferase AAT (AT2G22250), which is already known to be localized in the chloroplast and which was also identified in this study. Furthermore, the plastidiar isoform AAT3 (AT4G31990) was also detected. Besides localization in the chloroplast, ATF was also distributed within the cytoplasm. This is probably not an artifact due to overexpression because all other investigated chloroplast proteins did not show any background signal. Thus, ATF seems to have a specific function within the cytoplasm.

HAC (haloacid dehalogenase-like protein)

The haloacid dehalogenase-like protein HAC was exclusively localized in chloroplasts. It belongs to the HAD superfamily including enyzmes with a diversity of functions such as dehalogenases, phosphoesterases or sugar phosphomutases but the majority of members are phosphatases and P-type ATPases (Burroughs et al., 2006). Protein phosphatases are the well-known counterparts of protein kinases and are involved in many signaling processes. Thus, it would be interesting to elucidate the enzymatic activity of HAC regarding a putative function in chloroplast signaling.

PIF (PRL1-interacting factor)

PIF was identified in a screen for proteins interacting with the WD (tryptophan-aspartate)-repeat protein PRL1. This regulatory protein controls glucose and hormone responses and is imported into the nucleus (Nemeth et al., 1998). Since PIF was clearly localized in chloroplasts and probably mitochondria the interaction with PRL1 is much likely an experimental artifact.

According to TAIR PIF contains a CobW-like domain. CobW is involved in the biosynthesis of vitamin B_{12} (cobalamin) and contains a P-loop motif for nucleotide-binding (Rodionov et al., 2003), which explains binding to the affinity column. But it is known that only bacteria are capable of synthesizing cobalamin and in eukaryotes only three cobalamin dependent

enzymes have been described so far, which all are lacking in Arabidopsis (Herbert, 1988; Zhang et al., 2009). Thus, the function of PIF still remains elusive.

But PIF is present in the Chloroplast 2010 database, which collects information from screens for chloroplast-related phenotypes of thousands of experimentally verified or predicted chloroplast proteins (http://www.plastid.msu.edu). There, a knock-out of PIF is shown to result in altered plant morphology and chlorophyll fluorescence.

PGL (phosphoglycerate mutase)

Phosphoenolpyruvat (PEP), together with erythrose 4-phoshpate, is the precursor of aromatic amino acids synthesized via the shikimate pathway and is therefore a key metabolite in plants. Strikingly, in C_3 plants, PEP has to be imported into the chloroplast from the cytoplasm by a specific (PEP)/phosphate translocator of the plastid inner envelope membrane (Voll et al., 2003). A knock-out mutant of this transporter (*cue1*) exhibited a reticulate leaf phenotype and delayed chloroplast development, which could be rescued by overexpression of C_4-type pyruvate, orthophosphate dikinase. But the fact that the *cue1* mutant was still viable indicates that some capacity to produce PEP must also reside in plastids of C_3-plants.

In principle, PEP can be formed from 3-phoshoglycerate in two consecutive reaction steps involving a phosphoglycerate mutase and an enolase. In Arabidopsis the enolase ENO1 was already shown to be localized within the chloroplast (Prabhakar et al., 2009). In this study the missing phosphoglycerate mutase, PGL, could be identified and its chloroplast localization could be confirmed by YFP fusion protein analysis. Recently, PGL was also identified in another independent chloroplast proteomic study (Joyard et al., 2009).

Integrated data analysis of shotgun proteomics and RNA profiling indicated a significant molecular mass bias for the detection of proteins, which are expressed at very low levels (Baginsky et al., 2005). This seems to be the case for the plastidiar PGL, thus explaining why its detection by mass spectrometry had been so difficult. In contrast other metabolic enzymes like transketolase accumulate at much higher levels then it would be expected based on their transcripts (Baginsky et al., 2005), even enabling its protein purification from plant tissues (Teige et al., 1998).

PAP (P-type ATPase)

The protein PAP was already identified in a chloroplast proteomic study and its localization could be confirmed within this thesis. This protein belongs to the family of P-type ATPases, which are mostly membrane proteins that use the energy provided by ATP hydrolysis to pump

ions across membranes. They are usually specific for a single ion such as H^+, Ca^{2+}, Mg^{2+}, Na^+ or Cu^{2+} (Axelsen and Palmgren, 1998). Interestingly, the ATPase PAP seems to lack any transmembrane domains as analyzed by the programs TMHMM and HMMTOP (Sonnhammer et al., 1998; Tusnady and Simon, 1998). This is in contradiction to the expected function as a membrane-bound ion pump.

Pea database vs. Arabidopsis database

The pea EST database used for the identification of proteins from pea samples is known to have a lower coverage of sequences compared to a full genome database, as it is available for Arabidopsis. In order to assess the impact of the two different databases on the protein identification potential, the targeted proteomic approach using ATP as affinity ligand was repeated with Arabidopsis chloroplasts.

Although the same amount of chloroplasts (corresponding to 20 mg chlorophyll) was used, after gel filtration only 0.82 mg protein could be recovered compared to 1.5 mg with pea. This indicated that isolated pea chloroplasts contain almost the double amount of stromal proteins, probably because Arabidopsis chloroplasts partially lost their stromal content during the isolation procedure. Remarkably, although less protein was present in the sample, 365 proteins could be identified with Arabidopsis in contrast to 234 with pea. This was probably due to the low coverage of the pea EST database. It is possible that peptides present in the sample mixture were measured but could not be matched to a protein simply because the sequence is missing in the EST database.

Strikingly, although approximately 50% more proteins were identified with Arabidopsis only nine putative novel chloroplast proteins were found compared to 21 with pea. Only two of the putative novel proteins were identified in both organisms. Firstly, this indicates that the Arabidopsis chloroplast proteome is already quite exploited and that secondly, using pea gives rise to the identification of a different subset of chloroplast proteins. On the one hand this could be due to species-specific differences in the chloroplast protein content. On the other hand this could reflect differences in the developmental state of the analyzed chloroplasts, because for chloroplast isolation from pea seedlings were used, while in the case of Arabidopsis leaves of mature plants were used. This hypothesis is strengthened by the fact that the overlap of identified proteins between both organisms accounts for only 160 proteins.

The general weakness of the pea EST database further was revealed by the higher rate of non-chloroplast contaminants (4.7%) compared to Arabidopsis (3.6%; Fig.25). This is much likely not due to contaminations per se - pea chloroplast preparations are known to be highly pure -

but rather due to wrong protein assignments. As a consequence of the low coverage of the pea EST database it is possible that peptides of a chloroplast protein, which sequence is missing in the database, were measured in the sample. Subsequently the peptide may have been matched to a similar protein which is not localized in the chloroplast. Thus, a major improvement for the targeted affinity approach presented in this thesis would be the sequencing of the complete pea genome. I predict that usage of a whole genome database would result in the detection of more (putative novel) chloroplast proteins accompanied with a decrease of the contamination rate.

4.6. cKin18

cKin18 is the only novel chloroplast-localized protein kinase that has been identified within this work. Although it is lacking its active site according to Prosite, it was shown to be able to phosphorylate a substrate in a stromal but not in a thylakoid protein extract. Interestingly, phosphorylation of at least one thylakoid protein was inhibited upon the addition of cKin18. I assume that this is more likely a redox-dependent effect of glutathione, which was used to elute recombinant GST-tagged cKin18 during protein purification, than an effect of inherent phosphatase activity of cKin18. To address this question, protein kinase assays of thylakoid protein extracts in the presence or absence of glutathione should be performed. Nevertheless, redox sensitive protein phosphorylation in thylakoids has already been described (Vener et al., 1998). Concluding, it seems that cKin18 exhibits its activity exclusively within the chloroplast stroma.

In order to elucidate the function of cKin18, further investigations should focus on the identification of its substrate(s), for example by yeast two-hybrid assays or pull-down assays using a cKin18-specific antibody. Also, if becoming available, T-DNA insertion lines exhibiting a complete loss of cKin18 transcript should be analyzed regarding a phenotype which would help to clarify the physiological role of this protein kinase.

4.7. A revised survey of chloroplast protein kinases and phosphatases

In 2008 a survey of chloroplast protein kinases and phosphatases in Arabidopsis has been published (Schliebner et al., 2008). I integrated data extracted from publications, also from other organisms, and data obtained during this thesis and created a revised version of this survey (Tab.14).

