A serine/threonine protein kinase that is able to phosphorylate chloroplast-destined precursor proteins was purified from leaf extract of *Arabidopsis thaliana* and was identified by mass spectrometry. The protein kinase, encoded by *AT2G17700*, belongs to a small protein family comprising in addition *AT4G35780* and *AT4G38470*. All three proteins were expressed heterologously in *Escherichia coli* and characterized with regard to their properties in precursor protein phosphorylation. They were able to phosphorylate several chloroplast-destined precursor proteins within their cleavable presequences. In contrast, a mitochondria-destined precursor protein was not a substrate for these kinases. For all three enzymes, the phosphorylation reaction was specific for ATP with apparent *Km* values between 14 and 67 μM. They did not utilize other NTPs nor were those able to compete for ATP in the reaction. An excess of ADP was able to inhibit ATP-dependent phosphorylation. Furthermore, all three kinases exhibited autophosphorylation. The protein kinases described here could represent subunits of a regulatory network involved in the cytosolic events of chloroplast protein import.

The biogenesis and differentiation of chloroplasts as well as their function require a vast number of nuclear-encoded proteins. Chloroplasts must therefore import about 3000 different proteins from the cytosol in a posttranslational process (1). Most but not all precursor proteins use a standard import pathway, which requires an N-terminal, cleavable presequence and involves the Toc complex (translocon at the outer envelope of chloroplasts) and the Tic complex (translocon at the inner envelope of chloroplasts) at the chloroplast outer and inner envelope membranes, respectively (2).

Translocation of precursor proteins across the envelope membrane is a highly regulated process including GTP regulation by two prominent GTPases, Toc34 and Toc159, at the state of precursor recognition and translocation initiation (3). At the level of the Tic translocon several subunits exhibit redox properties (4–6), and therefore the import route of certain precursor proteins might depend on the redox status of the organelle as conveyed to the translocon by the ratio of NADPH/NADP in the stroma. Recently, calcium regulation of the translocation process has been described (7).

Very little is known about the events that occur between translation of precursor proteins in the cytosol and their arrival at the organellar surface. Once emerged from the ribosome, they must maintain their import competence until they have been transported to their final compartment within the eukaryotic cell (8, 9). Molecular chaperones of the Hsp70 and Hsp90 family have been shown to bind to precursor proteins to maintain an unfolded state compatible with translocation (10–14). This rather ubiquitous mechanism appears to be complemented, at least in plants, by a more specific precursor protein interaction with distinct soluble factors. It has been demonstrated that chloroplast precursor proteins can be phosphorylated by a serine/threonine-specific protein kinase, which is found in the cytosol of pea leaf mesophyll cells or in a translationally active wheat germ lysate (15, 16). The protein kinase activity is only detected in plant extracts but not in mammalian systems like reticulocyte lysate (15). Furthermore, mitochondrial precursor proteins were not accepted as substrates by the plant kinase, although they contained similar numbers of serine/threonine residues within their presequences. Phosphorylation of the chloroplast precursor proteins resulted in the binding of a cytosolic guidance complex, consisting of at least a 14-3-3 dimer and Hsp70 (15). Although phosphorylation of precursor proteins and their association with the guidance complex did not affect targeting specificity; it clearly influenced the rate of translocation. A guidance complex-associated precursor of the stroma localized small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU) imported with a 4–5-fold higher rate compared with its free form (15). These differences in import kinetics are most likely because of the fact that the primary import receptor Toc34 has a higher affinity for the phosphorylated presequence than for its nonphosphorylated form (17), thus discriminating against the latter. Although phosphorylation is not essential for targeting and translocation, it might be an important regulatory mechanism under certain developmental or environmental conditions that require the preferential import of one precursor protein over the other.

The nature of the protein kinase that phosphorylates chloroplast precursor proteins, however, has so far remained elusive. We have therefore undertaken a biochemical approach to purify this kinase from the model plant *Arabidopsis thaliana*. A serine/threonine-specific protein kinase was enriched several thousand-fold and identified by mass spectrometry. The substrate requirements of the kinase suggest its specific action in chloroplast precursor phosphorylation. Further analysis...

