Analysis of the Complexity of Protein Kinases within the Phloem Sieve Tube System

CHARACTERIZATION OF CUCURBITA MAXIMA CALMODULIN-LIKE DOMAIN PROTEIN KINASE 1

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In angiosperms, functional, mature sieve elements lack nuclei, vacuoles, ribosomes, and most of the endomembrane network. In this study, the complexity, number, and nature of protein kinases within the phloem sap of Cucurbita maxima were investigated to test the hypothesis that the enucleate sieve tube system utilizes a simplified signal transduction network. Supporting evidence was obtained in that only five putative protein kinases (three calcium-independent and two calcium-dependent protein kinases) were detected within the phloem sap extracted from stem tissues. Biochemical methods were used to purify one such calcium-dependent protein kinase. The gene for this C. maxima calmodulin-like domain protein kinase 1 (CmCPK1), was cloned using peptide microsequences. A combination of mass spectrometry, peptide fingerprinting, and amino-terminal sequencing established that, in the phloem sap, CmCPK1 exists as an amino-terminally cleaved protein. A second highly homologous isoform, CmCPK2, was identified, but although transcripts could be detected in the companion cells, peptide fingerprint analysis suggested that CmCPK2 does not enter the phloem sap. Potential substrates for CmCPK1, within the phloem sap, were also detected using an on-membrane phosphorylation assay. Entry of CmCPK1 into sieve elements via plasmodesmata and the potential roles played by these phloem protein kinases are discussed.

The phloem long-distance translocation system of plants plays an important role in whole-plant development, not only as a conduit for nutrient transport but also as a conduit for the delivery of signaling molecules. Recent advances in this area have reinforced the concept that plants function as supramolecular entities that can exert an influence over plant development (6). Where they can exert an influence over plant development (6). The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY072801 and AY072802.

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EXPERIMENTAL PROCEDURES

Plant Material—C. maxima Duch. cv. Big Max (pumpkin) plants were grown as described previously (15). Stem tissue was excised from...
6-week-old pumpkin plants, and vascular bundles were stripped (using surgical forceps) and immediately frozen in liquid N₂. Proteins in these vascular bundles were extracted into buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, and protease inhibitors (Complete™; Roche Molecular Biochemicals), using routine procedures for plant proteins. Phloem sap was then mixed with an equal volume of buffer containing 100 mM Tris, pH 7.5, 10 mM EDTA, 5 mM EGTA, 1% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, and protease inhibitors (Complete™) and stored at -80 °C. All purification procedures were performed at 4 °C. Phloem sap was dialyzed against buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, and 30 mM 2-mercaptoethanol), clarified by centrifugation (15,000 g for 20 min), and then applied to a buffer A-equilibrated HiTrap Q-Sepharose column (Amersham Biosciences) connected to an FPLC system (Amersham Biosciences). CmCPK was eluted with a gradient of 0–0.5 M NaCl in buffer A. Fractions containing CmCPK were identified by Western analysis with a soybean CDPKα antibody (17), pooled, and dialyzed against buffer B (50 mM Tris, pH 7.5, and 1 mM CaCl₂). Dialyzed samples were then applied to a HiTrap Q column equilibrated with buffer B and eluted with a linear gradient of 0–0.5 M NaCl in buffer B. Purified CmCPK was dialyzed against 25 mM Tris, pH 7.5, 100 mM NaCl, and 10% (v/v) glycerol and then stored at either 4 °C or -80 °C.