Tab. 14. Revised survey of chloroplast protein kinases and phosphatases.

name	organism(s)	chloroplast
conventional protein kinases		
STN7[a,b]	*Chlamydomonas reinhardtii*; Arabidopsis	yes
STN8[a,b]	Arabidopsis	yes
CKII[a]	*Sinapis alba*; Arabidopsis	yes
TAK1[a]	Arabidopsis	yes
CIPK13[a]	Arabidopsis	yes
AT1G51170[a]	Arabidopsis	yes
ABC1	Arabidopsis	yes
ABC2	Arabidopsis	yes
ABC4	Arabidopsis	yes
ABC5	Arabidopsis	yes
CDPK1	*Spirodela oligorrhiza*	disputed
MKK4	Arabidopsis	disputed
unusual protein kinases		
AtRP1[a,c]	Arabidopsis	yes
CSK	Arabidopsis	yes
NDPK2	Arabidopsis	yes
cKin18	Arabidopsis	yes
excluded protein kinases		
TAK2[a]	Arabidopsis	plasma membrane
TAK3[a]	Arabidopsis	plasma membrane
MSK4	*Medicago sativa*, Arabidopsis	cytoplasm
NtDSK1	*Nicotiana tabacum*	cytoplasm
protein phosphatases		
AtRP1[a,b]	Arabidopsis	yes
SEX4/DSP4[a]	Arabidopsis	yes
AT2G30020[a]	Arabidopsis	yes
AT4G33500[a]	Arabidopsis	yes
AT1G67820[a]	Arabidopsis	yes
AT2G30170[a]	Arabidopsis	yes
AT3G10940[a]	Arabidopsis	yes
AT4G03415[a]	Arabidopsis	yes
AT1G07160[a]	Arabidopsis	yes
TAP38	Arabidopsis	yes

[a]Protein is present in the original survey of Schliebner et al. (2008). [b]STN7 and STN8 both are homologs of the *Chlamydomonas reinhardtii* protein kinase STT7 (Bonardi et al., 2005). [c]Due to its activity AtRP1 was counted as protein kinase as well as a protein phosphatase.

In the original survey only the seven protein kinases STN7, STN8, CKII, TAK1, CIPK13, AT1G51170 and AtRP1 were contained. Interestingly, AtRP1, which was also identified in my proteomic experiments, was shown to possess both protein kinase and phosphatase activity on pyruvate, orthophosphate dikinase (Chastain et al., 2008). TAK2 and TAK3 were not accepted as chloroplast protein kinases, because they were only identified by sequence homology to TAK1. In the meantime, TAK2 and TAK3 have even been curated to be localized at the plasma membrane according to PPDB.

Based on the results of this thesis, ABC1, ABC2, ABC4, ABC5, NDPK2 and cKin18 were added to the complement of chloroplast-localized protein kinases. The ABC1 family kinases are supposed to be involved in quinone synthesis but their protein kinase activity still is disputed and has to be confirmed experimentally. Finally, the recently published chloroplast sensor kinase CSK, which was shown to undergo autophosphorylation and to be involved in the redox-dependent regulation of chloroplast gene expression, was included (Puthiyaveetil et al., 2008).

Chloroplast localization of the already published protein kinase MSK4 from was falsified in the course of this thesis and thus, it was excluded. I also excluded the protein kinase NtDSK1 from tobacco, because I was not able to confirm the chloroplast localization of its closest homolog in Arabidopsis, AtDSK1. In *Spirodela oligorrhiza* the Ca^{2+}-dependent protein kinase CDPK1 was shown to be chloroplast-localized by an *in vitro* chloroplast import assay (Raskind, 2001). But due to the bad quality of this assay, chloroplast localization of CDPK1 is disputed. Also, the MAPKK MKK4 was shown to be imported into chloroplasts in an *in vitro* assay (Samuel et al., 2008). Since this is in contradiction to the nuclear and cytoplasmic localization pattern observed in a previous study (Koroleva et al., 2005) and to its function in the cytoplasmic and nuclear localized MAPK signaling cascade, chloroplast localization of MKK4 is also disputed.

Together with the phosphatase TAP38, which recently has been shown to be chloroplast-localized (Pribil et al., 2010), the updated survey contains now 14 protein kinases and 10 phosphatases, whereas AtRP1 was counted for both categories.

I decided to divide the experimentally confirmed chloroplast protein kinases into two categories: conventional and unusual. Conventional protein kinases are containing a conserved protein kinase domain consisting of an ATP-binding region and an active-site signature according to Prosite. In contrast, the unusual protein kinase cKin18 is lacking its active site and AtRP1, NDPK2 and the recently discovered CSK are lacking the complete protein kinase domain. Nevertheless, they all retained protein kinase activity.

4.8. Conclusion - the mystery of chloroplast protein kinases

Based on the number of protein kinases present in the genome and the predicted number of chloroplast proteins at least 73 chloroplast-localized protein kinases are expected in Arabidopsis but up to date only 14 have been identified.

In the candidate approach out of 10 analyzed protein kinases, predicted to be chloroplast-localized by TargetP, not a single one could be confirmed in the chloroplast. This was

completely unexpected, since TargetP was shown to correctly predict chloroplast localization in 45% of all cases (Richly and Leister, 2004). Additionally, in the phylogenetic approach 465 proteins conserved between Arabidopsis and cyanobacteria were identified, whereas more than 60% of them were already known to be chloroplast-localized. Again, this approach failed to identify novel chloroplast protein kinases. Finally, a proteomic approach, involving the separation of protein extracts from isolated chloroplasts by different chromatographic techniques, was implemented. But also this approach completely failed to identify novel conventional protein kinases in the chloroplast. Incidentally, in the course of all experimental approaches I was able to identify conventional protein kinases localized to almost all subcellular compartments including plasma membrane, nucleus, cytoplasm, mitochondria, ER, oleosome and even cytoskeleton but not chloroplasts. All in all, despite the effort that has been made within this PhD thesis, only one novel unusual chloroplast protein kinase, cKin18, could be identified and the presence of the unusual protein kinases NDPK2 and AtRP1 in the chloroplast could be confirmed.

Considering the already identified proteins AtRP1, NDPK2, CSK and cKin18 and the unexpected results from protein kinase substrate and inhibitor experiments, evidences for unusual chloroplast protein kinases accumulate. Altogether, this suggests that unusual protein kinases are responsible for many if not most protein phosphorylation events occurring inside the chloroplast. If this is really the case, then it will be impossible to identify these protein kinases by directed approaches involving protein identification by MS, which relies on the functional annotation of proteins. The only chance would be to make use of known protein kinases substrates in order to identify the interacting protein kinases for example by co-immunoprecipitation or by biochemical fractionation using the substrates as markers.

Another explanation for the failure of the identification of novel, conventional chloroplast protein kinases is, that there number is simply overestimated. Out of 965 protein kinases (3.5% of the whole proteome; PlantsP) only 1% is already known to be chloroplast-localized compared to 4.6% of all 217 protein phosphatases (0.8% of the whole proteome)(Schliebner et al., 2008). This clearly indicates that protein kinases are underrepresented in the chloroplast. It is possible that chloroplasts evolved a mechanism of signaling different from the rest of the cell, where protein kinases are usually the key mediators. This could be established by general phosphorylation of all chloroplast substrates by only a few protein kinases such as CKII and eventual regulation of substrate activity by phosphatase-catalyzed dephosphorylation. This is in accordance with the finding of the previously mentioned chloroplast phosphoproteomic study that the CKII phosphorylation motif is enriched within the 174 identified chloroplast

phosphoproteins (Reiland et al., 2009). Hence, it was suggested that CKII is a central regulator of chloroplast processes. This could be elucidated, for example, by comparison of phosphorylation patterns in isolated chloroplasts of CKII knock-out and wild-type plants.

4.9. The impact of N-terminal acylation on chloroplast import

YFP analyses of cKin proteins and their G2A mutants clearly showed that myristoylation is affecting membrane localization, which confirmed previous findings that myristoylation is crucial for the membrane attachment of many proteins involved in signal transduction (Taniguchi, 1999).

But more interesting, YFP-localization studies on CPK16, FNR, Ruba and their acylation mutants revealed that myristoylation as well as palmitoylation is able to interfere with chloroplast import. However, it seems that this is not a general mechanism but has to be analyzed for each protein separately. In the case of CPK16 abolishing myristoylation in the G2A mutant led to chloroplast localization, but removal of only the palmitoylation site in the C4S mutant had no effect. Thus, myristoylation alone did clearly inhibit chloroplast import *in vivo*. In contrast, an artificial introduction of N-myristoylation sites in FNR and Ruba in the A2G mutants did only slightly influence chloroplast targeting. But additional introduction of palmitoylation sites in the A2GA4C mutants led to a strong accumulation of the proteins within the cytoplasm. In these cases inhibition of chloroplast import must primarily be attributed to palmitoylation. These conclusions were drawn based on *in vivo* data obtained from YFP studies. This is important because in an *in vitro* situation wild-type CPK16, FNR, Ruba, and all their acylation mutants, which were translated in a wheat germ extract system, were imported into isolated chloroplasts without significant differences in their import efficiencies.