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

1 To whom correspondence should be addressed: Dept. Biology I, Botanik, Menzinger Str. 67, D-80638 München, Germany. Tel.: 49-89-17861-245; Fax: 49-89-17861-185; E-mail: soll@lmu.de.
revealed that the protein was part of a small family, which might regulate chloroplast import at a cytosolic stage.

**EXPERIMENTAL PROCEDURES**

**Materials**—[³²P]ATP (3000 Ci/mmol) was obtained from Amersham Biosciences. All other chemicals were p.a. grade or of the highest purity available.

**Purification of a Cytosolic Precursor-phosphorylating Protein Kinase**—A cytosol-enriched protein fraction was isolated from 8-week-old leaves of *A. thaliana* (ecotype Columbia). Unless otherwise mentioned, all procedures were performed at 4 °C. Leaves were ground in a Waring blender in PS-A buffer (20 mM Tris/HCl, pH 7.5, 10 mM β-mercaptoethanol), and the extract was filtered through four layers of gauze and layers of Swiss cloth. The final leaf extract was obtained by two subsequent centrifugations for 15 min at 48,000 × g and 30 min at 150,000 × g, respectively.

For hydrophobic interaction chromatography (HIC) on Phenyl-Sepharose 6 Fast Flow HR 10/10 (GE Healthcare), the cytosol-enriched protein fraction was adjusted to 1 M NaCl in PS-A buffer. The column was pre-equilibrated in the same buffer. Chromatography was performed at a flow rate of 2 ml/min. Unbound protein was washed off the column with the equilibration buffer. Bound proteins were eluted by a linear gradient from 1 M NaCl to NaCl-free buffer over five-column volumes. The eluate was collected in 5-mI fractions and immediately assayed for kinase activity. Fractions with the highest enzyme activity were pooled and diluted 1:8 in MQ-A buffer (20 mM Tris/HCl, pH 8, 10 mM β-mercaptoethanol) to reach a final salt concentration under 50 mM NaCl.

Anion exchange chromatography was performed on a Mono Q HR 5/5 (GE Healthcare) equilibrated in buffer MQ-A with a flow rate of 1 ml/min. After loading of the column, the unbound protein was washed off with buffer MQ-A, and bound protein was eluted with a linear gradient from 0 to 0.5 M NaCl in MQ-A buffer over two-column volumes. The eluate was collected in 0.5-mI fractions and immediately assayed for enzyme activity.

The fractions with the highest enzyme activity were purified separately by size exclusion chromatography on Superose 6 10/300 GL (GE Healthcare) using SR6 buffer (50 mM NaCl, 20 mM Tris/HCl, pH 7.5, 10 mM β-mercaptoethanol) and a flow rate of 0.4 ml/min. 0.25 ml of fractions were collected and immediately assayed for enzyme activity. The fraction with the highest activity was pooled from all runs and used for SDS-PAGE analysis.

**Identification of the Purified Precursor Protein Kinase**—Proteins from the size exclusion chromatography purification were separated on SDS-PAGE and analyzed by staining with colloidal Coomassie. Protein bands, 50–65 kDa, were cut out of the gel and used for protein sequencing by mass spectrometry.

**Transient Expression in Tobacco Protoplasts**—The coding sequence of *AT2G17700* was cloned into the pOL-LP vector, N-terminal to the coding sequence for GFP, thereby creating the plasmids pAT2G17700-GFP (18). For the transient transformation seedlings of *Nicotiana tabacum* cv., petite Havana were germinated on B5-modified medium (19), and leaves of 3–4-week-old plants were used for protoplast isolation and transformation as described previously (20).