**Protein Microsequencing, PCR, and cDNA Cloning of CmCPK**—Phloem-purified CmCPK was separated by SDS-PAGE and subjected to internal microsequencing. Two microsequences were obtained: VI-AGELSEELIGL and EEHLVAAP. For cloning of CmCPK, stem poly(A)⁺ RNA isolated from 4-week-old pumpkin plants was used to synthesize double-stranded cDNA (Stratagene, La Jolla, CA), which was then employed as a template for PCR. Because the two microsequences were positioned within the same region of known CDPK genes, a second sequence, GGELEFDR, located within a well-conserved domain, was chosen to design the degenerate 5’ primer, 5’-GO/TAG/AA/GT/GC/G/A/G/T/CT/G/C/G/A/G/T/CT/G/C/G/3’. A degenerate 3’ primer, 5’-AANGCNGCNACNA/G/A/G/T/CT/G/C/G/CT/C/G/C-G/C-G/3’ was designed from the microsequence EEHLVAAF. The resultant 900-bp PCR product was then used as a probe to screen a pumpkin stem cDNA library from 6-week-old pumpkin. The library was constructed in Zap Express vector using the above-described double-stranded cDNA and packaged using Gigapack (Stratagene, La Jolla, CA) and E. coli C. Two positive plaques were purified from approximately 3 x 10⁶ plaque-forming units. The cDNA inserts were rescued in pBR-CMV by in vivo excision and sequenced. Sequencing analysis revealed that the positive clones belonged to two very closely related cDNAs, CmCPK1 and CmCPK2. However, the longest clone, 59-7, encoding CmCPK1, was not full-length, whereas a full-length clone for CmCPK2 was obtained (16) and independently verified using the MS-Digest program from Protein Prospector (20). Five cold-resistant clones (CmCPK1–5) were obtained. To determine the full-length clone for CmCPK2, 59-7, encoding CmCPK1, was not full-length, whereas a very closely related cDNAs, CmCPK1 and CmCPK1–5, were adapted from Lee et al. (19).

**Peptide Mass Mapping Analysis**—Highly purified CmCPK (phloem sap) was resolved on SDS-PAGE and subjected to in-gel trysin digestion. Proteolytic peptides were then extracted and processed for matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) on a Biflex III system (Bruker, Billerica, MA). These studies were carried out in the Protein Structure Laboratory at the University of California, Davis. In some cases, high pressure liquid chromatography-fractionated CmCPK trypsic peptides were subjected to MALDI-TOF MS. In *silico* trypsin digestion of CmCPK1 and CmCPK2 followed by calculation of the mass of each peptide was performed using the MS-Digest program from Protein Prospector (20).

**Mass Determination and Amino-terminal Sequencing**—The molecular mass of phloem-purified CmCPK was determined by MALDI-TOF planar laser desorption mass microsequencing, purified CmCPK was resolved by SDS-PAGE and then transferred to a Sequi-Blot™ polyvinylidene difluoride membrane (Bio-Rad). The membrane was washed extensively with Milli Q ultra-pure water (Millipore, Bedford, MA), stained with Ponceau S (Sigma-Aldrich), and subjected to microsequencing procedures.

**Generation and Affinity Purification of CmCPK1 Antibodies**—Two regions in CmCPK1 were selected, and corresponding oligopeptides (HHLIQAEFSKEN²⁶ and R7RNSSLNMSD-GPAG,²⁷) were synthesized (United Biochemical Research, Inc., Seattle, WA) and used to raise polyclonal antibodies in rabbits (Cocalico Biologicals, Inc., Reamstown, PA). For purification of antibodies, affinity chromatography was performed with columns prepared using the oligopeptides and an Ul trafLink EDC/diaminopropylamine immobilization kit (Pierce). Cross-reactivity of the purified antibody was verified using either recombinant GST-CmCPK1, GST-CmCPK1N, or phloem-purified CmCPK.

**In Situ RT-PCR**—Six-week-old pumpkin plants were sectioned and processed for in situ RT-PCR detection of CmCPK transcripts essentially as described previously (80).

**Western Analysis**—After fractionation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes and probed with the appropriate antibody. For visualization, peroxidase-conjugated secondary antibody and chemiluminescent reagents (Renaissance, PerkinElmer Life Sciences) were employed.

**Protein Kinase Assay**—Protein kinase activity assays were performed as described by Harmon et al. (21) with the modification that the assay buffer contained 50 mM HEPES, pH 7.0, 10 mM MgCl₂, and 2 mM EGTA: 2.2 mM CaCl₂.

