

# Plasmodesmal-Associated Protein Kinase in Tobacco and *Arabidopsis* Recognizes a Subset of Non-Cell-Autonomous Proteins <sup>W</sup>

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**Cell-to-cell communication in plants involves the trafficking of macromolecules through specialized intercellular organelles, termed plasmodesmata. This exchange of proteins and RNA is likely regulated, and a role for protein phosphorylation has been implicated, but specific components remain to be identified. Here, we describe the molecular characterization of a plasmodesmal-associated protein kinase (PAPK). A 34-kD protein, isolated from a plasmodesmal preparation, exhibits calcium-independent kinase activity and displays substrate specificity in that it recognizes a subset of viral and endogenous non-cell-autonomous proteins. This PAPK specifically phosphorylates the C-terminal residues of tobacco mosaic virus movement protein (TMV MP); this posttranslational modification has been shown to affect MP function. Molecular analysis of purified protein established that tobacco (*Nicotiana tabacum*) PAPK is a member of the casein kinase I family. Subcellular localization studies identified a possible *Arabidopsis thaliana* PAPK homolog, PAPK1. TMV MP and PAPK1 are colocalized within cross-walls in a pattern consistent with targeting to plasmodesmata. Moreover, *Arabidopsis* PAPK1 also phosphorylates TMV MP in vitro at its C terminus. These results strongly suggest that *Arabidopsis* PAPK1 is a close homolog of tobacco PAPK. Thus, PAPK1 represents a novel plant protein kinase that is targeted to plasmodesmata and may play a regulatory role in macromolecular trafficking between plant cells.**

## INTRODUCTION

Plasmodesmata represent special intercellular organelles that establish cytoplasmic and endomembrane continuity between neighboring plant cells (Robards and Lucas, 1990; Lucas, 1995). This feature allows small molecules, such as ions, metabolites, and hormones, to diffuse cell to cell and thereby enhance the coordination of biochemical and physiological processes (Lucas et al., 1993). A fundamental difference between the intercellular communication channels found in animals, the gap junctions, and plasmodesmata is that the latter acquired an additional capacity to mediate the selective cell-to-cell trafficking of proteins and protein/RNA complexes (Gilbertson and Lucas, 1996; Zambryski and Crawford, 2000; Haywood et al., 2002; Heinlein, 2002; Ding et al., 2003; Wu et al., 2003; Lucas and Lee, 2004; Oparka, 2004).

A significant body of evidence now supports the concept that plasmodesmata establish a unique control system by allowing a special class of proteins, including transcription factors, to

pass through fields of cells (Jackson, 2000; Hake, 2001; Wu et al., 2002; Cilia and Jackson, 2004). Examples of these transcription factors include KNOTTED1 (KN1), a homeobox protein in maize (*Zea mays*; Jackson et al., 1994; Lucas et al., 1995), LEAFY (LFY), the *Arabidopsis thaliana* homolog of the FLORICAULA that controls floral meristem identity (Weigel and Meyerowitz, 1993; Perbal et al., 1996; Sessions et al., 2000), SHORT-ROOT (SHR), a putative transcription factor required for endodermal specification (Helariutta et al., 2000; Nakajima et al., 2001; Gallagher et al., 2004), and CAPRICE, a protein central to hair cell formation (Schellmann et al., 2002; Wada et al., 2002; Pesch and Hulskamp, 2004). Many of these proteins appear to use the macromolecular trafficking capacity of plasmodesmata to act as non-cell-autonomous proteins (NCAPs), thereby regulating the biological events occurring outside the cells in which they are produced.

Given the roles played by these NCAPs, their cell-to-cell trafficking is likely to be a highly regulated process. Evidence consistent with this notion has been gained through genetic, molecular, and biochemical studies on the function of the 30-kD movement protein (MP) of *Tobacco mosaic virus* (TMV; Wolf et al., 1989; Citovsky, 1999) and other such viral MPs (Fujiwara et al., 1993; Noueiry et al., 1994; Rojas et al., 1998; Lazarowitz and Beachy, 1999; Heinlein, 2002; Waigmann et al., 2004). These viral-encoded proteins act as NCAPs, in that they mediate the cell-to-cell movement of infectious viral RNA (Lucas and Gilbertson, 1994; Carrington et al., 1996; Citovsky, 1999; Lazarowitz and Beachy, 1999; Tzfira et al., 2000; Zambryski and Crawford, 2000; Haywood et al., 2002). One form of regulation may well involve

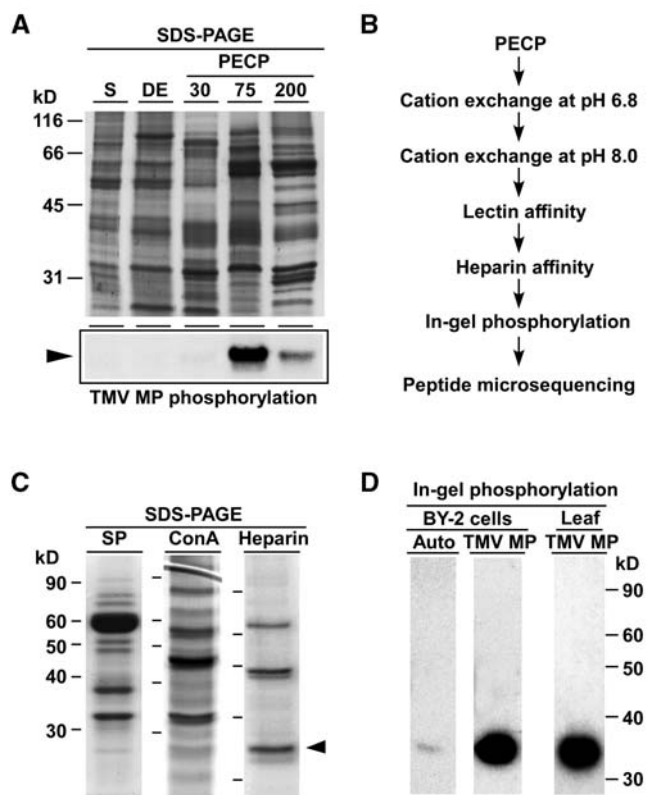
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**Figure 1.** Biochemical Isolation of a Tobacco PAK.

**(A)** Enrichment of PAK activity within the subfractions of a PECP preparation obtained using tobacco suspension cultured cells. Proteins from each fraction were resolved by electrophoresis in 10% SDS-PAGE gels and visualized by staining with GelCode Blue (top panel). The subcellular fractions include the supernatant (S), a 1% CHAPS extract of the cell wall pellets (DE), and PECP subfractions extracted with 30 mM (PECP30), 75 mM (PECP75), and 200 mM (PECP200)  $\text{Ca}^{2+}$  buffer. Each lane was loaded with 8  $\mu\text{g}$  of protein. PAK activity was determined by phosphorylation assays in which 1  $\mu\text{g}$  of each fraction was added to assay mixtures containing TMV MP. Kinase assay reactions were resolved in 10% SDS-PAGE gels and autoradiography used to detect TMV MP phosphorylation (bottom panel, arrowhead).

**(B)** Biochemical protocol developed for PAK purification. Phosphorylation assays were used to identify the chromatographic fractions containing peak enzyme activity for each step.

**(C)** Chromatographic purification of PAK. Pooled PECP70 peak fractions were separated by cation exchange (SP; pH 6.8), lectin (Con A), and heparin columns. Proteins were resolved by electrophoresis in 10% SDS-PAGE gels and then stained with GelCode Blue. Protein loading volumes in each gel were 18, 25, and 35  $\mu\text{L}$  for SP, Con A, and Heparin fractions, respectively. The arrowhead indicates  $\sim 34$ -kD protein corresponding to the PAK subsequently confirmed by in-gel phosphorylation assays.

**(D)** Purified heparin fraction, shown in **(C)**, was used for in-gel auto-phosphorylation and TMV MP phosphorylation assays. For in-gel auto-phosphorylation, 20  $\mu\text{L}$  of the heparin peak fraction was first resolved in 12% SDS-PAGE gels, then given denaturation/renaturation treatments, followed by incubation of the gel in complete assay buffer (2 mM DTT plus ATP mixture of 10  $\mu\text{Ci}/\text{mL}$  [ $\gamma^{32}\text{P}$ ]ATP) for 1 h. In-gel substrate phosphorylation assays were as above except that TMV MP was included at  $\sim 300$   $\mu\text{g}/\text{mL}$  gel. PAK was also purified from tobacco leaves using the same purification protocol as **(B)**. Peak fractions were

combined, concentrated to  $\sim 80$   $\mu\text{L}$ , and 5- $\mu\text{L}$  aliquots used for each in-gel TMV MP phosphorylation assay.