**In Vivo Expression of Various Constructs**—pLHCP2 (precursor of light harvesting complex protein 2), pCAO (precursor of chlorophyllide a oxygenase), pAPC1 (precursor of the ATP synthase γ-subunit of the plastidoplast), and pHCF136 (precursor of high chlorophyll fluorescence protein 136) were constructed by fusion of their transit peptides to mSSU. In all other cases, the full-length gene including presequence was utilized. For phosphorylation assays, all substrate proteins were expressed and purified as described previously (16).

*AT2G17700, AT4G35780,* and *AT4G38470* were cloned by PCR. *AT2G17700* and *AT4G38470* were amplified from cDNA isolated from *Arabidopsis* rosette leaves by the plant RNeasy kit (Qiagen, Hilden, Germany) and SuperScript III reverse transcriptase (Invitrogen). *AT4G35780* was amplified from the expressed sequence tag clone RAFL11-12-O20 (RIKEN Bioresource Center). The PCR products were cloned directly into pCRHIs2 TOPO (Invitrogen) in frame with a C-terminal His₆ tag, and all constructs were verified by DNA sequencing.

Heterologous expression of *AT2G17700, AT4G35780,* and *AT4G38470* was obtained in *E. coli* BL21(DE3) cell using 1 mM isopropyl 1-thio-β-D-galactopyranoside for 2.5 h at 37 °C. For purification of the proteins, the cells were harvested by centrifugation at 4000 × g and resuspended in 20 mM Tris/HCl, pH 7.5. The cells were ruptured by passage through a French pressure cell at 1200 psi. After removal of the cell debris by centrifugation at 12,000 × g, the supernatant was used for nickel-nitrilotriacetic acid affinity purification (Qiagen) following the manufacturer’s instructions. After the purification, the protein concentrations were determined and equalized for all preparations. The α-*AT2G17700* antiserum was prepared in rabbit using purified heterologously expressed *AT2G17700*-His₆.

**Precursor Protein Phosphorylation Assay**—*In vitro* phosphorylation of proteins was performed as described in Ref. 16. A standard phosphorylation (50 μl) was performed in 20 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM MnCl₂ with 2.5 μM ATP plus 2–5 μCi of [³²P]ATP (3000 Ci/mmol). Each assay contained 1–3 μg of heterologously expressed and purified substrate protein, which were dissolves in 8 μl urea prior to the experiment. The final urea concentration in the phosphorylation assay did not exceed 150 mM. The reaction was started by the addition of either protein from different column fractions or of the recombinant, nickel-nitrilotriacetic purified proteins. If not otherwise mentioned, the reaction was carried out for 10 min at 20 °C. The reactions were stopped by the addition of 12 μl of 4× SDS-gel buffer (21).

All phosphorylation assays were analyzed by SDS-gel electrophoresis and phosphorimaging on a Fuji film FLA-3000.
Quantification of the radioactivity signals was performed using the software included in the AIDA package version 3.52.046. For the antibody depletion assay either α-AT2G17700 or pre-immune serum was added to freshly prepared leaf extract in a 1:200 dilution and incubated for 2 h at 4 °C before being used in a standard phosphorylation assay.

RESULTS
Purification of a Precursor Protein Kinase from Arabidopsis Leaves—A soluble protein extract was obtained from 8-week-old Arabidopsis leaves. After clearing the extract by ultracentrifugation, it was passed over a HIC matrix in the presence of 1 M NaCl. Bound proteins were eluted from the matrix by a decreasing linear NaCl gradient (Fig. 1A). Although most proteins did not bind to the HIC matrix under these conditions, the flow-through did not contain any precursor-specific protein kinase activity (data not shown). All precursor protein kinase activity was detected in the eluate at a salt concentration of ~150 mM NaCl (Fig. 1A, upper panel). Active kinase fractions were detected by their capacity to phosphorylate pSSU (Fig. 1A, lower panel) and were pooled for further purification. The HIC resulted in a 50-fold enrichment of specific kinase activity compared with leaf extract (Table 1). The pooled enzyme fractions from the HIC were diluted 8-fold to lower the NaCl concentrations before they were passed over a Mono Q anion exchange matrix (Fig. 1B). Bound proteins were eluted by an increasing NaCl gradient. The precursor protein kinase eluted from the matrix at a NaCl concentration of ~200 mM in a peak far removed from the bulk of proteins (Fig. 1B), resulting in a 460-fold enrichment of specific activity (Table 1). The active enzyme fractions were further fractionated by size exclusion chromatography on Superose 6 (Fig. 1C). The kinase eluted in the shoulder of the major peak, resulting in ~3000-fold purification factor over the initial leaf extract (Table 1).