**Detection of Protein Kinase Autophosphorylation Activity in SDS-Polyacrylamide Gels**—A modification of the method of Kameshita and Fujisawa (22) was employed to detect protein kinase autophosphorylation in SDS-polyacrylamide gels. Proteins extracted from excised vascular bundles and phloem exudates were resolved by SDS-PAGE. In addition, phloem proteins were also FPLC-fractionated on an anion exchange column and then separated by SDS-PAGE. After removal of SDS, resolved proteins were denatured at 22 °C with 6 x guanidine-HCl in 50 mM HEPES, pH 7.5, and 5 mM 2-mercaptoethanol and subsequently renatured in 4 °C in a stepwise decreasing concentration of guanidine-HCl in the same buffer. The gel containing renatured proteins was then equilibrated with the above-described kinase assay buffer for 1 h at 22 °C. After incubation with [γ⁻³²P]ATP for 1 h, followed by extensive washing, the gel was stained with GelCode® Blue (Pierce) before being dried and exposed to x-ray film.

**Protein Kinase Activity Assay on Nitrocellulose Membrane**—A protein kinase assay was adapted from Verhey et al. (23) with the following modifications. FPLC-fractionated phloem proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane. Next, the membrane was treated with blocking/reanunaturatation buffer containing 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM dithiothreitol, 10 mM MgCl₂, and 1 mg/ml bovine serum albumin, for 16 h at 4 °C. Bound proteins were then equilibrated with the above-described protein kinase assay buffer supplemented with 0.1 mg/ml bovine serum albumin for 1 h at 22 °C. Potential sites for autophosphorylation were mapped by the addition of 20 μl ATP. The membrane was then bathed for 1 h in an aliquot (1 ml) of protein kinase assay buffer containing 0.1 mg/ml bovine serum albumin, 20 μl ATP, 20 μCi of [γ⁻³²P]ATP, and 2 μg of purified CmCPK at 22 °C. The protein kinase reaction was stopped by transferring the membrane into 100 ml of 20 mM Tris, pH 7.5, 0.5 M NaCl, 50 mM EDTA, and 10 mM EGTA. The membrane was then given five 30-min washes in the same buffer, dried, and exposed to x-ray film. Parallel experiments were performed in the presence and absence of calcium.

**Protein Assay**—The concentration of proteins was measured by the Bio-Rad dye binding assay, based on the method of Bradford (24). The concentration of syntide-2 was determined by analysis of amino acid composition.

**RESULTS**

**Complexity of Protein Kinases in the Cucurbit Phloem**—Experiments were first conducted to test the hypothesis that the enucleate sieve tube system of the angiosperms contains fewer...
protein kinases, as compared with normal nucleate plant cells. An in-gel autophosphorylation activity assay (22) was employed for these studies. Equal amounts of protein extracted from pumpkin phloem sap and vascular tissue were first resolved by SDS-PAGE (Fig. 1A), followed by further denaturation with 6 M guanidine-HCl, and finally renatured in the gel. Gels were then incubated with [γ-32P]ATP in either the absence or presence of Ca2⁺ (Fig. 1, B and C). The overall autophosphorylation activity associated with the phloem sap was significantly lower than that observed with vascular tissue extracts. Under the experimental conditions employed, the phloem sap yielded approximately 10-fold less phosphoproteins compared with the vascular tissue. As expected, the phosphorylation pattern of proteins from both the phloem sap and vascular tissues was affected by the presence of Ca2⁺ (Fig. 1, B and C). The overall autophosphorylation activity associated with the phloem sap was significantly lower than that observed with vascular tissue extracts. Under the experimental conditions employed, the phloem sap yielded approximately 10-fold less phosphoproteins compared with the vascular tissue. As expected, the phosphorylation pattern of proteins from both the phloem sap and vascular tissue was affected by the presence of Ca2⁺. Control experiments utilizing [α-32P]ATP failed to yield detectable phosphoproteins (data not shown).

**Calcium-dependent and -independent Protein Kinase Activity in the Pumpkin Phloem Exudate**—To better understand the heterogeneity of protein kinases in the phloem sap, extracted proteins were first fractionated using anion exchange chromatography (Fig. 2A) and then assayed by in-gel autophosphorylation (Fig. 2, B and C). These experiments yielded two significant findings: the phloem sap contains at least three phosphoproteins that displayed calcium-independent autophosphorylation activity, and it also contains two distinct, similarly sized (approximately 54 kDa), putative calcium-dependent protein kinases.