MP phosphorylation within the infected host cells (Watanabe et al., 1992; Citovsky et al., 1993; Haley et al., 1995; Kawakami et al., 1999). Direct support for the role of phosphorylation in regulating TMV MP movement was provided by Waigmann et al. (2000), who showed that mutant forms of TMV, carrying changes that mimicked phosphorylation of specific residues in the MP, could neither infect *Nicotiana tabacum* nor move cell to cell. Thus, MP phosphorylation, by a plant kinase, may well serve to block infection, perhaps by confining the MP within the plasmodesmata (Tomenius et al., 1987; Atkins et al., 1991; Ding et al., 1992; Moore et al., 1992), thereby preventing the spread of the infectious RNA.

Based on these findings, it would appear that plants likely evolved specific protein kinases that serve to regulate the cell-to-cell trafficking of both viral and endogenous NCAPs (Lee and Lucas, 2001; Lucas and Lee, 2004; Waigmann et al., 2004). Phosphorylation of such NCAPs, at or near plasmodesmata, could well play an important role in modulating the activity, extent of trafficking, and/or intracellular localization of these proteins within their target cells (Kawakami et al., 1999; Trutnyeva et al., 2005). Given that specific NCAPs are involved in the determination of cell fate and pattern formation in meristematic tissues (Zambryski and Crawford, 2000; Jackson, 2001; Haywood et al., 2002; Wu et al., 2002), one could argue that control over their spatial and temporal activation would be critical for precise developmental progression. A protein kinase having broad substrate specificity toward these NCAPs, that is itself positioned within or near plasmodesmata, might contribute to controlling the NCAP pathway (Lee and Lucas, 2001; Waigmann et al., 2004).

In this study, we provide the means to test this hypothesis through the development of a protocol that allowed us to isolate and characterize a plasmodesmal-associated protein kinase (PAK). Molecular and bioinformatic analyses of an  $\sim 34$ -kD PAK, purified from tobacco, established that this protein belongs to the casein kinase I (CKI) family. Consistent with a role in macromolecular trafficking through plasmodesmata, this PAK displayed substrate specificity toward a group of viral and plant NCAPs. Finally, colocalization of PAK with the TMV MP, a protein well known to be targeted to and sequestered in plasmodesmata, confirmed that members of this plant PAK family are similarly targeted to the same region of the cell periphery and presumably reside within plasmodesmata.

## RESULTS

### Purification of a Putative PAK Using TMV MP as an *In Vitro* Substrate

A biochemical approach was developed to identify the plasmodesmal-associated subcellular fraction that was enriched in kinase activity. For this purpose, we used a protocol recently developed for the isolation of a plasmodesmal-enriched cell wall protein (PECP) fraction from tobacco suspension-cultured cells (Lee et al., 2003). The TMV MP was used as substrate in phosphorylation assays performed on PECP subfractions (Figure 1A).

combined, concentrated to  $\sim 80$   $\mu\text{L}$ , and 5- $\mu\text{L}$  aliquots used for each in-gel TMV MP phosphorylation assay.

This viral NCAP was selected as it was previously shown to be phosphorylated by a kinase present in cell wall extracts (Citovsky et al., 1993; Waigmann et al., 2004), and this modification was correlated with its altered movement function (Waigmann et al., 2000; Trutnyeva et al., 2005). Our experiments indicated that the highest kinase activity was present in the 75 mM CaCl<sub>2</sub> (PECP-75) extracted proteins. It is important to note that NCAPP1 (Lee et al., 2003) was similarly highly enriched in this same PECP-75 subfraction (see Supplemental Figure 1 online). Importantly, the TMV MP was not phosphorylated by either the supernatant or the subcellular fraction containing detergent-extracted proteins from the cell wall pellet.

To identify the molecular nature of this putative PAPK, the enzyme was further purified by employing a combination of chromatographic protocols, including cation-exchange, lectin, and heparin columns (Figure 1B). The PECP-75 extracts from several preparations were pooled and proteins first fractionated, by cation-exchange chromatography, and their profile then resolved by SDS-PAGE. Fractions containing high levels of enzyme activity were again identified, based on our TMV MP phosphorylation assay. These peak fractions were again pooled, and a relatively high level of PAPK purification was achieved following lectin (concanavalin A [Con A]) and heparin chromatography. Coomassie blue-stained SDS-PAGE gels revealed the presence of several discrete bands within this purified and enriched PECP fraction (Figure 1C).

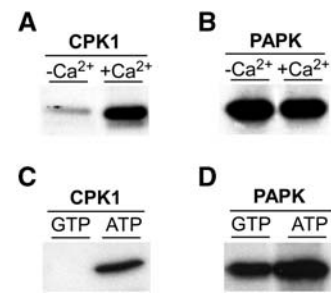
To determine the protein band corresponding to the putative PAPK, in-gel phosphorylation assays were next performed. These experiments established that the apparent molecular mass of the purified PAPK was ~34 kD (Figure 1D). PAPK activity was also detected in PECP fractions prepared from tobacco leaf tissues (data not shown), and in-gel phosphorylation assays similarly revealed kinase activity associated with an ~34-kD protein (Figure 1D).

### Biochemical Characteristics of PAPK

Biochemical assays were next performed to establish the functional characteristics of this ~34-kD putative PAPK. Experiments were first conducted to ascertain whether PAPK exhibited calcium-dependent protein kinase activity. Pumpkin (*Cucurbita maxima*) CPK1 is a calcium-dependent protein kinase identified from pumpkin phloem sap (Yoo et al., 2002), and it was used as a control for calcium-dependent phosphorylation of the TMV MP in vitro (Figure 2A). In contrast with pumpkin CPK1, the phosphorylation of TMV MP, by PAPK, was calcium independent (Figure 2B). In general, ATP is preferred as a phosphate donor by most protein kinases (Edelman et al., 1987). Consistent with this notion, pumpkin CPK1 did not appear to use GTP as a phosphate donor (Figure 2C). Interestingly, both GTP and ATP could be used as good phosphate donors for the tobacco PAPK (Figure 2D).

### PAPK Phosphorylates TMV MP at Its C Terminus

Recently, it was shown that a plant cell wall extract contained kinase activity that could phosphorylate TMV MP in a calcium-independent manner (Waigmann et al., 2000). The phosphoryla-



**Figure 2.** PAPK Exhibits Calcium-Independent Activity and Uses ATP or GTP as a Phosphate Donor.

**(A)** Control experiment employing pumpkin CPK1 demonstrated calcium-dependent phosphorylation of TMV MP. Kinase activity was assayed using 50  $\mu$ L reaction buffer, plus 20 ng of purified recombinant enzyme and 1  $\mu$ g of TMV MP. Reaction buffer contained 50 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM DTT, 2 mM EGTA, and 60  $\mu$ M ATP mixed with 4  $\mu$ Ci of [ $\gamma$ <sup>32</sup>P]ATP (3000 Ci/mmol), supplemented with 2.2 mM CaCl<sub>2</sub>.

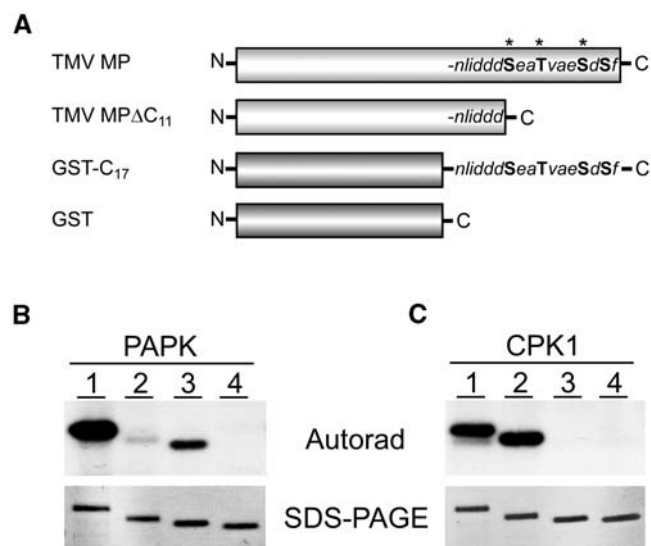
**(B)** PAPK phosphorylated TMV MP in a calcium-independent manner. Kinase assays were performed in 50  $\mu$ L of reaction buffer, plus 5  $\mu$ L of heparin peak fraction and 1  $\mu$ g of TMV MP  $\pm$  2.2 mM CaCl<sub>2</sub>.

**(C)** Pumpkin CPK1 uses ATP but not GTP as a phosphate donor. Reaction buffer was as above, except that it contained 4  $\mu$ Ci of [ $\gamma$ <sup>32</sup>P]GTP (3000 Ci/mmol) mixed with 60  $\mu$ M ATP.