There are two possible explanations: Either acylation does not inhibit the passage of proteins through the chloroplastic TOC-TIC apparatus per se, or it does rather direct proteins to different compartments before they can be recognized by chloroplast import components *in vivo*. For example it is possible that myristoylated CPK16 is recognized by SRP, co-translationally targeted to the ER and subsequently transported to its final destination. In contrast, non-myristoylated CPK16G2A would not be recognized by SRP and therefore, after completed translation, CPK16G2A would be available for components of the chloroplast import machinery. However, YFP studies with CPK16 and CPK16G2A carrying the ER retention signal KDEL, did not indicate involvement of the ER in CPK16 targeting. But since KDEL is only triggering retrograde transport of proteins from the Golgi to the ER, it still is

possible that CPK16 could directly be targeted from the ER to its final destination as it was previously described for the protein CBL1 (Batistic et al., 2008).

More likely, the observed differences between protein targeting *in vivo* and *in vitro* can be explained by a low efficiency of acylation during wheat germ extract mediated translation of proteins destined for chloroplast import assays. As a result, residual unmodified proteins would be able to be imported into isolated chloroplasts normally. This hypothesis is in accordance with a previous study on the effect of acylation on localization that included the analysis of myristoylation efficiency in wheat germ extract (Colombo et al., 2005). The protein b5R was translated in the presence of ^{35}S-methionine and ^{3}H-MyrCoA and consequently the amount of myristic acid attached to the synthesized protein was calculated from the ratio of incorporated ^{3}H to ^{35}S. It was shown that the efficiency of myristoylation increased with increasing concentration of myristic acid, reaching the maximum possible 1:1 stoichiometry at 120µM. This suggests that the myristate concentration of ~6.7µM used for the translation of CPK16, FNR, Ruba and their mutants, was indeed limiting. Consequently, only a small fraction of the myristoylable proteins actually became myristoylated explaining their chloroplast import competence in the *in vitro* assays.

The remaining question is, whether chloroplast localization of CPK16G2A has a physiological relevance or is just an experimental artifact. So far, it is not clear, if acylation affects 100% of the cellular pool of a protein with an acylation consensus motif *in vivo* or whether there's a stoichiometric distribution between acylated and unmodified subpools. Considering stoichiometry of acylation, it is expected that at least parts of the wild-type CPK16 protein pool are targeted to the chloroplast. However, this could not be confirmed by YFP localization studies, where CPK16 was actually overexpressed.

Furthermore, no other examples similar to CPK16 have been reported in the literature so far and even the most promising candidates CPK18 and PP1 did not localize to chloroplasts when their N-myristoylation was abolished. Altogether, this suggests that chloroplast localization of CPK16G2A is an experimental artifact. Nevertheless, CPK16 provides a good example to study the mechanism of chloroplast import impairment by N-terminal acylation.

5. REFERENCES

Abdallah F, Salamini F, Leister D (2000) A prediction of the size and evolutionary origin of the proteome of chloroplasts of Arabidopsis. Trends Plant Sci **5:** 141-142

Abu-Abied M, Avisar D, Belausov E, Holdengreber V, Kam Z, Sadot E (2009) Identification of an Arabidopsis unknown small membrane protein targeted to mitochondria, chloroplasts, and peroxisomes. Protoplasma **236:** 3-12

AGI (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408:** 796-815

Allen JF (2003) The function of genomes in bioenergetic organelles. Philos Trans R Soc Lond B Biol Sci **358:** 19-37; discussion 37-18

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653-657

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403-410

Annweiler A, Hipskind RA, Wirth T (1991) A strategy for efficient in vitro translation of cDNAs using the rabbit beta-globin leader sequence. Nucleic Acids Res **19:** 3750

Arnold T, Linke D (2008) The use of detergents to purify membrane proteins. Curr Protoc Protein Sci **Chapter 4:** Unit 4 8 1-4 8 30

Arnon DI (1949) Copper Enzymes in Isolated Chloroplasts. Polyphenoloxidase in Beta Vulgaris. Plant Physiol **24:** 1-15

Ashby MK, Houmard J (2006) Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. Microbiol Mol Biol Rev **70:** 472-509

Ashton AR, Hatch MD (1983) Regulation of C4 photosynthesis: regulation of pyruvate, Pi dikinase by ADP-dependent phosphorylation and dephosphorylation. Biochem Biophys Res Commun **115:** 53-60

Austin JR, 2nd, Frost E, Vidi PA, Kessler F, Staehelin LA (2006) Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. Plant Cell **18:** 1693-1703

Axelsen KB, Palmgren MG (1998) Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol **46:** 84-101

Baginsky S (2009) Plant proteomics: concepts, applications, and novel strategies for data interpretation. Mass Spectrom Rev **28:** 93-120

Baginsky S, Gruissem W (2009) The chloroplast kinase network: new insights from large-scale phosphoproteome profiling. Mol Plant **2:** 1141-1153

Baginsky S, Kleffmann T, von Zychlinski A, Gruissem W (2005) Analysis of shotgun proteomics and RNA profiling data from Arabidopsis thaliana chloroplasts. J Proteome Res **4:** 637-640

Bairoch A (2000) The ENZYME database in 2000. Nucleic Acids Res **28:** 304-305

Balmer Y, Koller A, del Val G, Manieri W, Schurmann P, Buchanan BB (2003) Proteomics gives insight into the regulatory function of chloroplast thioredoxins. Proc Natl Acad Sci U S A **100:** 370-375

Batistic O, Kudla J (2009) Plant calcineurin B-like proteins and their interacting protein kinases. Biochim Biophys Acta **1793:** 985-992

Batistic O, Sorek N, Schultke S, Yalovsky S, Kudla J (2008) Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK Ca2+ signaling complexes in Arabidopsis. Plant Cell **20:** 1346-1362

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

Bennett J (1977) Phosphorylation of chloroplast membrane polypeptides. Nature **269:** 344-346

Bevan M (1984) Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res **12:** 8711-8721

Beven L, Adenier H, Kichenama R, Homand J, Redeker V, Le Caer JP, Ladant D, Chopineau J (2001) Ca2+-myristoyl switch and membrane binding of chemically acylated neurocalcins. Biochemistry **40:** 8152-8160

Bhattacharya D, Archibald JM, Weber AP, Reyes-Prieto A (2007) How do endosymbionts become organelles? Understanding early events in plastid evolution. Bioessays **29:** 1239-1246

Blum H, Beier H, Gross H (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93-99

Bologna G, Yvon C, Duvaud S, Veuthey AL (2004) N-Terminal myristoylation predictions by ensembles of neural networks. Proteomics **4:** 1626-1632

Bonardi V, Pesaresi P, Becker T, Schleiff E, Wagner R, Pfannschmidt T, Jahns P, Leister D (2005) Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. Nature **437:** 1179-1182

Bousquet I, Dujardin G, Slonimski PP (1991) 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 J **10:** 2023-2031

Brautigam A, Shrestha RP, Whitten D, Wilkerson CG, Carr KM, Froehlich JE, Weber AP (2008) Low-coverage massively parallel pyrosequencing of cDNAs enables proteomics in non-model species: comparison of a species-specific database generated by pyrosequencing with databases from related species for proteome analysis of pea chloroplast envelopes. J Biotechnol **136:** 44-53

Bruce BD (2000) Chloroplast transit peptides: structure, function and evolution. Trends Cell Biol **10:** 440-447

Buchanan B, Gruissem W, Jones RL (2000) Biochemistry & Molecular Biology of Plants. Wiley & Sons

Burroughs AM, Allen KN, Dunaway-Mariano D, Aravind L (2006) Evolutionary genomics of the HAD superfamily: understanding the structural adaptations and catalytic diversity in a superfamily of phosphoesterases and allied enzymes. J Mol Biol **361:** 1003-1034

Bussemer J, Vothknecht UC, Chigri F (2009) Calcium regulation in endosymbiotic organelles of plants. Plant Signal Behav **4:** 805-808

Campbell NA, Reece JB (2009) Biology, Ed 8

Carrie C, Kuhn K, Murcha MW, Duncan O, Small ID, O'Toole N, Whelan J (2009) Approaches to defining dual-targeted proteins in Arabidopsis. Plant J **57:** 1128-1139

Chaga GS, Ersson B, Porath JO (1996) Isolation of calcium-binding proteins on selective adsorbents. Application to purification of bovine calmodulin. J Chromatogr A **732:** 261-269

Chastain CJ, Xu W, Parsley K, Sarath G, Hibberd JM, Chollet R (2008) The pyruvate, orthophosphate dikinase regulatory proteins of Arabidopsis possess a novel, unprecedented Ser/Thr protein kinase primary structure. Plant J **53:** 854-863

Chen JJ, Jiang CZ, Britt AB (1996) Little or No Repair of Cyclobutyl Pyrimidine Dimers Is Observed in the Organellar Genomes of the Young Arabidopsis Seedling. Plant Physiol **111:** 19-25

Chevalier D, Walker JC (2005) Functional genomics of protein kinases in plants. Brief Funct Genomic Proteomic **3:** 362-371