The two putative calcium-dependent protein kinases were eluted from the anion exchange column at different salt concentrations and shown to be immunologically dissimilar (Fig. 2D). The soybean CDPKs antibody, which has been used to identify CDPK isoforms from various plants (17, 25, 26), recognized only the protein kinase eluted at the higher salt concentration. To confirm that this protein kinase is a CDPK, we next tested its Ca2⁺ binding capacity by gel mobility shift assay. As demonstrated with other CDPKs (27, 28), this phloem protein kinase also displayed a significant mobility shift upon binding to Ca2⁺ (Fig. 3). In addition to the fast-migrating major band, in the presence of Ca2⁺, three retarded minor bands were also detected. These bands likely represent different calcium binding states of this protein kinase. Collectively, these results established that the phloem sap protein kinase eluted under high salt concentration is a CDPK. Consequently, this protein was named CmCPK.

**Purification of CmCPK from C. maxima Phloem Sap—Ca2⁺ binding alters the elution pattern of CDPK in anion exchange chromatography without affecting that of other proteins (19). This property was utilized to purify CmCPK from phloem sap. First, dialyzed phloem sap proteins were applied to the anion exchange column in the presence of EDTA. Fractions containing CmCPK, identified by Western analysis using the soybean CDPKs antibody, were pooled and dialyzed against Ca2⁺-containing buffer. Subsequently, this dialyzed sample was applied to the second anion exchange column in the presence of Ca2⁺. By this two-step purification, CmCPK was purified to near homogeneity (Fig. 4A). Immunological analysis with the soybean CDPKs antibody verified that the purified protein was CmCPK with an apparent molecular mass of 54 kDa (Fig. 4B). In addition, this purified CmCPK exhibited calcium-dependent kinase activity as determined using syntide-2 as substrate; the measured specific activities were 1.10 ± 0.12 μmol min⁻¹ mg⁻¹ (+ Ca2⁺) and 0.007 ± 0.003 μmol min⁻¹ mg⁻¹ (− Ca2⁺).**

**cDNA Cloning and Analysis of CmCPK Expression Pattern**—To clone the cDNA encoding CmCPK, purified protein was subjected to in-gel trypsin digestion followed by internal microsequencing. Two resultant microsequences, VIAESLSEE-LAGL and EEEHLVAFAF, identified homologous regions within a calmodulin-like domain present in the CDPK gene family. Two degenerate primers, one derived from the microsequence EEHLVAFAF and one from a well-conserved sequence among known CDPKs (GGELFDR), were used in PCR reactions. A pumpkin stem cDNA library was then screened with the amplified PCR product as a specific probe. Two full-length cDNA clones were isolated and found to...
encode CDPKs containing both microsequences (Fig. 5A). *CmCPK1* (2270 bp) had an open reading frame of a 572-amino acid polypeptide with a predicted molecular mass of 64 kDa, whereas *CmCPK2* (2214 bp) encoded a 559-amino acid polypeptide with a predicted molecular mass of 62.5 kDa. The predicted molecular mass for both protein kinases was approximately 10 kDa larger than the apparent molecular mass of phloem-purified CmCPK. Both CmCPK1 and CmCPK2 contained four domains, characteristic of CDPKs: an amino-terminal variable domain, a catalytic domain, a junction domain, and a calmodulin-like domain (29, 30) (Fig. 5B). Exclusion of the amino-terminal variable domains from sequence comparisons indicated that these CDPKs share 98% amino acid identity, differing by only 10 residues (Fig. 5A).

The observed 10-kDa difference between the experimental and predicted molecular masses for CmCPK may have been due to proteolytic degradation during the process of phloem sap protein preparation. This possibility was investigated by collecting phloem sap directly into collection buffer that contained 4% SDS and 0.2 mM dithiothreitol. By using very short collection times and the SDS + dithiothreitol collection buffer, potential protease activity would be greatly reduced. Western analysis of phloem proteins obtained using control and SDS + dithiothreitol collection buffer revealed the presence of a single 54-kDa immunoreactive band (data not shown). The complete absence of any signal at the 64-kDa region of the gel argues strongly against the involvement of proteolytic degradation.