**(D)** PAPK can use GTP or ATP as the phosphate donor. Reaction buffer was as above, except that it contained 4  $\mu$ Ci of [ $\gamma$ <sup>32</sup>P]GTP (3000 Ci/mmol) mixed with 60  $\mu$ M ATP.

tion sites were mapped to the three residues, Ser258, Thr261, and Ser265, within the C-terminal end of the TMV MP. As PAPK is enriched in our PECP fraction and phosphorylates TMV MP, we next investigated whether this enzyme might be the corresponding endogenous kinase. For these studies, a deletion mutant that lacked the C-terminal 11 amino acid residues (TMV MP $\Delta$ C<sub>11</sub>) and a recombinant glutathione S-transferase (GST) fusion protein containing the C-terminal 17 residues of TMV MP (GST-C<sub>17</sub>) were constructed (Figure 3A). In contrast with the full-length TMV-MP, the engineered TMV MP $\Delta$ C<sub>11</sub> served as a poor substrate for phosphorylation by the purified PAPK (Figure 3B). Densitometric analysis indicated that the level of phosphorylation of TMV MP $\Delta$ C<sub>11</sub> by PAPK was 10% of that observed for the TMV MP. This result indicated that the major phosphorylation site for PAPK is located within the deleted region of the TMV MP. As the expression of this mutant was as stable as that of full-length TMV MP, protein instability is unlikely to be responsible for this reduction in phosphorylation by the PAPK. Consistent with this notion, the TMV MP $\Delta$ C<sub>11</sub> served as a comparable substrate to the full-length TMV MP for phosphorylation by pumpkin CPK1 (Figure 3C).

GST-C<sub>17</sub> also served as a substrate for phosphorylation by PAPK; controls using GST alone demonstrated the specificity of this reaction (Figure 3B). However, in comparison with the full-length TMV MP, GST-C<sub>17</sub> appeared to serve as a less favorable substrate. The level of phosphorylation of GST-C<sub>17</sub> by PAPK was 30% of that observed for the TMV MP based on densitometric analysis. Specificity of the phosphorylation reaction, mediated by PAPK, toward the C terminus of TMV MP was



**Figure 3.** The TMV MP C-Terminal Domain Contains Major Phosphorylation Sites Recognized by PAPK.

**(A)** Schematic diagram illustrating the recombinant proteins used to identify the phosphorylation sites, present in TMV MP, recognized by PAPK. TMV MP: general structure indicating the positions of Ser/Thr residues in the C terminus (bold), including those previously identified as being *in vivo* phosphorylation sites (asterisks; Ser258, Thr261, and Ser265; Waigmann et al., 2000). TMV MP $\Delta$ C<sub>11</sub>: deletion mutant lacking the 11 C-terminal residues. GST-C<sub>17</sub>: GST fusion protein containing the 17 C-terminal residues of the TMV MP. GST: control protein for our phosphorylation reactions.

**(B)** PAPK recognized, specifically, the Ser/Thr residues within the C terminus of TMV MP. Lane 1, TMV MP (30 kD); lane 2, TMV MP $\Delta$ C<sub>11</sub> (29 kD); lane 3, GST-C<sub>17</sub> (28 kD); lane 4, GST (26 kD). Each phosphorylation assay contained 5  $\mu$ L of heparin column-purified PAPK fraction and 1  $\mu$ g of substrate protein in a 50- $\mu$ L reaction volume.

**(C)** TMV MP C terminus lacked phosphorylation substrate sites for pumpkin CPK1. Parallel assays to those in **(B)** performed using 20 ng of purified recombinant pumpkin CPK1. Substrate phosphorylation visualized by autoradiography (top panels) and purified substrate proteins used in these assays (1  $\mu$ g each) are shown in GelCode Blue-stained SDS-PAGE gels (bottom panels).

supported by the finding that pumpkin CPK1 failed to phosphorylate the GST-C<sub>17</sub>. Taken together, our results indicate that the major PAPK phosphorylation site resides within the C-terminal 11 amino acid residues of TMV MP. These findings support the hypothesis that PAPK is a plant protein kinase that recognizes NCAPs, such as the TMV MP.

### PAPK Phosphorylates a Subset of Endogenous and Viral NCAPs

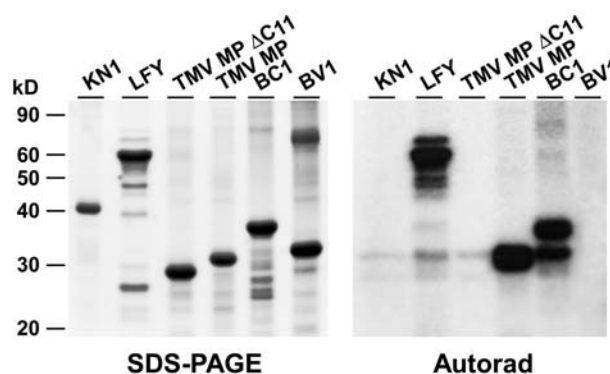
It is possible that PAPK might be an endogenous kinase that modulates, specifically, TMV MP upon viral infection. If this were the case, PAPK would likely be expressed in TMV host plants, and its expression might well be affected by viral infection. Moreover, the substrate specificity of PAPK could be quite narrow, if not exclusive to TMV MP. Alternatively, PAPK could play a role in controlling NCAP trafficking during normal plant

growth and development (i.e., viruses could have exploited this host mechanism) (Lucas and Wolf, 1993; Oparka, 2004; Waigmann et al., 2004). Under this scenario, PAPK would likely show broad substrate specificity toward both viral and endogenous NCAPs. To test between these two possibilities, we next examined the substrate specificity of PAPK.

For this purpose, we selected four additional viral MPs and two well-characterized endogenous NCAPs. BC1 and BV1 represent MPs encoded by a DNA virus, *Bean dwarf mosaic virus* (Noueiry et al., 1994; Rojas et al., 1998), while the MPs of *Cucumber mosaic virus* (CMV) and *Red clover mosaic virus* (RCNMV) were selected as examples of RNA viruses (Fujiwara et al., 1993; Ding et al., 1995; Canto et al., 1997; Itaya et al., 1997; Wang et al., 1998). KN1 and LFY are plant transcription factors that are well characterized for their non-cell-autonomous activities (Lucas et al., 1995; Sessions et al., 2000; Kim et al., 2002, 2003; Lucas and Lee, 2004). Under the *in vitro* conditions used for our experiments, PAPK displayed substrate specificity, in that it phosphorylated LFY, BC1, and TMV MP, but not KN1 or BV1 (Figure 4). The MPs of CMV and RCNMV were also examined and found to serve as poor substrates for PAPK (see Supplemental Figure 2 online). These studies established that PAPK likely functions in the recognition and phosphorylation of a specific subset of NCAPs of both plant and viral origin.

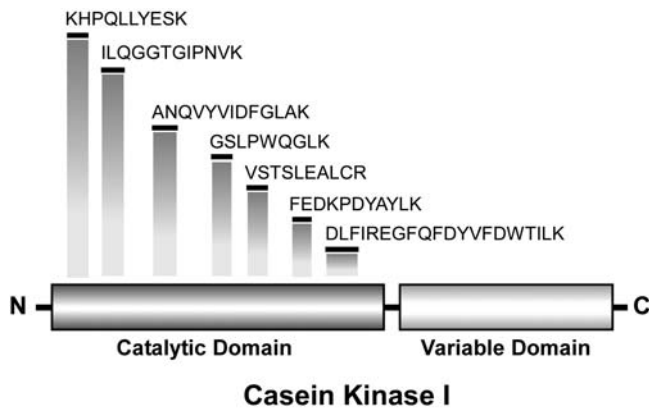
### PAPK Is a Member of the CKI Gene Family

To identify the tobacco gene encoding PAPK, the  $\sim$ 34-kD protein band (Figure 1C) was excised and subjected to conventional internal peptide microsequencing. A National Center for Biotechnology Information BLAST database search was conducted using the resultant peptide sequences, and this identified PAPK as a member of the CKI gene family (Figure 5). CKI is a Ser/Thr protein kinase family found in eukaryotes (Gross



**Figure 4.** PAPK Displays Substrate Specificity for Viral and Endogenous Non-Cell-Autonomous Proteins.

Substrate specificity of PAPK tested using two plant NCAPs, KN1 and LFY, and two viral MPs, BC1 and BV1. TMV MP and TMV MP $\Delta$ C<sub>11</sub> were included as controls. Each *in vitro* phosphorylation assay contained 5  $\mu$ L of heparin column-purified PAPK fraction and 1  $\mu$ g of substrate protein in a 50- $\mu$ L reaction volume. Note that LFY, TMV MP, and BC1 served as specific substrates for PAPK, whereas KN1 and BV1 were not phosphorylated.