When we analyzed the most active kinase fraction by SDS-PAGE and silver staining, it still contained a number of different proteins in the range expected for the precursor protein kinase (Fig. 2A). Earlier studies had indicated that the kinase from pea as well as wheat germ has a molecular mass slightly above 50 kDa (15, 16). Therefore polypeptides were fractionated by SDS-PAGE, and the gel was cut into multiple pieces covering the molecular mass range from 50 to 65 kDa. Polypeptides present in each slice were identified by tandem mass spectrometry. This approach resulted in a peptide fingerprint with seven distinct peptides matching the deduced amino acid sequence of AT2G17700 from Arabidopsis, a protein that had been tentatively identified as a serine/threonine/tyrosine-specific kinase in the databases (Fig. 2B). The peptides were identified in the slice corresponding to about 60 kDa, which correlated well to the estimated size of the AT2G17700 gene product (Mₚ, 61,509.26).

Arabidopsis Encodes a Small Precursor Protein Kinase Family—Blast searches against the complete Arabidopsis genome revealed the presence of two more genes, namely AT4G35780 and AT4G38470, that encoded proteins with a very high identity to AT2G17700 at the primary amino acid sequence level (Fig. 2C). Domain analysis clearly identified all three proteins as dual-specificity protein kinases (Sty kinases) containing the typical motifs found in this protein family (22). A number of other proteins possess similarity to AT2G117700 within the C-terminal kinase domain but only AT4G35780 and AT4G38470 showed high similarity also within the N-terminal part of the protein sequence. Furthermore, a bioinformatic analysis of protein kinases by Rajasekharan and co-workers (22) placed AT4G35780 and AT4G38470 into a separate subfamily.
together with AT2G17700. Thus it appears that these three genes represent a small gene family within *Arabidopsis*. Even though the overall identity between these three proteins is very high, all except one of the peptides identified by mass spectrometry matched exclusively to AT2G17700. Only a single peptide could be matched to AT4G38470 as well as AT2G17700. All Three Precursor Protein Kinases Have Similar Enzymatic Properties for pSSU Phosphorylation—To perform a biochemical characterization of AT2G17700 and a comparison of its properties with AT4G35780 and AT4G38470, we expressed all three proteins heterologously in *E. coli*. The corresponding cDNA was obtained by PCR either from mRNA or from an expressed sequence tag clone (AT4G35780). The genes were cloned into the expression vector pDest17 allowing expression via a T7 promotor as well as adding a C-terminal His tag for purification. For all three constructs, soluble expression of the protein was obtained. They were subsequently purified to apparent homogeneity by immobilized metal affinity chromatography (supplemental Fig. 1 and Fig. 4A). The purified enzymes retained their activity for several days at 4 °C but were inactivated by freezing and thawing.

In general, nonspecific model substrates like casein, phosvitin, histone, or myelene basic protein are used to characterize the biochemical properties of protein kinases (23). All three heterologously expressed kinases could utilize these substrates as do other serine/threonine kinases from plants or mammalian systems (data not shown). Nevertheless, to study the
enzymatic properties in more detail we used pSSU as a substrate. Equal amounts of freshly purified enzymes were used in each assay. All three proteins phosphorylated pSSU in a time-dependent manner (Fig. 3A), while at the same time displaying autophosphorylation (data not shown and compare Fig. 5). Phosphorylation of pSSU required ATP, which could not be substituted for by GTP as a phosphate donor. Phosphorylation was furthermore dependent on ATP concentration, and the $S_{0.5}$ values were calculated from the Lineweaver-Burk plot. C, nucleotide specificity of pSSU phosphorylation. Kinase assays were performed in the presence of 2.5 $\mu$M ATP and competed for by increasing amounts of unlabeled ADP, GTP, CTP, and UTP as indicated.