Cho HS, Yoon GM, Lee SS, Kim YA, Hwang I, Choi D, Pai HS (2001) A novel dual-specificity protein kinase targeted to the chloroplast in tobacco. FEBS Lett **497:** 124-130

Cohen P (2002) Protein kinases--the major drug targets of the twenty-first century? Nat Rev Drug Discov **1:** 309-315

Colombo S, Longhi R, Alcaro S, Ortuso F, Sprocati T, Flora A, Borgese N (2005) N-myristoylation determines dual targeting of mammalian NADH-cytochrome b5 reductase to ER and mitochondrial outer membranes by a mechanism of kinetic partitioning. J Cell Biol **168:** 735-745

Cotton DW (1993) Intimate relations: the serial endosymbiotic theory of the origin of eukaryotes. J Pathol **169:** 189-190

Deusch O, Landan G, Roettger M, Gruenheit N, Kowallik KV, Allen JF, Martin W, Dagan T (2008) Genes of cyanobacterial origin in plant nuclear genomes point to a heterocyst-forming plastid ancestor. Mol Biol Evol **25:** 748-761

Duchene AM, Giritch A, Hoffmann B, Cognat V, Lancelin D, Peeters NM, Zaepfel M, Marechal-Drouard L, Small ID (2005) Dual targeting is the rule for organellar aminoacyl-tRNA synthetases in Arabidopsis thaliana. Proc Natl Acad Sci U S A **102:** 16484-16489

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol **300:** 1005-1016

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci **8:** 978-984

Farazi TA, Waksman G, Gordon JI (2001) The biology and enzymology of protein N-myristoylation. J Biol Chem **276:** 39501-39504

Ferro M, Brugiere S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Ramus C, Miras S, Mellal M, Le Gall S, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, Rolland N (2010) AT_CHLORO: A comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Mol Cell Proteomics

Fling SP, Gregerson DS (1986) Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. Anal Biochem **155:** 83-88

Foyer CH (1985) Stromal protein phosphorylation in spinach (Spinacia oleracea) chloroplasts. Biochem J **231:** 97-103

Gray NS, Wodicka L, Thunnissen AM, Norman TC, Kwon S, Espinoza FH, Morgan DO, Barnes G, LeClerc S, Meijer L, Kim SH, Lockhart DJ, Schultz PG (1998) Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. Science **281:** 533-538

Grefen C, Harter K (2004) Plant two-component systems: principles, functions, complexity and cross talk. Planta **219:** 733-742

Gribskov M, Fana F, Harper J, Hope DA, Harmon AC, Smith DW, Tax FE, Zhang G (2001) PlantsP: a functional genomics database for plant phosphorylation. Nucleic Acids Res **29:** 111-113

Hadlington JL, Denecke J (2000) Sorting of soluble proteins in the secretory pathway of plants. Curr Opin Plant Biol **3:** 461-468

Halliwell B (1978) The chloroplast at work. A review of modern developments in our understanding of chloroplast metabolism. Prog Biophys Mol Biol **33:** 1-54

Hanks SK (2003) Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. Genome Biol **4:** 111

Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. Faseb J **9:** 576-596

Haynes PA, Roberts TH (2007) Subcellular shotgun proteomics in plants: looking beyond the usual suspects. Proteomics **7:** 2963-2975

Heazlewood JL, Tonti-Filippini J, Verboom RE, Millar AH (2005) Combining experimental and predicted datasets for determination of the subcellular location of proteins in Arabidopsis. Plant Physiol **139:** 598-609

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH (2004) Experimental analysis of the Arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell **16:** 241-256

Herbert V (1988) Vitamin B-12: plant sources, requirements, and assay. Am J Clin Nutr **48:** 852-858

Hrabak EM, Chan CW, Gribskov M, Harper JF, Choi JH, Halford N, Kudla J, Luan S, Nimmo HG, Sussman MR, Thomas M, Walker-Simmons K, Zhu JK, Harmon AC (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant Physiol **132:** 666-680

Hulo N, Bairoch A, Bulliard V, Cerutti L, Cuche BA, de Castro E, Lachaize C, Langendijk-Genevaux PS, Sigrist CJ (2008) The 20 years of PROSITE. Nucleic Acids Res **36:** D245-249

Hunter T (1991) Protein kinase classification. Methods Enzymol **200:** 3-37

Hunter T (2000) Signaling--2000 and beyond. Cell **100:** 113-127

Huo L, Lee EK, Leung PC, Wong AO (2004) Goldfish calmodulin: molecular cloning, tissue distribution, and regulation of transcript expression in goldfish pituitary cells. Endocrinology **145:** 5056-5067

Hwang I, Chen HC, Sheen J (2002) Two-component signal transduction pathways in Arabidopsis. Plant Physiol **129:** 500-515

Ichimura K (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci **7:** 301-308

Ishitani M, Liu J, Halfter U, Kim CS, Shi W, Zhu JK (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. Plant Cell **12:** 1667-1678

Ito J, Heazlewood JL, Millar AH (2006) Analysis of the soluble ATP-binding proteome of plant mitochondria identifies new proteins and nucleotide triphosphate interactions within the matrix. J Proteome Res **5:** 3459-3469

Jan van Wijk K (2000) Proteomics of the chloroplast: experimentation and prediction. Trends Plant Sci **5:** 420-425

Jarvis P (2004) Organellar proteomics: chloroplasts in the spotlight. Curr Biol **14:** R317-319

Jarvis P (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants. New Phytol **179:** 257-285

Jones K (1991) A review of biotechnology and large scale affinity chromatography Chromatographia **32:** 469-480

Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N (2009) Chloroplast proteomics highlights the subcellular compartmentation of lipid metabolism. Prog Lipid Res **49:** 128-158

Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM, Zarrinkar PP (2008) A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol **26:** 127-132

Karniely S, Pines O (2005) Single translation--dual destination: mechanisms of dual protein targeting in eukaryotes. EMBO Rep **6:** 420-425

Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem **74:** 5383-5392

Kempa S, Rozhon W, Samaj J, Erban A, Baluska F, Becker T, Haselmayer J, Schleiff E, Kopka J, Hirt H, Jonak C (2007) A plastid-localized glycogen synthase kinase 3 modulates stress tolerance and carbohydrate metabolism. Plant J **49:** 1076-1090

Kishimoto K, Ishijima S, Ohnishi M (2003) ATP-binding proteins of spinach chloroplast membranes. J. Biol. Macromol. **3:** 69-74

Kitajima A, Asatsuma S, Okada H, Hamada Y, Kaneko K, Nanjo Y, Kawagoe Y, Toyooka K, Matsuoka K, Takeuchi M, Nakano A, Mitsui T (2009) The rice alpha-amylase glycoprotein is targeted from the Golgi apparatus through the secretory pathway to the plastids. Plant Cell **21:** 2844-2858

Kleffmann T, Hirsch-Hoffmann M, Gruissem W, Baginsky S (2006) plprot: a comprehensive proteome database for different plastid types. Plant Cell Physiol **47:** 432-436

Kleinschmidt JH (2003) Membrane Proteins – Introduction. Cellular and Molecular Life Sciences **60:** 1527-1528

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489-503

Knight H, Trewavas AJ, Knight MR (1997) Calcium signalling in Arabidopsis thaliana responding to drought and salinity. Plant J **12:** 1067-1078

Koroleva OA, Tomlinson ML, Leader D, Shaw P, Doonan JH (2005) High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant J **41:** 162-174

Krebs EG (1983) Historical perspectives on protein phosphorylation and a classification system for protein kinases. Philos Trans R Soc Lond B Biol Sci **302:** 3-11

Kunst L (1998) Preparation of physiologically active chloroplasts from Arabidopsis. Methods Mol Biol **82:** 43-48

Laing WA, Christeller JT (1984) Chloroplast phosphoproteins: Distribution of phosphoproteins within spinach chloroplasts. Plant Science Letters **36:** 99-104

Laurie S, Halford NG (2001) The role of protein kinases in the regulation of plant growth and development. Plant Growth Regulation **34:** 253-265

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. Biotechnology (N Y) **9:** 963-967

Lee DW, Kim JK, Lee S, Choi S, Kim S, Hwang I (2008) Arabidopsis nuclear-encoded plastid transit peptides contain multiple sequence subgroups with distinctive chloroplast-targeting sequence motifs. Plant Cell **20:** 1603-1622

Li HM, Chiu CC (2010) Protein transport into chloroplasts. Annu Rev Plant Biol **61:** 157-180

Lilley KS, Dupree P (2007) Plant organelle proteomics. Curr Opin Plant Biol **10:** 594-599

Makarova KS, Aravind L, Koonin EV (2000) A novel superfamily of predicted cysteine proteases from eukaryotes, viruses and Chlamydia pneumoniae. Trends Biochem Sci **25:** 50-52

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science **298:** 1912-1934

Martin DM, Miranda-Saavedra D, Barton GJ (2009) Kinomer v. 1.0: a database of systematically classified eukaryotic protein kinases. Nucleic Acids Res **37:** D244-250