**Figure 5.** Peptide Microsequence Analysis Identifies PAPK as a Member of the CKI Gene Family.

All microsequences matched to the highly conserved catalytic domain of CKI; vertical bars indicate the relative locations within this domain.

and Anderson, 1998). The kinase domains of plant CKI isoforms that were identified in the *Arabidopsis* and rice (*Oryza sativa*) genome databases shared 86% similarity at the amino acid sequence level (see Supplemental Figure 3 online). Interestingly, all PAPK-derived peptide microsequences were found to match this highly conserved kinase domain (Figure 5). This result prevented us from determining the actual tobacco CKI isoform corresponding to the biochemically purified PAPK. As both N- and C-terminal regions of purified PAPK were found to be blocked, we designed degenerate primers, based on the identified peptide sequences, to amplify PCR products that were then used to probe a tobacco cDNA library. A number of cDNA clones that contained regions closely matching the peptide microsequences were isolated. This indicated that the catalytic domain of CKI is highly conserved. However, we were unable to obtain any cDNA clones that completely matched all seven

peptide microsequences and also encoded a 34-kD protein, the apparent molecular mass of purified PAPK.

To resolve this situation, we adopted an alternative approach to identify the *PAPK* gene, based on the expectation that *PAPK* would be localized to, or near, plasmodesmata. The *Arabidopsis* genome was found to contain some 14 *Casein Kinase I-like* (*CKL*) genes (Table 1), and within the conserved kinase domains, they shared 89% sequence similarity at the amino acid level. Of these genes, one (At4g08800) appeared to be a pseudogene, based on the fact that it contained an in-frame stop codon and/or short deletions within the conserved kinase subdomains; no reliable ESTs have been reported, and RT-PCR amplification of the putative transcript failed to yield any product. By contrast, all other members were successfully amplified by RT-PCR. As two isoforms, CKL9 $\alpha$  and CKL9 $\beta$ , are apparently derived from alternative splicing, we concluded that 13 genes give rise to 14 CKL isoforms.

### Subcellular Localization Patterns of *Arabidopsis* CKL Isoforms

In yeast and humans, CKI isoforms contain variable domains, either at the N or C terminus (Gross and Anderson, 1998); in *Arabidopsis*, all CKL isoforms appeared to contain C-terminal extensions with variable lengths from ~20 to 180 amino acid residues. Three out of four yeast isoforms, YCK1, 2, and 3, contain isoprenylation sites at their C termini and are associated with the plasma membrane (Wang et al., 1992; Vancura et al., 1994). However, no significant sequence conservation, predictable motifs, or domains for specific intracellular targeting/localization were detected within any of the *Arabidopsis* CKL extensions.

In view of this finding, the intracellular localization/targeting of each CKL isoform was determined by expressing N- or C-terminal green fluorescent protein (GFP) fusion proteins in plant cells. For transient expression of CKL-GFP fusion proteins, appropriate plasmid constructs were biolistically bombarded into tobacco suspension-cultured cells (Lee et al., 2003). We divided these CKL isoforms into three groups on the basis

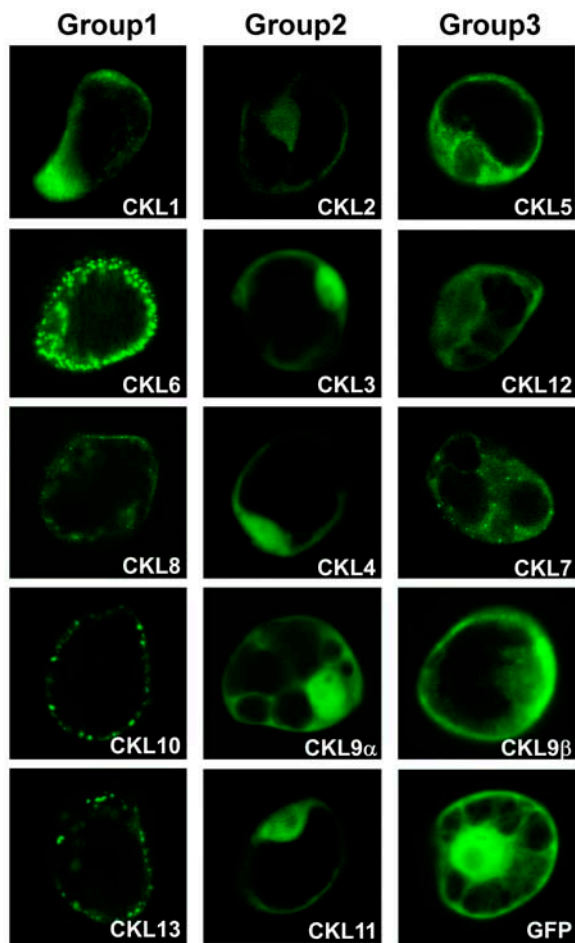
**Table 1.** Molecular Information and Intracellular Localization of CKL Isoforms

Isoform	AGI Locus Identifier <sup>a</sup>	GenBank Accession No.	No. of Amino Acid Residues	kD <sup>b</sup>	Intracellular Localization
CKL1	At4g26100	AY943842	450	51.1	Punctate particles
CKL2	At1g72710	AY943843	465	52.0	Cytoplasm/nucleus
CKL3	At4g28880	AY943852	415	47.0	Cytoplasm/nucleus
CKL4	At4g28860	AY943853	414	46.8	Cytoplasm/nucleus
CKL5	At2g19470	AY943844	433	49.0	Strongly cytoplasm
CKL6	At4g28540	AY943845	479	54.1	Punctate particles
CKL7	At5g44100	AY943846	476	53.7	Strongly cytoplasm
CKL8	At5g43320	AY943854	480	54.5	Punctate particles
CKL9 $\alpha$	At1g03930	AY943847	319	36.8	Cytoplasm/nucleus
CKL9 $\beta$	At1g03930	AY943848	471	53.1	Strongly cytoplasm
CKL10	At3g23340	AY943849	442	50.5	Punctate particles
CKL11	At4g14340	AY943850	457	52.0	Cytoplasm/nucleus
CKL12	At5g57015	AY943855	435	49.5	Strongly cytoplasm
CKL13	At1g04440	AY943851	468	53.3	Punctate particles

<sup>a</sup> AGI, Arabidopsis Genome Initiative.

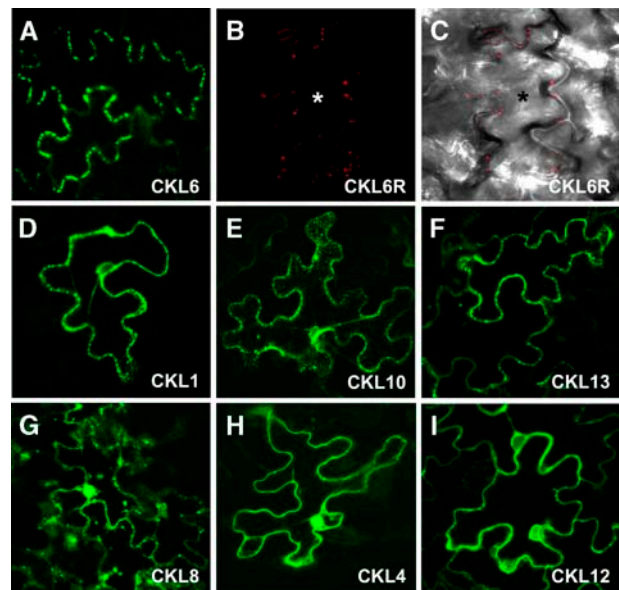
<sup>b</sup> Calculated molecular mass.

of localization patterns determined by fluorescence microscopy (Figure 6). Group 1 isoforms exhibited a predominantly punctate fluorescence pattern at the cell periphery; group 2 isoforms had strong fluorescent signals within the nucleus, but signal was also present in the cytoplasm; and for group 3 isoforms, signal was located predominantly in the cytoplasm, with only a low level detected in the nucleus. Parallel expression studies were also performed using tobacco leaf tissues. Here, similar results were obtained with these same *CKL-GFP* constructs when we analyzed epidermal cells of mature tobacco leaves (Figure 7).



**Figure 6.** Subset of *Arabidopsis* CKL Isoforms Localizes to the Plasmodesmata/Cell Periphery.

The *Arabidopsis* genome contains 14 members of the CKL gene family (CKL1 to 14). Putative *Arabidopsis* PPKs were identified on the basis of intracellular localization patterns observed for these CKL-GFP fusion proteins. Transient expression assays, using tobacco suspension culture cells, indicate the presence of three localization patterns, represented by genes in groups 1, 2, and 3. Fluorescent patterns were collected by laser scanning confocal microscopy 16 h after biolistic bombardment. Cytoplasmic and nuclear localization of GFP was used as a control. Based on the similarity in fluorescence patterns exhibited by group 1 isoforms and other plasmodesma-targeted NCAPs, these are likely candidates for *Arabidopsis* PPKs. Group 2 represents CKL isoforms that localize to the nucleus and cytoplasm. Group 3 CKLs accumulate mainly in the cytoplasm.