FIGURE 3. Enzymatic properties of pSSU phosphorylation by AT2G17700, AT4G35780, and AT4G38470. A, time course of pSSU phosphorylation. B, ATP dependence of pSSU phosphorylation. Phosphorylation assays were performed with increasing amounts of ATP. $S_{0.5}$ values were calculated from the Lineweaver-Burk plot. C, nucleotide specificity of pSSU phosphorylation. Kinase assays were performed in the presence of 2.5 $\mu$M ATP and competed for by increasing amounts of unlabeled ADP, GTP, CTP, and UTP as indicated.

of precursor proteins in vivo, we raised an antiserum against heterologously expressed AT2G17700. The antibody predominantly recognized its antigen, AT2G17700, as shown by immunoblot analysis using equal amounts of heterologously expressed AT2G17700, AT4G35780, AT4G38470, and AT2G43790 indicates that $\alpha$-AT2G17700 recognizes predominantly its own antigen, whereas the other two kinases are only weakly immunodecorated by this antibody. The unrelated serine/threonine kinase AT2G43790 is not recognized at all. The upper panel shows a Coomassie staining indicating the amounts of proteins used for the immunoblot analysis shown in the lower panel. B, immunoblot analysis of different cell fractions. A single, immunoreactive band of the correct size can be observed in total leaf extract as well as in fractions containing only soluble proteins and in the cytosol fraction. C, fluorescence micrographs of protoplasts transformed with pGFP (upper panel) and pAT2G17700-GFP (lower panel). An overlay of chlorophyll fluorescence and GFP fluorescence is shown. D, soluble leaf extract of Arabidopsis was incubated with $\alpha$-AT2G17700 or pre-immune serum (PIS) prior to the phosphorylation assays. Addition of $\alpha$-At2g17700 inhibited the phosphorylation of pSSU by over 50%.

$\alpha$-AT2G17700 Antiserum Inhibits Precursor Phosphorylation in Leaf Extracts—To test whether the kinase purified from Arabidopsis leaves was indeed responsible for the phosphorylation of precursor proteins in vivo, we raised an antiserum against heterologously expressed AT2G17700. The antibody predominantly recognized its antigen, AT2G17700, as shown by immunoblot analysis using equal amounts of heterologously expressed AT2G17700, AT4G35780, AT4G38470, and AT2G43790 protein (Fig. 4A). The antiserum did not recognize an unrelated serine/threonine kinase (AT2G43790) and furthermore showed only one immunoreactive band of the right size when tested against
complete leaf extracts of *Arabidopsis* (Fig. 4A). When the antiserum was used in immunoblot analysis of different protein fractions, the protein was found to be soluble and present in the cytosol. The cytosolic localization of AT2G17700 was confirmed by transient expression of an AT2G17700-GFP fusion protein in tobacco protoplasts (Fig. 4C). The antiserum was then used to deplete the precursor protein kinase activity of a soluble leaf extract from *Arabidopsis* (Fig. 4D). Immunodepletion resulted in a more than 50% decrease of phosphorylation activity. Failure to completely inhibit pSSU phosphorylation was most likely because of the presence of the other two kinases in the leaf extract, which were not well detected by the antiserum against AT2G17700. Therefore, some enzyme activity remained in the immunodepleted extract. These results strongly support the idea that AT2G17700 indeed is a cytosol-localized precursor protein kinase of *Arabidopsis*.