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M, Penny D (2002) Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci U S A **99:** 12246-12251

Maurer-Stroh S, Eisenhaber B, Eisenhaber F (2002) N-terminal N-myristoylation of proteins: prediction of substrate proteins from amino acid sequence. J Mol Biol **317:** 541-557

Meggio F, Donella Deana A, Ruzzene M, Brunati AM, Cesaro L, Guerra B, Meyer T, Mett H, Fabbro D, Furet P, et al. (1995) Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. Eur J Biochem **234:** 317-322

Mehlmer N (2009) Ca2+ Dependent Protein Kinases in Arabidopsis thaliana. PhD thesis. University of Vienna, Vienna

Mehlmer N, Wurzinger B, Stael S, Hofmann-Rodrigues D, Csaszar E, Pfister B, Bayer R, Teige M (2010) The Ca2+-dependent protein kinase CPK3 is required for MAPK-independent salt-stress acclimation in Arabidopsis. The Plant Journal. **PMID: 20497378**

Mereschkowsky KS (1905) Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Biol. Centralbl. **25:** 593–604

Minet M, Dufour ME, Lacroute F (1992) Complementation of Saccharomyces cerevisiae auxotrophic mutants by Arabidopsis thaliana cDNAs. Plant J **2:** 417-422

Miras S, Salvi D, Ferro M, Grunwald D, Garin J, Joyard J, Rolland N (2002) Non-canonical transit peptide for import into the chloroplast. J Biol Chem **277:** 47770-47778

Moore LL, Fulton AM, Harrison ML, Geahlen RL (2004) Anti-sulfonylbenzoate antibodies as a tool for the detection of nucleotide-binding proteins for functional proteomics. J Proteome Res **3:** 1184-1190

Motohashi K, Kondoh A, Stumpp MT, Hisabori T (2001) Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A **98:** 11224-11229

Munoz-Bertomeu J, Cascales-Minana B, Mulet JM, Baroja-Fernandez E, Pozueta-Romero J, Kuhn JM, Segura J, Ros R (2009) Plastidial glyceraldehyde-3-phosphate dehydrogenase deficiency leads to altered root development and affects the sugar and amino acid balance in Arabidopsis. Plant Physiol **151:** 541-558

Murray D, Ben-Tal N, Honig B, McLaughlin S (1997) Electrostatic interaction of myristoylated proteins with membranes: simple physics, complicated biology. Structure **5:** 985-989

Nada A, Soll J (2004) Inner envelope protein 32 is imported into chloroplasts by a novel pathway. J Cell Sci **117:** 3975-3982

Nair JS, Ramaswamy NK (2004) Chloroplast Proteases. Biologia Plantarum **48:** 321-326

Nakao M, Okamoto S, Kohara M, Fujishiro T, Fujisawa T, Sato S, Tabata S, Kaneko T, Nakamura Y (2009) CyanoBase: the cyanobacteria genome database update 2010. Nucleic Acids Res **38:** D379-381

Nanjo Y, Oka H, Ikarashi N, Kaneko K, Kitajima A, Mitsui T, Munoz FJ, Rodriguez-Lopez M, Baroja-Fernandez E, Pozueta-Romero J (2006) Rice plastidial N-glycosylated nucleotide pyrophosphatase/phosphodiesterase is transported from the ER-golgi to the chloroplast through the secretory pathway. Plant Cell **18:** 2582-2592

Nelson BK, Cai X, Nebenfuhr A (2007) A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J **51:** 1126-1136

Nemeth K, Salchert K, Putnoky P, Bhalerao R, Koncz-Kalman Z, Stankovic-Stangeland B, Bako L, Mathur J, Okresz L, Stabel S, Geigenberger P, Stitt M, Redei GP, Schell J, Koncz C (1998) Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in Arabidopsis. Genes Dev **12:** 3059-3073

Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem **75:** 4646-4658

Nussaume L (2008) Chloroplast SRP takes another road. Nat Chem Biol **4:** 529-531

Ogrzewalla K, Piotrowski M, Reinbothe S, Link G (2002) The plastid transcription kinase from mustard (Sinapis alba L.). A nuclear-encoded CK2-type chloroplast enzyme with redox-sensitive function. Eur J Biochem **269:** 3329-3337

Orchard S, Hermjakob H, Apweiler R (2005) Annotating the human proteome. Mol Cell Proteomics **4:** 435-440

Parker PJ (1993) Antibodies to fluorylsulfonylbenzoyladenosine permit identification of protein kinases. FEBS Lett **334:** 347-350

Parkinson JS (1993) Signal transduction schemes of bacteria. Cell **73:** 857-871

Peitzsch RM, McLaughlin S (1993) Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. Biochemistry **32:** 10436-10443

Podell S, Gribskov M (2004) Predicting N-terminal myristoylation sites in plant proteins. BMC Genomics **5:** 37

Prabhakar V, Lottgert T, Gigolashvili T, Bell K, Flugge UI, Hausler RE (2009) Molecular and functional characterization of the plastid-localized Phosphoenolpyruvate enolase (ENO1) from Arabidopsis thaliana. FEBS Lett **583:** 983-991

Pribil M, Pesaresi P, Hertle A, Barbato R, Leister D (2010) Role of plastid protein phosphatase TAP38 in LHCII dephosphorylation and thylakoid electron flow. PLoS Biol **8:** e1000288

Prins A, van Heerden PD, Olmos E, Kunert KJ, Foyer CH (2008) Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. J Exp Bot **59:** 1935-1950

Pujol C, Marechal-Drouard L, Duchene AM (2007) How can organellar protein N-terminal sequences be dual targeting signals? In silico analysis and mutagenesis approach. J Mol Biol **369:** 356-367

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF (2008) The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proc Natl Acad Sci U S A **105:** 10061-10066

Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc Natl Acad Sci U S A **99:** 8436-8441

Raskind A (2001) Calcium dependent protein phosphorylation in the chloroplast thylakoid membrane. PhD thesis. Rehovot

Reiland S, Messerli G, Baerenfaller K, Gerrits B, Endler A, Grossmann J, Gruissem W, Baginsky S (2009) Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiol **150:** 889-903

Reinbothe C, Lebedev N, Apel K, Reinbothe S (1997) Regulation of chloroplast protein import through a protochlorophyllide-responsive transit peptide. Proc Natl Acad Sci U S A **94:** 8890-8894

Renzone G, Salzano AM, Arena S, D'Ambrosio C, Scaloni A (2006) Selective ion tracing and MSn analysis of peptide digests from FSBA-treated kinases for the analysis of protein ATP-binding sites. J Proteome Res **5:** 2019-2024

Reumann S, Ma C, Lemke S, Babujee L (2004) AraPerox. A database of putative Arabidopsis proteins from plant peroxisomes. Plant Physiol **136:** 2587-2608

Richly E, Leister D (2004) An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of Arabidopsis and rice. Gene **329:** 11-16

Rintamaki E, Salonen M, Suoranta UM, Carlberg I, Andersson B, Aro EM (1997) 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. J Biol Chem **272:** 30476-30482

Rochaix JD (2007) Role of thylakoid protein kinases in photosynthetic acclimation. FEBS Lett **581:** 2768-2775

Rodionov DA, Vitreschak AG, Mironov AA, Gelfand MS (2003) Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. *In*, Vol 278, pp 41148-41159

Rudrabhatla P, Reddy MM, Rajasekharan R (2006) Genome-wide analysis and experimentation of plant serine/ threonine/tyrosine-specific protein kinases. Plant Mol Biol **60:** 293-319

Sai J, Johnson CH (2002) Dark-stimulated calcium ion fluxes in the chloroplast stroma and cytosol. Plant Cell **14:** 1279-1291

Sakamoto K, Briggs WR (2002) Cellular and subcellular localization of phototropin 1. Plant Cell **14:** 1723-1735

Sakurai T, Satou M, Akiyama K, Iida K, Seki M, Kuromori T, Ito T, Konagaya A, Toyoda T, Shinozaki K (2005) RARGE: a large-scale database of RIKEN Arabidopsis resources ranging from transcriptome to phenome. Nucleic Acids Res **33:** D647-650

Salinas P, Fuentes D, Vidal E, Jordana X, Echeverria M, Holuigue L (2006) An extensive survey of CK2 alpha and beta subunits in Arabidopsis: multiple isoforms exhibit differential subcellular localization. Plant Cell Physiol **47:** 1295-1308

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2nd edition

Samuel MA, Chaal BK, Lampard G, Green BR, Ellis BE (2008) Surviving the passage: Non-canonical stromal targeting of an Arabidopsis mitogen-activated protein kinase kinase. Plant Signal Behav **3:** 6-12

Sato N, Ishikawa M, Fujiwara M, Sonoike K (2005) Mass identification of chloroplast proteins of endosymbiont origin by phylogenetic profiling based on organism-optimized homologous protein groups. Genome Inform **16:** 56-68