An initial analysis of the expression pattern for *CmCPK* was conducted using full-length *CmCPK1* as a probe in Northern blot assays (Fig. 6A). The results demonstrated that transcripts of the expected size were expressed in stem, root, and floral tissues at a higher level than in leaves. In contrast, transcripts were not detected in the vegetative apex. Next, the differential expression of *CmCPK1* and *CmCPK2* was investigated in RT-PCR experiments using isoform-specific primers and total RNA extracted from stem tissue (Fig. 6B). Identities of the RT-PCR products were verified by DNA sequencing. *In situ* RT-PCR experiments (5) demonstrated that both transcripts were expressed in mature CCs and developing CC-SE complexes in petiole and stem vascular tissues (data not shown).

*CmCPK1* but not *CmCPK2* Is Present in Phloem Sap—To determine which isoforms are present within the phloem sap, highly purified CmCPK was subjected to peptide mass fingerprint analysis by in-gel trypsin digestion and MALDI-TOF MS. The mass information obtained for these tryptic peptides was compared with that of *in silico* tryptic peptides of CmCPK1 and CmCPK2 amino acid sequences. Fig. 7 demonstrates a representative MALDI-TOF MS spectrum obtained with CmCPK and *in silico* tryptic peptide mass information for both CmCPK1 and CmCPK2. Each mass value marked in the MS spectrum was matched only to the theoretical mass from the respective CmCPK1 tryptic peptide. These results provided direct evidence that the purified form of CmCPK was CmCPK1. Furthermore, these data suggested that CmCPK2 does not enter the phloem translocation stream.

Amino-terminal Processing of CmCPK1—To further investigate the molecular basis for the discrepancy between the apparent molecular mass (54 kDa) and the predicted molecular mass (64 kDa) of CmCPK, the molecular mass of the phloem-purified CmCPK1 was first determined by MALDI-TOF MS. The resultant mass value was 53.4 kDa, which closely matched the observed molecular mass of 54 kDa. This result confirmed that abnormal migration of CmCPK1 on the SDS gel was not responsible for the observed molecular mass discrepancy. In addition, peptide mass fingerprint analysis failed to yield any peptide masses derived from the amino-terminal variable domain of CmCPK1 (data not shown). Finally, amino-terminal sequencing of purified CmCPK1 revealed that its amino terminus was processed at three different sites: K87-S88, S88-A89, and N91-Q92 (Fig. 5A). Collectively, these results established that CmCPK1 in the phloem sap is proteolytically modified within the amino-terminal variable domain.

An immunological approach was next used to examine the proteolytic processing of CmCPK1. Two polyclonal peptide antibodies to CmCPK1 were raised; one against a unique region in the amino-terminal variable domain and a second against the conserved carboxyl terminus (Fig. 5A). The specificity of each polyclonal antibody preparation was confirmed by Western analysis using recombinant GST-CmCPK1 and an amino-terminal variable domain deletion mutant, GST-CmCPK1ΔN (data not shown). Proteins within the phloem sap and soluble and membrane fractions prepared from excised stems/stripped vascular bundles were analyzed for the detection of full-length/processed CmCPK1 using the above-described antibodies (Fig. 8). The soybean CDPKα antibody, used as a control, cross-reacted with CmCPK1 in the phloem sap (Fig. 8A, lane 1). As expected, this antibody also recognized multiple CDPKs that were present in either the soluble or membrane fractions obtained from the vascular bundles (Fig. 8A, lanes 2 and 3). The carboxyl-terminal antibody detected CmCPK1 in the phloem sap but not in either of the extracts prepared from the vascular bundles (Fig. 8B). This result indicated that CmCPK1 must be highly enriched in the phloem sap. As anticipated, the amino-terminal antibody failed to recognize CmCPK1 in the phloem sap (Fig. 8C, lane 1). Because neither antibody gave a signal with the vascular proteins (Fig. 8, B and C), the relative levels of CmCPK1 and CmCPK2 must be extremely low in these tissues.
Detection of Putative Substrates of Phloem Protein Kinases—To identify potential protein kinase substrates present within the phloem sap, individual FPLC fractions of phloem sap proteins were first incubated in protein kinase assay buffer containing \([\gamma-32P]ATP\) and Ca\(^{2+}\). SDS-PAGE analysis of the resultant phosphoproteins identified numerous potential substrates for these phloem sap protein kinases (Fig. 9, A and B).