**Substrate Requirements of the Heterologously Expressed Precursor Protein Kinases**—To further establish the substrate specificity of the three kinases we heterologously expressed and purified several chloroplast-destined precursor proteins, their mature forms without the presequence or mutant proteins, which contained specific amino acid exchanges at the precursor phosphorylation site (Fig. 5, A and B, top panels). A mitochondrial precursor protein (pF1β) was included into the set as a further control for phosphorylation specificity. We then tested the selectivity of the soluble leaf extract in comparison to all three heterologously expressed kinases (Fig. 5, A and B, panels 2–5). Because the leaf extract was not purified further, a number of radioactive protein bands were observed that were independent from the substrate protein and occurred also in assays without the substrate (data not shown). In case of the heterologously expressed kinases, a radioactive protein band at around 65 kDa is because of autophosphorylation of the proteins. The position of the substrate used in each assay is thus marked by an asterisk.

The leaf extract phosphorylated the transit sequence of several chloroplast precursor proteins such as pSSU, pLHCP2, pNDPK2, pAPC1, pCHPSP70B, pF1β, mSSU, pHCF136, pOE23 (precursor of the oxygen evolving protein of 23 kDa), iOE23 (OE23 without chloroplast targeting peptide but with thylakoid targeting peptide). pLHCP2, pNDPK2, and pCHPSP70 but neither the chloroplast precursor protein PCAO nor the mitochondrial precursor protein pF1β (Fig. 5, A (all lanes) and B (lanes 1, 4, and 6)). Further analysis showed that phosphorylation was confined to distinct phosphorylation sites within the transit peptide (Fig. 5B, lanes 2, 3, 5, 7, and 8). Neither the mature form of pSSU, i.e. mSSU nor iOE23, which contained only the thylakoid transfer signal but no longer the chloroplast targeting signal, were phosphorylated by the leaf extract. Phosphorylation occurs at specific residues within the transit peptide, as established before for
Chloroplast Precursor Protein Kinases in Arabidopsis

pSSU-M31/34-S/A and pOE23-M22-S/A (16). In addition, we determined the phosphorylation site in pHCF136. Exchange of serine residues at positions 52 and 54 by threonine drastically reduced (>90%) the extent of phosphorylation by leaf extract (Fig. 5A, panel 2). These results clearly demonstrate the substrate requirements of the crude leaf extract system.

In case of the heterologously expressed kinases, all three isoforms exhibited autophosphorylation capacity in the presence (Fig. 5, A and B, panels 3–5, see arrows) or absence of exogenous protein substrates (data not shown). With regard to the substrates tested, AT2G17700 showed a specificity similar to the leaf extract with the only exception that pOE23 was not phosphorylated by the purified enzyme (Fig. 4, compare panel 2 with panel 3). Because of the nature of the exchange (threonine for serine) and because pHCF136 is such a good substrate for AT2G17700, some phosphorylation (<10%) occurred also in the pHCF136-M52/54-S/T mutant in the purified in vitro system (Fig. 5B). AT4G35780 and AT4G35780 were comparable in their specificity to AT2G17700 except that pOE23 was phosphorylated (Fig. 5, compare panels 3–5) by both proteins. However, because iOE23 and pOE23-M22-S/A also became phosphorylated by these enzymes, this phosphorylation did not appear to be specific for the transit peptide but occurred instead somewhere within the mature part. In case of pSSU or pHCF136, the phosphorylation was specific to the precursor protein with no phosphorylation of the mature form or the phosphorylation site mutants.

It cannot be excluded that AT4G38470 and AT4G35780 might require a specificity factor for pOE23, which is present in the leaf extract but absent from the reconstituted in vitro system consisting solely of the kinase and its substrate. Although AT4G38470 behaved very much like AT4G35780 in its selectivity properties, the overall yield of phosphorylation was significantly lower. This is most likely because of the fact that all assays were conducted at an ATP concentration of 2.5 μM, which was close to the S0.5 determined for AT2G17700 and AT4G35780 but much below the one for AT4G38470. Taken together, our data clearly demonstrate that all three members of this kinase family can phosphorylate chloroplast precursor proteins. Further factors might be involved in the regulation of the kinase activity and its selectivity in vivo.