Schimper AFW (1885) "Untersuchungen über die Chlorophyllkörper und die Ihnen Homologen Gebilde. Jahrbuch für wissenschaftliche Botanik**: 1-247

Schinkmann K, Blenis J (1997) Cloning and characterization of a human STE20-like protein kinase with unusual cofactor requirements. J Biol Chem **272:** 28695-28703

Schleiff E, Soll J, Kuchler M, Kuhlbrandt W, Harrer R (2003) Characterization of the translocon of the outer envelope of chloroplasts. J Cell Biol **160:** 541-551

Schliebner I, Pribil M, Zuhlke J, Dietzmann A, Leister D (2008) A Survey of Chloroplast Protein Kinases and Phosphatases in Arabidopsis thaliana. Curr Genomics **9:** 184-190

Schwacke R, Fischer K, Ketelsen B, Krupinska K, Krause K (2007) Comparative survey of plastid and mitochondrial targeting properties of transcription factors in Arabidopsis and rice. Mol Genet Genomics **277:** 631-646

Schwacke R, Schneider A, van der Graaff E, Fischer K, Catoni E, Desimone M, Frommer WB, Flugge UI, Kunze R (2003) ARAMEMNON, a novel database for Arabidopsis integral membrane proteins. Plant Physiol **131:** 16-26

Scott MS, Calafell SJ, Thomas DY, Hallett MT (2005) Refining protein subcellular localization. PLoS Comput Biol **1:** e66

Second TP, Blethrow JD, Schwartz JC, Merrihew GE, MacCoss MJ, Swaney DL, Russell JD, Coon JJ, Zabrouskov V (2009) Dual-pressure linear ion trap mass spectrometer improving the analysis of complex protein mixtures. Anal Chem **81:** 7757-7765

Seger R, Krebs EG (1995) The MAPK signaling cascade. Faseb J **9:** 726-735

Seki M, Carninci P, Nishiyama Y, Hayashizaki Y, Shinozaki K (1998) High-efficiency cloning of Arabidopsis full-length cDNA by biotinylated CAP trapper. Plant J **15:** 707-720

Seki M, Narusaka M, Kamiya A, Ishida J, Satou M, Sakurai T, Nakajima M, Enju A, Akiyama K, Oono Y, Muramatsu M, Hayashizaki Y, Kawai J, Carninci P, Itoh M, Ishii Y, Arakawa T, Shibata K, Shinagawa A, Shinozaki K (2002) Functional annotation of a full-length Arabidopsis cDNA collection. Science **296:** 141-145

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol **22:** 1567-1572

Shen Y, Kim JI, Song PS (2006) Autophosphorylation of Arabidopsis nucleoside diphosphate kinase 2 occurs only on its active histidine residue. Biochemistry **45:** 1946-1949

Smirnoff N (2000) Ascorbate biosynthesis and function in photoprotection. Philos Trans R Soc Lond B Biol Sci **355:** 1455-1464

Smith JA, Francis SH, Corbin JD (1993) Autophosphorylation: a salient feature of protein kinases. Mol Cell Biochem **127-128:** 51-70

Snyders S, Kohorn BD (1999) TAKs, thylakoid membrane protein kinases associated with energy transduction. J Biol Chem **274:** 9137-9140

Snyders S, Kohorn BD (2001) Disruption of thylakoid-associated kinase 1 leads to alteration of light harvesting in Arabidopsis. J Biol Chem **276:** 32169-32176

Soll J (1988) Purification and Characterization of a Chloroplast Outer-Envelope-Bound, ATP-Dependent Protein Kinase. Plant Physiol **87:** 898-903

Soll J, Fischer I, Keegstra K (1988) A guanosine 5'-triphosphate-dependent protein kinase is localized in the outer envelope membrane of pea chloroplasts. Planta **176:** 488-496

Soll J, Schleiff E (2004) Protein import into chloroplasts. Nat Rev Mol Cell Biol **5:** 198-208

Sonnhammer EL, von Heijne G, Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol **6:** 175-182

Sorek N, Bloch D, Yalovsky S (2009) Protein lipid modifications in signaling and subcellular targeting. Curr Opin Plant Biol **12:** 714-720

Stone JM, Walker JC (1995) Plant protein kinase families and signal transduction. Plant Physiol **108:** 451-457

Subramani S (1996) Protein translocation into peroxisomes. J Biol Chem **271:** 32483-32486

Sugiyama N, Nakagami H, Mochida K, Daudi A, Tomita M, Shirasu K, Ishihama Y (2008) Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in Arabidopsis. Mol Syst Biol **4:** 193

Sun Q, Zybailov B, Majeran W, Friso G, Olinares PD, van Wijk KJ (2008) PPDB, the Plant Proteomics Database at Cornell. Nucleic Acids Res

Sutherland EW, Jr., Wosilait WD (1955) Inactivation and activation of liver phosphorylase. Nature **175:** 169-170

Swarbreck D, Wilks C, Lamesch P, Berardini TZ, Garcia-Hernandez M, Foerster H, Li D, Meyer T, Muller R, Ploetz L, Radenbaugh A, Singh S, Swing V, Tissier C, Zhang P, Huala E (2008) The Arabidopsis Information Resource (TAIR): gene structure and function annotation. Nucleic Acids Res **36:** D1009-1014

Taniguchi H (1999) Protein myristoylation in protein-lipid and protein-protein interactions. Biophys Chem **82:** 129-137

Teige M, Melzer M, Suss KH (1998) Purification, properties and in situ localization of the amphibolic enzymes D-ribulose 5-phosphate 3-epimerase and transketolase from spinach chloroplasts. Eur J Biochem **252:** 237-244

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J **37:** 914-939

Towler DA, Gordon JI, Adams SP, Glaser L (1988) The biology and enzymology of eukaryotic protein acylation. Annu Rev Biochem **57:** 69-99

Trojanek JB, Klimecka MM, Fraser A, Dobrowolska G, Muszynska G (2004) Characterization of dual specificity protein kinase from maize seedlings. Acta Biochim Pol **51:** 635-647

Tusnady GE, Simon I (1998) Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J Mol Biol **283:** 489-506

Vener AV, Ohad I, Andersson B (1998) Protein phosphorylation and redox sensing in chloroplast thylakoids. Curr Opin Plant Biol **1:** 217-223

Vidi PA, Kanwischer M, Baginsky S, Austin JR, Csucs G, Dormann P, Kessler F, Brehelin C (2006) Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. J Biol Chem **281:** 11225-11234

Villarejo A, Buren S, Larsson S, Dejardin A, Monne M, Rudhe C, Karlsson J, Jansson S, Lerouge P, Rolland N, von Heijne G, Grebe M, Bako L, Samuelsson G (2005) Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. Nat Cell Biol **7:** 1224-1231

Voll L, Hausler RE, Hecker R, Weber A, Weissenbock G, Fiene G, Waffenschmidt S, Flugge UI (2003) The phenotype of the Arabidopsis cue1 mutant is not simply caused by a general restriction of the shikimate pathway. Plant J **36:** 301-317

Wahlroos T, Soukka J, Denesyuk A, Wahlroos R, Korpela T, Kilby NJ (2003) Oleosin expression and trafficking during oil body biogenesis in tobacco leaf cells. Genesis **35:** 125-132

Wakasugi T, Tsudzuki T, Sugiura M (2001) The genomics of land plant chloroplasts: Gene content and alteration of genomic information by RNA editing. Photosynth Res **70:** 107-118

Walter P, Blobel G (1983) Signal recognition particle: a ribonucleoprotein required for cotranslational translocation of proteins, isolation and properties. Methods Enzymol **96:** 682-691

Wang D, Harper JF, Gribskov M (2003) Systematic trans-genomic comparison of protein kinases between Arabidopsis and Saccharomyces cerevisiae. Plant Physiol **132:** 2152-2165

Weber CN (2006) Molekulare Determinanten für die Palmitoylierung integraler Membranproteine. PhD thesis. Freie Universität Berlin, Berlin

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot **92:** 487-511

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One **2:** e718

Wise RR, Hoober JK (2007) The Structure and Function of Plastids, Vol 23. Springer

Wissing J, Jansch L, Nimtz M, Dieterich G, Hornberger R, Keri G, Wehland J, Daub H (2007) Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. Mol Cell Proteomics **6:** 537-547

Wolters DA, Washburn MP, Yates JR, 3rd (2001) An automated multidimensional protein identification technology for shotgun proteomics. Anal Chem **73:** 5683-5690

Yalovsky S, Rodr Guez-Concepcion M, Gruissem W (1999) Lipid modifications of proteins - slipping in and out of membranes. Trends Plant Sci **4:** 439-445

Yang SD, Fong YL, Yu JS, Liu JS (1987) Identification and characterization of a phosphorylation-activated, cyclic AMP and Ca2+-independent protein kinase in the brain. J Biol Chem **262:** 7034-7040