**Figure 7.** *Arabidopsis* CKL6 Localizes to Discrete Regions at the Cell Periphery of Tobacco and *Arabidopsis* Epidermal Cells.

Tobacco leaf tissue transfected using *Agrobacterium tumefaciens* transformed with GFP-tagged CKL isoforms ([A] and [D] to [I]) was analyzed 48 h after agroinfiltration. *Arabidopsis* leaf tissue biolistically bombarded with plasmid DNA expressing RFP-tagged CKL6 ([B] and [C]) was analyzed 24 h after biolistic DNA delivery. Confocal microscopic images represent paradermal regions of epidermal cells expressing these fluorescently tagged constructs.

(A) CKL6-GFP develops a punctate fluorescence pattern reminiscent of plasmodesmal pit field distribution within epidermal cell walls.  
 (B) CKL6-RFP signal in an *Arabidopsis* epidermal cell shows punctate fluorescence signals similar to those observed in tobacco cells.  
 (C) Same image shown in (B) but combined with the transmitted light image to show the contour of the bombarded cell (indicated by an asterisk).  
 (D) to (F) CKL1-, CKL10-, and CKL13-GFP exhibit punctate fluorescence signals.  
 (G) CKL8-GFP signal appears to label cytoplasm and nucleus and to form punctate particles.  
 (H) CKL4-GFP signal accumulates in the nucleus and cytoplasm; note the absence of a punctate pattern over the cell wall.  
 (I) CKL12-GFP signal was strongest in the cytoplasm but was also present in the nucleus; note the absence of a punctate pattern over the cell wall.

Fusion of GFP either at the CKL N or C terminus did not appear to make a difference in terms of the observed localization patterns (data not shown). These results indicate that the specific localization of the CKL isoforms is not dependent on either terminus being exposed. Among the CKL genes, group 1 isoforms were considered most likely to contain the PPK homolog(s), as they formed punctate fluorescent signals along the periphery of both suspension cultured and epidermal cells. Indeed, in epidermal cells, the fluorescence pattern associated with group 1 isoforms, including CKL1, CKL6, CKL10, and CKL13, was very similar to those earlier observed for viral MPs (Oparka et al., 1997; Itaya et al., 1998; Boyko et al., 2000;

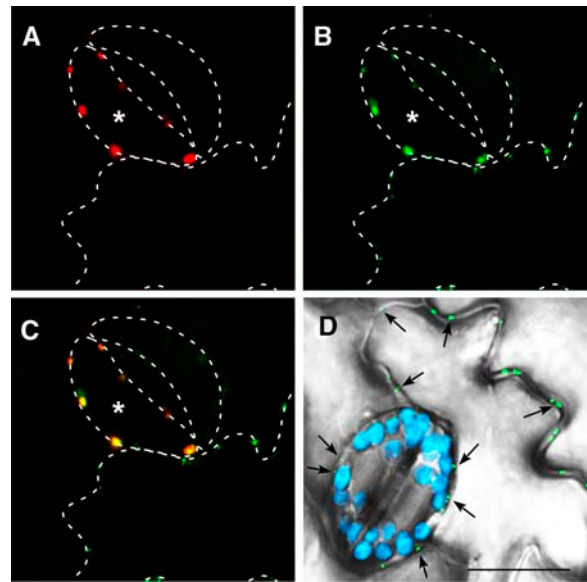


Crawford and Zambryski, 2001; Kotlizky et al., 2001; Lawrence and Jackson, 2001; Roberts et al., 2001; Gorshkova et al., 2003). The CKL8-associated fluorescence pattern appeared rather distinct, in that although the signal appeared punctate in nature the protein was also detected in both the cytoplasm and nucleus. Among the group 1 isoforms, CKL6 gave rise to the most stable expression and, therefore, was selected for further characterization as a putative *Arabidopsis* PAPK1. We first tested whether the localization pattern of CKL6 was consistent between *Arabidopsis* and tobacco. The experiments demonstrated that transient expression of CKL6 in *Arabidopsis* and tobacco leaves yielded similar punctate patterns (Figures 7A to 7C).

### PAPK1 Colocalizes with TMV MP

To address more directly the association of PAPK with plasmodesmata, we next conducted colocalization studies using PAPK1 and TMV MP. To this end, PAPK1 was transiently expressed, as a translational fusion to red fluorescent protein (RFP), in transgenic tobacco plants expressing TMV MP-GFP driven by the 35S constitutive promoter. To ascertain whether PAPK1 and TMV MP use the same intracellular delivery pathway (Ruiz-Medrano et al., 2004; Haupt et al., 2005), we first examined the fluorescence patterns present within specialized epidermal cells, the guard cells. These cells were chosen because, at maturity, they are symplasmically isolated as they do not have functional plasmodesmata (Wille and Lucas, 1984; Palevitz and Hepler, 1985; Ding et al., 1997). As such, these guard cells provide a single-cell-based assay within the context of an intact leaf. Expression of PAPK1-RFP, in individual guard cells, gave rise to strong punctate red fluorescent signals (Figure 8A). A similar green punctate pattern was detected for TMV MP-GFP within the same cell (Figure 8B). However, the strong accumulation of TMV MP-GFP signal within the target guard cell is apparently induced by the ectopic expression of PAPK1, as equivalent TMV MP-GFP signal is not detected within the member of the guard cell pair nor in other guard cells. Note the punctate green fluorescent signals within the epidermal cell walls (Figure 8D), previously shown to reflect the localization of TMV MP-GFP within plasmodesmal pit fields (i.e., the regions of high plasmodesma density; Robards and Lucas, 1990). Importantly, PAPK1-RFP and TMV MP-GFP signals were found to be colocalized (Figure 8C, yellow signals), consistent with the hypothesis that both proteins engage a similar intracellular delivery pathway.

Epidermal pavement cells exhibited a similar pattern of overlapping signals between PAPK1-RFP and TMV MP-GFP (Figure 9). In these experiments, the TMV MP-GFP was detected within the cross-walls as a green punctate signal. Again, it is important to note that, within individually bombarded target cells, punctate signals produced by PAPK1-RFP almost always overlapped with those of TMV MP-GFP (Figures 9A to 9D). These results provide strong support for the hypothesis that PAPK1 is targeted to plasmodesmata, or it is associated with novel plasmodesmal-targeting machinery. Moreover, these studies imply that the *in vivo* interaction between PAPK1 and TMV MP likely occurs either during targeting to and/or within plasmodesmata.



**Figure 8.** Colocalization of PAPK1 and TMV MP in Symplasmically Isolated Guard Cells.

PAPK1-RFP was transiently expressed, by particle bombardments, in transgenic tobacco leaves expressing TMV MP-GFP.

**(A)** Fluorescent signals, produced by PAPK1-RFP in a target guard cell (asterisk), detected in the red channel of the confocal microscope. Contours of the epidermal cells, drawn with white dashed lines, allow identification of the target cell in the context of the epidermal layer.

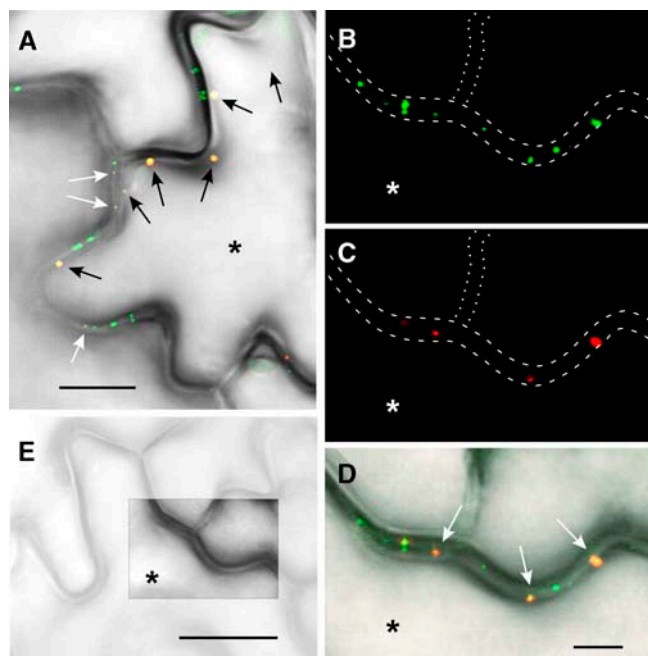
**(B)** Fluorescent signals, produced from TMV MP-GFP, were observed in the periphery of all epidermal cells, detected in the green channel of the confocal microscope. Note that the strong accumulation of TMV MP-GFP signal is only detected in one guard cell that ectopically expressed PAPK1-RFP, which is in contrast with the neighboring guard cell in which TMV MP-GFP was not detectable.

**(C)** Combined image of **(A)** and **(B)** showing overlapping signals between the red and green channels. Note the presence of strong yellow signals confined to the bombarded guard cell.