DISCUSSION

Chloroplast differentiation in young, rapidly expanding leaves requires the massive influx of polypeptides to build up the thylakoid membrane and establish the full photosynthetic and CO2 fixation capacity of the organelle (1). Similarly, during normal day/night cycles the maintenance of the photosynthetic redox chemistry, which is highly prone to polypeptide damage and turnover, requires a constant but strictly regulated replenishment of the organelle with nuclear-encoded proteins (25–27). Therefore, a tight regulatory network exists between chloroplasts and the nucleus to coordinate gene expression, most likely via metabolite and redox signaling (28–30). Additionally, post-transcriptional processes influence mRNA processing, stability, and hence translation efficiency. It therefore appears highly unlikely that a regulatory “no man’s land” exists between completion of translation and recognition of the protein at the organellar surface. Controlled phosphorylation of chloroplast-destined precursor proteins might be such a mechanism, working at the post-translational level. Although phosphorylation is essential neither for chloroplast targeting nor for conveying organelle (sorting) specificity within the cell (16, 31), it increases the rates of chloroplast import by several folds (15). Therefore phosphorylated precursor proteins are imported preferentially (have the right of way) over nonphosphorylated precursor proteins. This differential import kinetics are established through the import receptor Toc34, which binds phosphorylated precursor proteins with much higher affinity than nonphosphorylated precursor proteins (17).

Using a biochemical approach we were able to identify AT2G17700 as a precursor protein phosphorylating kinase of Arabidopsis. This protein was the only potential serine/threonine kinase detected in a fraction highly enriched for pSSU phosphorylation activity obtained from a cytosol-enriched leaf extract. Its deduced amino acid sequence contains all signature sequences of protein kinases, and the protein has been tentatively classified by a bioinformatical approach as one of about 60 dual Sty kinases and is termed Sty8 (22). Dual-specificity kinases contain domains characteristic for both serine/threonine as well as tyrosine kinases and are believed to possess both activities. Our characterization of the heterologously expressed and purified AT2G17700 shows that the protein is indeed a bona fide serine/threonine kinase, which phosphorylates chloroplast precursor proteins within their transit sequence. Because no phosphorylation on tyrosine residues has been detected so far for precursor proteins, our results suggest that the tyrosine protein kinase activity does not play an important role in this case. The genome of Arabidopsis contains two other protein kinases, AT4G35780 and AT4G38470, with high sequence similarity to AT2G17700. These three are the only Arabidopsis proteins sharing significant amino acid homology over the whole of their sequence. Because all the heterologously expressed proteins have serine/threonine kinase activities as well as similar substrate requirements as AT2G17700, we conclude that the three proteins represent a small family of chloroplast precursor phosphorylating kinases. This is supported by the fact that a phylogenetic analysis placed these three proteins in a small subfamily of Sty kinases.

According to array data of the AtGenExpress consortium, all three genes were ubiquitously expressed in all the tissues. Although it is most likely from the peptides obtained by mass spectrometry that we only detected AT2G17700 in our biochemical purification, the fact that the α-AT2G17700 antiserum could not completely deplete pSSU phosphorylation in leaf extracts indicates that all three proteins were present in the cytosol of mesophyll cells at the same time. Because building and refurbishing an organelle with proteins is an extremely complex process, which requires feedback and “feed forward” control circuits at all levels, having three precursor protein kinases present simultaneously would allow for differential regulation of the import process. Organellar needs for selective protein replacement, changes in metabolic conditions, or environmental adaptation and differentiation are signaled to the cytosol where they could influence either directly or indirectly...
the precursor protein kinase activity and specificity. Precursor protein phosphorylation could then serve as a process to adapt and fine-tune protein import rates to the requirement of the organelles.

Specificity as well as differential regulation could be aided by the cytosolic factors that still have to be determined. Certain differences in the phosphorylation properties between the leaf extract and the purified kinases suggest the existence of such factors. The identification of these three precursor protein kinases will allow us to identify and characterize further components of this regulatory network.

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