Naturally, some of the phosphoproteins located in the 40–70-kDa region of these autoradiographs will represent autophosphorylated protein kinases (see Fig. 2, B and C). Potential substrates for CmCPK1 were detected in the 30-kDa region of the autoradiographs (compare Fig. 2, C and D, lanes 8 and 9; Fig. 9, B, lanes 4–6). A modified protein kinase assay, on-membrane, was employed to further explore likely substrates for CmCPK1. Here, nitrocellulose membranes containing FPLC-fractionated phloem proteins were incubated (with \([\gamma-32P]ATP\) and Ca\(^{2+}\)) with purified CmCPK1 (Fig. 9, C and D). These experiments further confirmed both the calcium-dependent activity of CmCPK1 and the presence of putative substrates in the 30-kDa size range. Three additional substrates were also detected in the 60–80-kDa size range. Control experiments performed in the absence of CmCPK1 and preincubation of the membrane with ATP (to allow autophosphorylation before the addition of \([\gamma-32P]ATP\)) demonstrated that these bands resulted from phosphorylation by CmCPK1 (data not shown). Finally, because each FPLC fraction contained many proteins (Fig. 2 A), but only five phosphoproteins were detectable, these results are consistent with a specific interaction between CmCPK1 and these phloem proteins.

DISCUSSION

In this study, the complexity, number, and nature of the protein kinases present within the phloem sap of pumpkin were investigated. The limited number of proteins detected within the phloem sap that were capable of autophosphorylation (Figs. 1 and 2), thereby likely representing potential pro-
tein kinases, is consistent with the hypothesis that functional, enucleate SEs utilize a simplified signal transduction network. Here, it is important to stress that only soluble SE proteins were examined, and thus future studies will need to focus on the presence of integral membrane or membrane-associated protein (receptor) kinases. Because the endomembrane network of the mature SEs is highly reduced (9), such protein kinases should be located predominantly in the plasma membrane. Protein kinases can be present in cells at very low concentrations, and others require activation as a prerequisite for autophosphorylation (32); thus the possibility also exists that a number went undetected in the present study. Furthermore, our analyses were conducted on phloem exudates collected from stem tissues engaged in long-distance translocation. It will be important to ascertain whether the phloem sap of SEs located in sites of loading and unloading contains the same or a different complement of protein kinases (see Fig. 6A); the effects of the influence of plant development and abiotic and biotic stresses on these phloem proteins should also prove insightful.

Based on in-gel autophosphorylation, patterns of elution on anion exchange chromatography, and immunological analysis (Figs. 2 and 9), the pumpkin phloem sap was shown to contain at least two classes of protein kinases, one calcium-independent class (three proteins) and one calcium-dependent class (two proteins). The presence of CDPKs in the pumpkin phloem sap is consistent with earlier biochemical studies performed on the phloem of rice and cucumber plants (33–36). Thus, the current study now establishes an experimental foundation for the elucidation of the roles played by these protein kinases in signal transduction within the phloem.

To further investigate the function of the phloem CDPKs, a protocol was developed that allowed for the purification of one member of this CmCPK family. Two highly homologous isoforms, CmCPK1 and CmCPK2, were identified by using cDNA cloning and RT-PCR analysis, and both transcripts were detected in CCs by in situ RT-PCR analysis of stem tissues. However, peptide mass fingerprint analysis of phloem-purified CmCPK clearly demonstrated that only CmCPK1 was present in the phloem sap (Fig. 7). Hence, as with many other phloem proteins (16, 37, 38), CmCPK1 appears to be capable of traf-
ficking from the CC into the SE via the interconnecting plasmodesmata. This is an intriguing situation, given the high degree of identity observed between CmCPK1 and CmCPK2 (Fig. 5A). This selective recognition and cell-to-cell transport of CmCPK1 may be mediated by additional residues (potential targeting motifs) located in the amino-terminal variable domain of CmCPK1 but absent from CmCPK2 (Fig. 5A). This possibility could be tested by the exchange of specific regions within the amino-terminal variable domains of these two proteins.