**(D)** Control image showing guard cells and surrounding epidermal cells in a TMV MP-GFP transgenic tobacco leaf. Picture represents a combined image of signals collected from three different channels. Punctate green signal, representing TMV MP-GFP, accumulates in plasmodesmata (arrows). Note that hardly any signals are detected within guard cells. Blue signal (false color): chloroplast autofluorescence restricted to the guard cell pair. Gray image: epidermal cells collected in the transmitted-light channel. Bar = 20  $\mu$ m for **(A)** to **(D)**.

### *Arabidopsis* PAPK1 Exhibits Similar Enzymatic Properties as Tobacco PAPK

Colocalization data strongly suggested that *Arabidopsis* PAPK1 represents a homolog of the PAPK purified from tobacco. However, stronger evidence would be provided if these two isoforms shared similar enzymatic properties. To this end, full-length *Arabidopsis* PAPK1 was expressed as a GST fusion protein in *Escherichia coli*, affinity purified, and tested for phosphorylation activity. As shown in Figure 10, recombinant *Arabidopsis* PAPK1 phosphorylated TMV MP and its derivatives, BDMV BC1 and LFY, in a similar manner to that observed with the tobacco PAPK (see Figures 3B and 4). Interestingly, recombinant



**Figure 9.** PAPK1 and TMV MP Colocalize within the Cross-Walls of Neighboring Epidermal Cells.

Leaves of a TMV MP-GFP-expressing transgenic tobacco line were bombarded with PAPK1-RFP plasmid and observed by confocal microscopy 24 h after bombardment.

**(A)** Overlapping TMV MP-GFP and PAPK1-RFP signals (yellowish-orange; white arrows) detected within epidermal cross-walls; additional overlapping signals are also detected near the cell periphery (black arrows). Image represents the combination of green, red, and transmitted-light channels. Asterisk indicates the bombarded epidermal cell that is connected to its neighbors by plasmodesmata.

**(B) to (D)** High-resolution images show overlapping signals at the cross-wall of neighboring epidermal cells. Green channel, TMV MP-GFP **(B)**; red channel, PAPK1-RFP **(C)**; combined image demonstrates the overlapping nature of signals **(D)**. Note the confinement of strong yellow-orange signals over the target cell wall (asterisk). Dashed lines in **(B)** and **(C)** illustrate the cell contour, whereas dotted lines indicate the position of a cross-wall slightly out of the focal plane.

**(E)** Epidermal cells imaged at lower magnification by transmitted light; boxed region represents the area shown in **(B)** to **(D)**.

Bars = 10  $\mu\text{m}$  in **(A)**, 5  $\mu\text{m}$  in **(D)**, and 20  $\mu\text{m}$  in **(E)**. Bar in **(D)** is common to **(B)** and **(C)**.

*Arabidopsis* PAPK1 appeared to have relatively stronger substrate preference toward BC1 than LFY. Densitometric analysis indicated that the levels of phosphorylation of TMV MP $\Delta\text{C}_{11}$  and GST-C17 by *Arabidopsis* PAPK1 were 8 and 30% of that observed for the TMV MP, respectively, which is comparable to the tobacco PAPK result (see Figure 3). In 10 replicate experiments, KN1 and BDMV BV1 were not phosphorylated by recombinant *Arabidopsis* PAPK1, but all other proteins showed consistent patterns of phosphorylation. Taken together, the results for substrate specificity, as demonstrated by these experiments, provided strong support for the hypothesis that *Arabidopsis* PAPK1 is a close homolog of tobacco PAPK.

## DISCUSSION

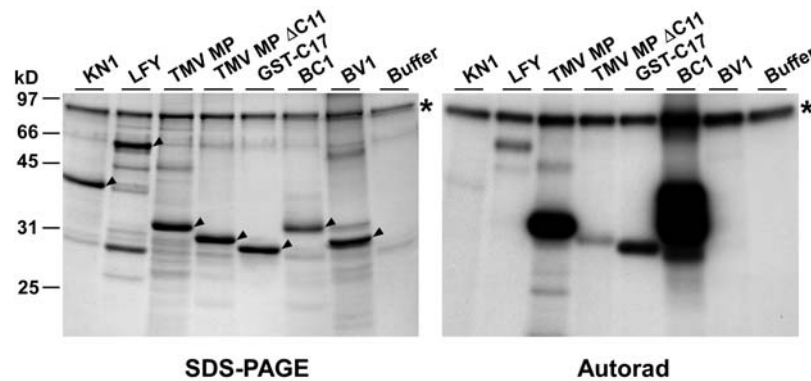
In this study, we describe the molecular characterization of a PAPK. This was achieved using a combination of biochemical enrichment of plasmodesmal proteins and purification methods using TMV MP as a substrate for this plant kinase (Figure 1). Tobacco PAPK was expressed in both suspension cells and leaf tissues. The purified PAPK exhibited calcium-independent kinase activity and could use both ATP and GTP as phosphate donors (Figure 2). PAPK appeared to display substrate specificity in terms of its recognition of viral and plant non-cell-autonomously acting proteins (Figures 3 and 4). Peptide microsequence analysis identified PAPK as a member of the CKI gene family (Figure 5). Punctate localization patterns of group 1 *Arabidopsis* CKL isoforms suggested that they might be closely related to the tobacco PAPK, as they both appear to be targeted to plasmodesmata (Figures 6 and 7). Colocalization studies using PAPK1-RFP and TMV MP-GFP provided support for the notion that these proteins might interact within the cytoplasm and are targeted to plasmodesmata (Figures 8 and 9). Phosphorylation of TMV MP by *Arabidopsis* PAPK1 in a similar manner to tobacco PAPK further strengthens our argument. Although a number of putative plasmodesmal proteins have been identified in recent studies (Escobara et al., 2003; Lucas and Lee, 2004; Oparka, 2004), *PAPK1* is a novel gene to be cloned and characterized. Furthermore, our studies allowed us to assign a novel function for one member of the plant CKI gene family.

### PAPK Phosphorylates TMV MP

Phosphorylation of TMV MP, at multiple Ser/Thr residues, has been observed in both in vitro and in vivo studies (Watanabe et al., 1992; Citovsky et al., 1993; Haley et al., 1995; Karpova et al., 1999; Waigmann et al., 2000; Lee and Lucas, 2001; Karger et al., 2003). Among these various TMV MP phosphorylation sites, the C-terminal three Ser/Thr residues were previously shown to be phosphorylated in vivo, and this posttranslational modification was important for movement function in a host-dependent manner (Waigmann et al., 2000; Trutnyeva et al., 2005). The translatability of a TMV MP-viral RNA complex also appears to be strongly affected by phosphorylation, and this posttranslational modification is correlated with trafficking through plasmodesmata (Karpova et al., 1997, 1999). These attributes made this viral NCAP an ideal substrate for our study. Based on our experiments, the major phosphorylation sites for this PAPK reside within the 11 C-terminal residues of the TMV MP (Figure 3), including the three earlier established in vivo phosphorylation sites (Waigmann et al., 2000). Importantly, this region contains the consensus phosphorylation sites of CKI (i.e., Ser/Thr immediately following a cluster of negatively charged residues; Gross and Anderson, 1998). Previous studies have demonstrated that substrate peptides of 10 to 18 amino acid residues are sufficient for recognition and phosphorylation by CKI (Agostinis et al., 1989; Flotow and Roach, 1991). Consistent with this observation, the GST fusion peptide containing the TMV MP C-terminal 17 residues served as a specific substrate for PAPK (Figure 3).

In our experiments, both PAPK and pumpkin CPK1 could phosphorylate the TMV MP in vitro. However, these kinases





**Figure 10.** PAPK1 Phosphorylates TMV MP in a Similar Manner to Tobacco PAPK.

Recombinant *Arabidopsis* PAPK1 phosphorylates full-length TMV MP and BDMV BC1 strongly and LFY less extensively. The C-terminal deletion mutant (TMV MP $\Delta$ C<sub>11</sub>) and the C-terminal peptide (GST-C<sub>17</sub>) derivative of TMV MP showed reduced phosphorylation by *Arabidopsis* PAPK1 in a manner similar to that observed for tobacco PAPK. Among the tested proteins, KN1 and BDMV BV1 were not phosphorylated by *Arabidopsis* PAPK1. Negative control: phosphorylation reaction in the absence of substrate (buffer) shows GST-PAPK1 (indicated by asterisk) as an autophosphorylating band. Predicted molecular mass of GST-PAPK1 is ~82 kD. Target substrate protein in each lane is marked by arrowheads. Phosphorylation assays were performed essentially as described for Figures 3 and 4, except that 0.4  $\mu$ g of purified GST-PAPK1 was used in 50  $\mu$ L of kinase reaction mixture. The assay was terminated by adding 10  $\mu$ L of 6 $\times$  SDS sample buffer, and 40  $\mu$ L of each reaction was resolved on 12% SDS-PAGE. The gel was stained with GelCode Blue (SDS-PAGE), dried, and exposed to x-ray film (Autorad).

clearly recognize distinct sites, as PAPK phosphorylated the C-terminal residues, whereas pumpkin CPK1 did not. These findings are consistent with the presence of multiple phosphorylation sites on TMV MP (Watanabe et al., 1992; Citovsky et al., 1993; Haley et al., 1995; Karpova et al., 1999; Waigmann et al., 2000; Lee and Lucas, 2001; Karger et al., 2003). The role of additional protein kinases, in terms of modulating the functions performed by TMV MP, will require identification and characterization of such entities. In this regard, it is of interest to note that a plant kinase associated with the endoplasmic reticulum was recently shown to phosphorylate TMV MP at Thr104 (Karger et al., 2003).