Ytterberg AJ, Peltier JB, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiol **140:** 984-997

Zhang FL, Casey PJ (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem **65:** 241-269

Zhang X, Zhao F, Guan X, Yang Y, Liang C, Qin S (2007) Genome-wide survey of putative serine/threonine protein kinases in cyanobacteria. BMC Genomics **8:** 395

Zhang XP, Glaser E (2002) Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone. Trends Plant Sci **7:** 14-21

Zhang Y, Rodionov DA, Gelfand MS, Gladyshev VN (2009) Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. BMC Genomics **10:** 78

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

6. APPENDIX

6.1. Cloned genes and used primers

gene name	AGI code		primer sequence
A	AT4G25000	fw.:	5'-TAATGGGCCCATGACATCTCTCCATACGTTAC-3'
		rev.:	5'-CATAGCGGCCGCACTTCTTCTCCCAGACAGCAAAGTC-3'
ABC1	AT1G71810	fw.:	5'-GGGCCCATGCTTCGGCTTCCTTCTTCT-3'
		rev.:	5'-GCGGCCGCAAAGAAAAATCCTGCGAAT-3'
ABC2	AT1G79600	fw.:	5'-GGGCCCATGAGTCTGGTGGTTGGTCAGTCT-3'
		rev.:	5'-GCGGCCGCATGGGGATGGTGCAGAAGAT-3'
ABC4	AT4G31390	fw.:	5'-GGGCCCATGGAGTCAATCCACTGCAATAGT-3'
		rev.:	5'-GCGGCCGCCTCTGTCGGATAATACTGAGTTGC-3'
AGUK	AT3G18680	fw.:	5'-GGGCCCATGGCAATTCCGTTGCCTCTTACC-3'
		rev.:	5'-GCGGCCGCATGAGGTTGTTGTAACAATGGAGTTCC-3'
AtDSK1	AT3G13690	fw.:	5'-TAATGGGCCCATGAGTCGACTACAGAAGCGAGGG-3'
		rev.:	5'-CATAGCGGCCGCAGTATTGCTTGTTATGGTTTAGTTCAAACC-3'
ATF	AT1G77670	fw.:	5'-AAGGGCCCATGTACCTGGACATAAATGGTG-3'
		rev.:	5'-TTGCGGCCGCCGATTTTTCTCTTAAGCTTCTG-3'
AtMSK4	AT1G09840	fw.:	5'-GGGCCCATGGCATCCTCTGGACTGGGAAATGG-3'
		rev.:	5'-GCGGCCGCACGAATGCAAAGCCATGAAGAGGT-3'
B	AT3G53180	fw.:	5'-TAATGGGCCCATGGAGTTTAGTGAGTTAAAGGAAGC-3'
		rev.:	5'-CATAGCGGCCGCTCTCAGAACCATAGAAATAACCACC-3'
C	AT1G43710	fw.:	5'-TAATGGGCCCATGGTTGGATCTTTGGAATCTG-3'
		rev.:	5'-CATAGCGGCCGCACTTGTGAGCTGGACAGATGC-3'
CAT	AT2G04620	fw.:	5'-TAATGGGCCCATGGTGGATCATCATCATCA-3'
		rev.:	5'-CATAGCGGCCGCATGAGTTAACAGATTCCACC-3'
CKII (cKin5)	AT2G23070	fw.:	5'-AGGGCCCATGGCCTTAAGGCCTTGTACTGGATTC-3'
		rev.:	5'-GCGGCCGCCCTGGCTGCGCGGCGTACGGCTGCTC-3'
cKin1	AT2G02800	fw.:	5'-AACCATGGGTAATTGCTTAGATTCATCAGC-3'
		rev.:	5'-TTGCGGCCGCGTACACGAGGAGAGTGATTGTGAG-3'
cKin10	AT1G69790	fw.:	5'-ACCATGGGGAATTGCTTGGACTC-3'
		rev.:	5'-GCGGCCGCTCGACATATGAGATGAAGGAGACATGAC-3'
cKin11	AT1G71530	fw.:	5'-AACCATGGGTTGTATTTGTGCC-3'
		rev.:	5'-GCGGCCGCTCTTTCTTGCTTCTTCGTTGCGAAGC-3'
cKin16	AT2G01210	fw.:	5'-GGGCCCATGTTGGCCTCGCTGATCATCTTCG-3'
		rev.:	5'-GCGGCCGCAATCGCCGGCCACGGGTAATCTGTCG-3'
cKin17	AT3G09010	fw.:	5'-GGGCCCATGCGTTATAATTGCTTCGGAC-3'
		rev.:	5'-GCGGCCGCATCTAGGAGCCAACTCTGTGATGC-3'
cKin18	AT5G16810	fw.:	5'-GGGCCCATGAATCTGGTCGCTATTCATCGCG-3'
		rev.:	5'-GCGGCCGCAAACTACGCCATTCAAAAATCGATGAC-3'
cKin19	AT3G04810	fw.:	5'-GGGCCCATGGAGAATTACGAGGTTCTTGAGC-3'
		rev.:	5'-GCGGCCGCAATCTCCCAGCTTAGTAGTAGTGG-3'
cKin2	AT1G53050	fw.:	5'-AAGGGCCCATGGGTTGTGTTTGTGGTAAGCCTTCTGC-3'
		rev.:	5'-TTGCGGCCGCGGCTAGAAACAGATGAGGGATGATTGG-3'
cKin20	AT1G53440	fw.:	5'-GGGCCCATGGGTTTCTTTTTCTCGACCC-3'
		rev.:	5'-GCGGCCGCGTCCAGAGAGTCGGTTCCGGTTAC-3'
cKin21	AT1G73460	fw.:	5'-GGGCCCATGACGGACCAGAGCTCTGTTG-3'
		rev.:	5'-GCGGCCGCTACTACTACTATCATCAATG-3'
cKin22	AT2G40860	fw.:	5'-GGGCCCATGGTGATGGAAATTGTGAAACC-3'
		rev.:	5'-GCGGCCGCGCAAATTCTTCTTGTATTCAGC-3'
cKin23	AT5G25110	fw.:	5'-TAATGGGCCCATGGGATCCAAACTTAAACTTTACCC-3'