A combination of MALDI-TOF MS, peptide mass fingerprinting, and amino-terminal sequencing established that, in the phloem sap, CmCPK1 exists as an amino-terminally cleaved protein. A similar situation was recently reported for CmPP36, in which only the amino terminally truncated form of the protein (ΔN-CmPP36) was detected in the pumpkin phloem sap (38). Here, it was also demonstrated that full-length CmPP36 was unable to mediate its own transport through plasmodesmata, whereas ΔN-CmPP36 was shown to move from cell to cell in microinjection experiments. These similarities between CmPP36 and CmCPK1 implicate a role for amino-terminal proteolytic cleavage in the control of protein transport through the CC–SE plasmodesmata. Because CmPP36 was shown to have an amino-terminally located membrane-targeting domain, and CmCPK1 has a putative myristoylation motif (‘MGNTCRGS8’) at its amino terminus, proteolytic processing of these proteins within the CC may involve proteases anchored to the plasma membrane in close proximity to CC–SE plasmodesmata. In support of this hypothesis, membrane association of CDPKs through myristoylation has recently been demonstrated (38, 39). Mutagenesis of this putative motif in the CmCPK1 will test the possible role of myristoylation in membrane targeting, amino-terminal cleavage, and cell-to-cell transport of CmCPK1.

Calcium-dependent protein kinases act as major mediators in Ca\textsuperscript{2+} signaling in plants through the direct interaction of Ca\textsuperscript{2+} with the calmodulin-like regulation domain (29, 30, 40). In most biological systems, the level of free Ca\textsuperscript{2+} within the cytoplasm is maintained at a resting level that is in the nanomolar range, whereas upon activation by numerous stimuli, the concentration can rise into the micromolar range (13–60 μM; Ref. 42). If these measurements were correct, Ca\textsuperscript{2+}-regulated proteins in the phloem sieve tube system would need to be able to respond to much higher Ca\textsuperscript{2+} concentrations than those present in other cell types. Alternatively, the microenvironment of the sieve tube system (43) may preclude accurate determination of Ca\textsuperscript{2+} levels in the enucleate SEs. Because CmCPK1 is the first identified Ca\textsuperscript{2+}-regulated protein in the phloem sap, determining its Ca\textsuperscript{2+} concentrations for half maximal activity (K\textsubscript{m,50}) may well provide important insight into the nature of Ca\textsuperscript{2+} signaling in the phloem.

Elucidation of the role of CmCPK1 in signaling within the functioning of the CC–SE complex/sieve tube system requires the identification and characterization of its substrate(s). To this end, five putative substrates were detected within the phloem sap (Fig. 9). The specificity of these substrates can be argued on the basis that the phloem sap used in these experiments contained more than 200 proteins, with this number likely being in the range of 500 (data not shown). The identification and characterization of the second phloem CmCPK (Fig. 2C, lanes 4–6), which phosphorylated a number of phloem proteins (Fig. 9, A and B), as well as the three calcium-independent protein kinases and their substrates, will aid in the dissection of the signal cascades within the sieve tube system.

It is now established that the phloem translocation stream serves as a conduit for the long-distance delivery of proteins and ribonucleoprotein complexes that have been demonstrated to play a role in control over developmental programs within sink organs (2, 5, 6, 44). In addition, phosphorylation of viral movement proteins has been shown to influence viral infectivity in a species-specific manner at the level of movement protein-viral nucleic acid transport through plasmodesmata (45–47). In this regard, we speculate that the phloem protein kinases may be similarly involved in the control of ribonucleoprotein complex exchange through the CC–SE plasmodesmata.

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Analysis of the Complexity of Protein Kinases within the Phloem Sieve Tube System: CHARACTERIZATION OF CUCURBITA MAXIMA CALMODULIN-LIKE DOMAIN PROTEIN KINASE 1
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