#### PAPK Substrate Specificity for Viral and Endogenous NCAPs

It is well established that viral MPs interact with components of the plant NCAP machinery to traffic the infectious material into neighboring cells (Lucas and Gilbertson, 1994; Carrington et al., 1996; Gilbertson and Lucas, 1996; Lazarowitz and Beachy, 1999; Waigmann et al., 2004). Furthermore, cell biological studies have revealed the operation of distinct pathways engaged by various viral systems (Kragler et al., 1998, 2000; Haywood et al., 2002). Thus, an alteration in a particular pathway would affect a subset of viral MPs as well as any NCAPs using this same cell-to-cell trafficking pathway (Lee et al., 2003). Consistent with this notion, our phosphorylation experiments demonstrated that LFY, TMV MP, and BV1 acted as good *in vitro* substrates of PAPK, whereas KN1 and BV1 (Figure 4), as well as CMV MP and RCNMV MP, were either not recognized or served as poor substrates for this kinase.

This level of substrate specificity, as a means of regulating a particular intercellular communication pathway, was earlier demonstrated by our study of NCAPP1 (Lee et al., 2003). Here,

a mutant form of NCAPP1 was shown to block the trafficking of the TMV MP and LFY, but not KN1 nor the CMV MP. Identification of PAPK now provides us with an important tool for the further molecular dissection of the components that operate in the LFY/TMV MP trafficking pathway. Importantly, as LFY has been suggested to move cell to cell via the nontargeted plasmodesmata pathway (Crawford and Zambryski, 2001; Wu et al., 2003; Lucas and Lee, 2004), it will be of considerable interest to ascertain whether trafficking of this NCAP is regulated by phosphorylation. Finally, the TMV MP-based assay used to purify PAPK can now be expanded to screen for kinases that recognize other NCAPs, such as KN1, SHR, CMV MP, and so forth.

#### Colocalization of PAPK1 and TMV MP

The assignment of CKL6 as PAPK1 was based on a number of features. First, it accumulated within the periphery of the cytoplasm, as distinct fluorescent foci (punctuate pattern) (Figures 6 and 7). This pattern is identical to that observed for TMV MP, for which accumulation within plasmodesmata has long been known (Waigmann et al., 2004). Second, in regions where plasmodesmata are grouped into pit fields, TMV MP-GFP fluorescence has been shown to colocalize with a callose-based immunofluorescence signal (Oparka et al., 1997). Callose is a wall material that forms an extracellular collar around plasmodesmata (Northcote et al., 1989; Trethewey and Harris, 2002). Our finding that PAPK1-RFP becomes colocalized with TMV MP-GFP, in connecting cell walls (Figure 9), provides evidence consistent with the hypothesis that it is a plasmodesmal-targeted/localized protein. Third, *Arabidopsis* PAPK1 phosphorylates TMV MP at its C terminus (Figure 10), which suggests that PAPK1 has similar enzymatic properties to that of tobacco

PAPK. The fact that PAPK1 can phosphorylate TMV MP *in vitro* and that they colocalize within cells strongly suggest that they also interact *in vivo*. Direct evidence for an *in vivo* interaction between PAPK1 and TMV MP remains to be obtained.

Colocalization of PAPK1-RFP and TMV MP-GFP, within the periphery of mature guard cells that lack functional plasmodesmata, suggests they use a common pathway for delivery to and accumulation at a region near the plasma membrane (Figure 8). Additionally, the fact that TMV MP-GFP accumulates to higher levels in PAPK1-RFP expressing guard cells (Figure 8) supports the notion that PAPK1 in some way acts to stabilize the TMV MP. This is a stark contrast because normally the TMV MP-GFP signals within the guard cells of transgenic tobacco plants are under detection limit (see Figure 8 and legend). For colocalization studies, we used DsRed2 (Clontech) to label PAPK1 because this version of RFP is modified to reduce the aggregation problem of the original DsRed. However, it still forms tetramers that might prevent PAPK1 from associating properly within plasmodesmata. In this regard, the use of monomeric RFP might be helpful. In any event, localization of PAPK1, at the transmission electron microscopic level, would provide additional information on PAPK1 localization within or near plasmodesmal channels. Generation of PAPK1-specific antibodies would provide the tool for these studies. Finally, based on the similarity in localization patterns observed among group 1 CKL isoforms, these proteins might well be homologs with similar function. The possibility exists, however, that these isoforms exhibit distinct substrate and kinetic characteristics and/or that temporal/spatial expression patterns govern substrate specificity in plants.

### CKI Attributes Are Consistent with PAPK Function

CKI is a unique Ser/Thr protein kinase family highly conserved in all eukaryotes from yeast to humans and plants. All CKI isoforms contain a highly conserved kinase domain, flanked by a variable domain. The kinase domain is basic in nature and distinct in sequence when compared with other Ser/Thr protein kinase gene families (Gross and Anderson, 1998; Vielhaber and Virshup, 2001). In addition, members of the CKI family display broad substrate specificities and are targeted to a range of subcellular domains. These characteristics contribute to the involvement of CKI isoforms in a wide array of regulatory events; recent advances in diverse fields strongly suggested that this kinase family is important in regulating critical signaling processes (Peters et al., 1999; Vielhaber and Virshup, 2001; Price and Calderon, 2002; Eichwald et al., 2004; Jia et al., 2004; Preuss et al., 2004). In yeast, CKI isoforms are genetically linked to vesicular trafficking, DNA repair, cell cycle progression, and cytokinesis (Vancura et al., 1994; Gross and Anderson, 1998; Kafadar et al., 2003; Moriya and Johnston, 2004; Sun et al., 2004).

Although relatively little information is available as to the cellular function of CKI isoforms in metazoans and plants, they have been implicated in regulating Wnt signaling, circadian rhythms, and nuclear import (Peters et al., 1999; Vielhaber and Virshup, 2001; Price and Calderon, 2002; Eichwald et al., 2004; Jia et al., 2004; Preuss et al., 2004). Here, it is noteworthy that a rice CKI isoform was recently linked to control over root development (Liu et al., 2003). In any event, the two most

important properties of CKI family members are that subcellular localization is pivotal to their function, and they appear to be constitutively active and not subject to regulation by classical second messengers (Edelman et al., 1987; Gross and Anderson, 1998). These operational features make CKI family members ideal candidates for conscription into plasmodesmata to exert control over cell-to-cell trafficking of specific macromolecules.

Finally, such PAPKs could well provide an additional level of regulation, as CKI can phosphorylate substrates that have already been modified by other kinases (Flotow et al., 1990; Meggio et al., 1991; Xu et al., 1995). This form of control, in which multiple levels of protein phosphorylation are required for both targeting and modulation of protein function, is emerging as a paradigm for NCAP trafficking in plants (Ivanov et al., 2003; Lucas and Lee, 2004; Waigmann et al., 2004; Trutnyeva et al., 2005). Our observation that both the tobacco and *Arabidopsis* PAPK1 phosphorylate TMV MP suggests that phosphorylation is a common mechanism involved in TMV infection in both species. Previous studies demonstrated that TMV infects *Arabidopsis* and that the ability of TMV to move cell to cell is important for the spread of TMV infection (Dardick et al., 2000; Golem and Culver, 2003). Further investigation of the functional relationship between PAPK1 phosphorylation and TMV infection will provide important insights into the role of PAPK1 in NCAP trafficking.

## METHODS

### PECP Preparations

Maintenance of BY-2 tobacco (*Nicotiana tabacum*) suspension culture cells and preparation of PECP proteins were performed essentially as previously described (Lee et al., 2003). Briefly, cells were harvested 5 d after transfer by filtering 4 liters of culture through Miracloth (Calbiochem). Cells were then given a nondisrupting wash in 75 mM calcium solution to remove noncovalently bound proteins. PECP preparations were also obtained using young leaves of 3-week-old tobacco plants; this tissue was first chopped into small pieces using a BioHomogenizer (BioSpec Products) and then homogenized (BeadBeater; BioSpec Products) using 2.5-mm beads. BY-2 cells or leaf tissue were homogenized, at 4°C, in buffer H (40 mM HEPES, pH 6.8, 20 mM KCl, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, and 10% glycerol) containing a mixture of protease inhibitors (10 µg/mL each of leupeptin and aprotinin and 10 mM phenylmethylsulfonyl fluoride). Cell wall pellets were collected by low-speed (5 min at 300g) centrifugation of cell homogenates and were repeatedly subjected to resuspension in buffer H and low-speed centrifugation until a white-colored pellet was obtained. This pellet was then washed with 1% CHAPS followed by extraction of PECP proteins; subfractions were extracted using a step gradient of 30, 75, and 200 mM calcium.