		rev.:	5'-CATAGCGGCCGCAGCAGTCACTACCAGAATTTTCATCAC-3'
cKin24	AT5G47750	fw.:	5'-TAATGGGCCCATGGCGTCCACTCGTAAACCCAGTGG-3'
		rev.:	5'-CATAGCGGCCGCAGAAGAAATCAAATTCCAAATAG-3'
cKin25	AT5G50180	fw.:	5'-TAATGGGCCCATGGATTCTTTGACTGGATTTAGAATGG-3'
		rev.:	5'-CATAGCGGCCGCAATAACATTGATTGAAGCAGAAAAAC-3'
cKin3	AT2G17220	fw.:	5'-AACCATGGGTCTTTGTTGGGGATCTCCATC-3'
		rev.:	5'-TTGCGGCCGCCGTGAGCTCGAGACAGCTTTTGTCTAGG-3'
cKin4	AT1G14370	fw.:	5'-AAGGGCCCATGGGTAATTGTTTAGATTCATCAGC-3'
		rev.:	5'-TTGCGGCCGCCTCTTACACGAGGAGATTGAGTGTAAGAAGG-3'
cKin6	AT4G35600	fw.:	5'-ATCCATGGGTGCTTGTATTTCGTTC-3'
		rev.:	5'-AAGCGGCCGCATTTTTCTACTGATCCAAACCGTCC-3'
cKin7	AT1G26970	fw.:	5'-AACCATGGGAAATTGCTTTGGG-3'
		rev.:	5'-GCGGCCGCGAACACGTCGACATCGTCTAC-3'
cKin9	AT1G72540	fw.:	5'-ACCATGGGATTTTCTTGGAAGAATATATGTCTTC-3'
		rev.:	5'-GCGGCCGCTGGCTGGATTATATAAACTGGTTCC-3'
CPK16	AT2G17890	fw.:	5'-TTGGATCCGGGCCCATGGGTCTCTGTTTCTCCTCCGCCGCC-3'
		rev.:	5'-TTGCGGCCGCCCTTGCGAGAAATAAGAT-3'
CPK18G2A	AT4G36070	fw.:	5'-TTGGGCCCATGGCTCTCTGTTTCTCGTCT-3'
		rev.:	5'-GCGGCCGCCCATCTTTTGTGAAAGCTGGT-3'
CPK3	AT4G23650	fw.:	5'-GGGCCCATGGGCCACAGACACAGCAAGTCCAAATCCTCCG-3'
		rev.:	5'-GCGGCCGCACATTCTGCGTCGGTTTGGCACCAATTCTGGATTTCCC-3'
D	AT1G55880	fw.:	5'-TAATGGGCCCATGGCGCCTGTTAATATGACTGGCGC-3'
		rev.:	5'-CATAGCGGCCGCCCGCTTCTTTATCAGTCCCACG-3'
E	AT4G02610	fw.:	5'-TAATGGGCCCATGGATCTTCTCAAGACTCCTTCC-3'
		rev.:	5'-CATAGCGGCCGCAAGAGACAAGAGCAGACTTCAAAG-3'
F	AT5G21060	fw.:	5'-TAATGGGCCCATGAAGAAGATCCCGGTGCTTCTC-3'
		rev.:	5'-CATAGCGGCCGCAGTGAAAAAGATCCTGCAAGTCG-3'
FAK	AT3G61080	fw.:	5'-GGGCCCATGGCGGTGGCTTCTCTTAGTAT-3'
		rev.:	5'-GCGGCCGCAAGCTTTGAGCATCCGTAGAT-3'
FNR	AT5G66190	fw.:	5'-CCATGGCTGCTGCTATAAGTGCTGC-3'
		rev.:	5'-GCGGCCGCAGACTTCAACATTCCACTGTTCACTCC-3'
G	AT5G57040	fw.:	5'-TAATGGGCCCATGGCTTCTATTTTCAGACCTTC-3'
		rev.:	5'-CATAGCGGCCGCAAACTTGGGTGAACTCAAGAGCG-3'
GDH	AT4G33670	fw.:	5'-AAGGGCCCATGACGAAAATAGAGCTTCGAGC-3'
		rev.:	5'-TTGCGGCCGCTCTGATGGATTCCACTTGGCC-3'
HAC	AT2G25870	fw.:	5'-AAGGGCCCATGCTCTCTCGTGTCTGCCC-3'
		rev.:	5'-TTGCGGCCGCCGAATGCGTAGCGGTAGATGGC-3'
HSK	AT2G17265	fw.:	5'-GGGCCCATGGCAAGTCTTTGTTTCCA-3'
		rev.:	5'-GCGGCCGCATCTGGAGACGCTGTTGACAAG-3'
MPP	AT1G06900	fw.:	5'-AAGGGCCCATGTCTTCAATGAAATCCGTCTCG-3'
		rev.:	5'-CATAGCGGCCGCCCTTGTGCCTCCGGAGGATCC-3'
NADK1	AT3G21070	fw.:	5'-GGGCCCATGTCGTCGACCTACAAGCTC-3'
		rev.:	5'-GCGGCCGCAAGGTCCATCAGCAGATTGAGTCTTTC-3'
NDPK2	AT5G63310	fw.:	5'-GGGCCCATGGTGGGAGCGACTGTAGTTAG-3'
		rev.:	5'-GCGGCCGCACTCCCTTAGCCATGTAGCTAG-3'
OTL	AT3G57810	fw.:	5'-AAGGGCCCATGATGATTTGTTACTCTCCAATT-3'
		rev.:	5'-TTGCGGCCGCCTTTAGATTTTGGAATCGAAGC-3'
PAP	AT1G06190	fw.:	5'-TAATGGGCCCATGGCGATGTCGGGAACTTTCCAT-3'
		rev.:	5'-CATAGCGGCCGCAGCTGGAATCACTACCAAGC-3'
PGL	AT5G22620	fw.:	5'-AAGGGCCCATGATTTCTCTACCACTCACTACAC-3'
		rev.:	5'-ATGCGGCCGCTGGCGGGTTTTGTGATAGG-3'
PHR2	AT2G47590	fw.:	5'-GGGCCCATGGATTCTTCGAATGTTGAAG-3'

		rev.:	5'-GCGGCCGCAAGCAAAGGCACCGGTACAGGC-3'
PlDPK	AT3G10540	fw.:	5'-GGGCCCATGTTGACAATGGACAAGG-3'
		rev.:	5'-GCGGCCGCAACGGTTTTGAAGAGTTTCG-3'
PIF	AT1G15730	fw.:	5'-AAGGGCCCATGGCGACGCTGTTGAAACTCG-3'
		rev.:	5'-TTGCGGCCGCTCAAGCAAGCTCTGAAACCC-3'
plpKin2	AT3G44610	fw.:	5'-GGGCCCATGGAGCCATGGATTGACGAAT-3'
		rev.:	5'-GCGGCCGCAATAATAGTCGACATGGGTTTCCGG-3'
PP1G2A	AT3G02750	fw.:	5'-GGGCCCATGGCGTCCTGTTTATCTGCAGAGAGC-3'
		rev.:	5'-GCGGCCGCACTTTCCAGGCACAAATCTTGGTAAG-3'
PPR	AT3G49140	fw.:	5'-AAGGGCCCATGAAACGCATCAATGTCGATCTCC-3'
		rev.:	5'-TTGCGGCCGCAAGAACACGCCTTTAGAACACACG-3'
RUBA	AT2G39730	fw.:	5'-CCATGGCCGCCGCAGTTTCCACCG-3'
		rev.:	5'-GCGGCCGCAAAAGTTGTAGACACAGGTTCCATCG-3'
UMP	AT3G54470	fw.:	5'-AAGGGCCCATGTCAGCCATGGAAGCACTG-3'
		rev.:	5'-TTGCGGCCGCTCTGAGAGCATTTCTCCAAGTATGC-3'
WKin1	AT4G23160	fw.:	5'-GGGCCCATGGTATCTGATGCAGATGC-3'
		rev.:	5'-GCGGCCGCAGCGCGGATATAAATCAG-3'
WKin2	AT3G51990	fw.:	5'-GGGCCCATGGGTTATCTCTCCTGCAAAGC-3'
		rev.:	5'-GCGGCCGCATTCCACGGGTCGACCACCC-3'
WKin3	AT4G10730	fw.:	5'-GGGCCCATGGTGTCTCGGTTTCGTCTTGC-3'
		rev.:	5'-GCGGCCGCACAATTGCTCGCGACCGG-3'
YFP-KDEL	-	fw.:	5'-CTCGCAAGGGCGGCCGCATGG-3'
		rev.:	5'-GCCCTGTACACTACAGCTCGTCCTTGCCGAGAGTG-3'

6.2. Primers for T-DNA insertion line genotyping

primer name	primer sequence
ACT3-3'	5'-AGCACAATACCGGTAGTACG-3'
ACT3-5'	5'-ATGGTTAAGGCTGGTTTTGC-3'
c18Nt	5'-GGGCCCATGAATCTGGTCGCTATTCATCGCG-3'
c18RTrev	5'-GGCGTTCCTCGAAATGGACCC-3'
HSF3RTfw	5'-CGTCGTCTGGAGTGCCCCGG-3'
HSF3RTrev	5'-GCCTTTGCTCCATCACCTG-3'
LB	5'-CGCTGGACCGCTTGCTGCAACT-3'

6.3. Abbreviations

ATP	Adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmatic reticulum
FNR	ferredoxin-NADP+ reductase
FSBA	fluorosulfonylbenzoyladenosine
GFP/YFP	green/yellow fluorescent protein

GST	glutathione S-transferase
HK	histidine kinase
HY	hybrid kinase
kDa	kilodalton
LC	liquid chromatography
LL	Lacroute library
LTQ	linear trap quadrupole
MBP	myelin basic protein
MS	mass spectrometry
NLS	nuclear localization signal
o/n	overnight
PurB	Purvalanol B
RR	response regulator
RT	room temperature
Ruba	Rubisco activase
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
S200	Superdex 200 column
S75	Superdex 75 column
SPP	stromal processing peptidase
SRP	signal recognition particle
STK	serine/threonine-specific protein kinase
TFA	trifluoroacetic acid

7. CURRICULUM VITAE

personal information

name   Roman Gerhard Bayer

date of birth   05.11.1981

place of birth   Vienna, Austria

education

09/1988 – 06/1992   primary school Odoakergasse, Vienna

09/1992 – 05/2000   grammar school BG/BRG Maroltingergasse, Vienna

10/2001 – 06/2006   studies of biology with emphasis on genetics and microbiology at the University of Vienna

03/2005 – 06/2006   diploma thesis „Identification of chloroplast targeted protein kinases in *Arabidopsis thaliana*" at the department of biochemistry under supervision of Dr. Markus Teige

07/2006 – 07/2010   doctoral thesis at the department of biochemistry under supervision of Dr. Markus Teige

24.-29.03.2007   FEBS Advanced Lecture Course „Origin and Evolution of Mitochondria and Chloroplasts"

05.-07.08.2009   Workshop and Symposium „Proteomic, Redox, and Metabolomic Networks"

professional activities

2007 - 2010   tutorship and lectureship for the student practical course „Hefe als Modellorganismus für die Signaltransduktion in eukaryotischen Zellen"

8. LIST OF PUBLICATIONS

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

2) **Bayer R. G.**, Stael S., Csaszar E., Teige M. (2010). Mining the chloroplast proteome by affinity chromatography. **PROTEOMICS (in preparation)**.