Proteins contained in each subcellular fraction were resolved by electroporesis in SDS-PAGE gels and stained with GelCode Blue (Pierce) to reveal their protein profiles. Protein levels were determined using the Bradford assay (Bio-Rad).

### PAPK Purification

All subsequent purification steps were performed at 4°C. Proteins were concentrated using Centricon Plus-80 (Millipore) and dialyzed against buffer A (40 mM HEPES, pH 6.8, 2 mM EDTA, and 10% glycerol). Samples were then loaded onto a HiTrap-SP column (Amersham Biosciences)

equilibrated with buffer A, and 1 mL fractions were collected chromatographically in an increasing salt gradient with 1 M NaCl. Peak fractions with TMV MP-phosphorylating activity were pooled, dialyzed, and then loaded onto a new cation exchange column equilibrated with buffer A at pH 8.0. Next, a Con A Sepharose affinity column, equilibrated with buffer C (20 mM Tris-HCl, pH 7.2, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 0.5 M NaCl), was employed. Finally, pooled proteins were purified using a HiTrap Heparin column (Amersham Biosciences) equilibrated with 40 mM HEPES, pH 6.8, and 2 mM EDTA. Bound proteins were eluted with 1 M NaCl in a gradient and collected in 0.5-mL fractions.

### Phosphorylation Assays

Kinase activity assays contained 5  $\mu$ L of a chromatographic fraction plus 1  $\mu$ g of TMV MP (or other purified proteins) in a 50- $\mu$ L reaction volume: assay buffer contained 50 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM DTT, 2 mM EGTA, and 60 to 100 mM ATP mixed with 2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; Amersham Biosciences). For calcium-dependent phosphorylation assays, the reaction buffer was supplemented with 2.2 mM CaCl<sub>2</sub> (Lee et al., 1998). In assays employing *Cucurbita maxima* CPK1, 20 ng of purified recombinant enzyme was used. For kinase assays involving recombinant *Arabidopsis thaliana* PAPK1, 0.4  $\mu$ g of purified GST-PAPK1 was added to the kinase assay mixture. In some assays, [ $\gamma$ -<sup>32</sup>P]GTP (3000 Ci/mmol) was used as the phosphate donor. Kinase assay components were assembled in each tube, on ice, and following transfer to 30°C, the ATP mixture was added and incubated for 10 min. Reactions were stopped by adding 10  $\mu$ L of SDS sample loading buffer. Aliquots (15 to 30  $\mu$ L) of each reaction mixture were resolved in 10 to 12% SDS-PAGE gel and autoradiographed to identify kinase activity. Densitometry analysis of autoradiograph was performed using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

### In-Gel Phosphorylation

In-gel autophosphorylation assays were performed as previously described (Yoo et al., 2002). Briefly, following electrophoresis, the SDS-PAGE gels were washed (2  $\times$  1 h) in buffer D (20% 2-propanol and 40 mM HEPES, pH 7.5). Gels were then incubated for 1 h in buffer D containing 5 mM 2-mercaptoethanol before protein denaturation (1 h) in the same buffer containing 6 M guanidine-HCl. Proteins were renatured by incubation in buffer D containing 3 and 1 M guanidine-HCl (3 h each step). Gels were then incubated (3 h) in buffer D containing 0.04% Tween 20, followed by equilibration (2  $\times$  15 min) in phosphorylation assay buffer containing 50 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, and 2 mM EGTA.

In-gel phosphorylation was performed by incubating the conditioned gel in a complete assay buffer including 2 mM DTT and ATP mixture (10  $\mu$ Ci/mL [ $\gamma$ -<sup>32</sup>P]ATP) for 1 h. Phosphorylation reactions were stopped by rinsing the gels with water and then incubating (4  $\times$  1 h) in 5% trichloroacetic acid and 1% sodium pyrophosphate. In-gel substrate phosphorylation, in which TMV MP was polymerized to a final concentration of  $\sim$ 300  $\mu$ g/mL gel, was performed as for in-gel autophosphorylation assays.

### Expression and Purification of Recombinant Proteins

LFY was expressed in and purified from *Escherichia coli*. pLG2-8 containing LFY cDNA in frame to GST was digested with HindIII and EcoRI, blunt-ended, and self-ligated to produce a C-terminal deletion mutant whose expression in *E. coli* yields functional LFY (Parcy et al., 2002). Soluble GST fusion protein was purified using glutathione-agarose (Sigma-Aldrich) following a standard protocol. Other proteins used in phosphorylation assays were prepared by renaturing inclusion bodies. Briefly, protein expression in actively growing BL21(DE3, pLys) cells (Invitrogen) was induced by treatment with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside for 3 h at 37°C. Cells were then lysed in B-Per bacterial lysis buffer (Pierce) by sonication, and inclusion bodies were pelleted by

centrifugation (5000g, 15 min). Pellets were then washed several times and resuspended in denaturation buffer (2% SDS and 0.1 M NaHCO<sub>3</sub>, pH 9.0), boiled for 4 min, and cooled on ice for 2 min. Renaturation of proteins was performed by a stepwise dialysis in which the ratio between renaturation (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 2 mM DTT) and denaturation buffers was gradually increased from 20:80 to 100:0.

PAPK1 was recalcitrant to expression in *E. coli*, possibly due to its toxic effect to the bacteria. However, the following protocol allowed the production of active full-length enzyme. Briefly, the *Arabidopsis* PAPK1 open reading frame was fused to GST, in frame, using pGEX-KG plasmid (Guan and Dixon, 1991) by PCR cloning. Following DNA sequence confirmation, pGST-PAPK1 was transformed into an *E. coli* host, Rosetta (DE3) pLys (Novagen). Transformed cells were streaked onto Luria-Bertani plates containing chloramphenicol and carbenicillin and grown overnight at 37°C. Cells were then transferred to 750 mL Luria-Bertani media containing antibiotics and grown at 25°C by shaking at 250 rpm until an OD of  $\sim$ 0.8 was attained. Protein expression was induced by adding 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside to the culture and incubating for 2 h at 18°C with gentle shaking. Cells were harvested and lysed with B-PER *E. coli* extraction buffer (Pierce) according to the manufacturer's protocol. Cell extracts were briefly sonicated and centrifuged for 15 min at 4°C. The supernatant was then added to 1 mL of glutathione-agarose beads pre-equilibrated with 50 mM Tris (pH 7.5) and 150 mM NaCl and incubated for 30 min at 4°C while rotating the beads end over end. Following four times of wash in equilibration buffer, GST-PAPK1 was eluted from beads with 10 mM glutathione buffer, pH 8.0, quantified, and used in phosphorylation assays.

### *Arabidopsis* CKL cDNA Cloning and Plasmid Fusion Construction

Plasmid vectors, pdGN, pdGC, and pDR2N, were constructed for the expression of GFP or RFP fusion proteins in plants. The multicloning site (MCS) of pdGN was constructed by transferring the enhanced GFP (EGFP) from pEGFP-PL (Lee et al., 2003) to the expression cassette of a plant expression vector, pN6. First, pN6 was modified to remove the SacI site by blunt-ending the site and self-ligation. The vector was then digested with BamHI followed by blunt-ending and XhoI digestion to remove an intron sequence between the 35S promoter and Ocs terminator. The MCS-EGFP fragment, released from the pEGFP-PL by restriction enzyme digestions with NotI followed by blunt-ending and XhoI, was inserted into the expression cassette by ligation. pdGC was constructed in a similar manner such that it contained the same MCS as that of pdGN but allowed for the formation of EGFP fusion protein in the reverse orientation (i.e., C-terminal GFP-fusion). In pDR2N, EGFP was replaced with DsRed2 (Clontech) to allow the expression of N-terminal RFP fusion proteins.

CKL open reading frames, or cDNAs, were cloned by RT-PCR. For this purpose, total RNA and mRNA were prepared from *Arabidopsis* root tissue using Trizol (Gibco BRL) and the RNeasy (Qiagen) mRNA purification kit, following the protocols provided by the suppliers. Following the RT reactions, employing PowerScript reverse transcriptase (Clontech), PCR amplification was performed with gene-specific primers and a high-fidelity Taq-polymerase (Stratagene). PCR products were then cloned into pdGN, pdGC, or pDR2C, via TOPO cloning kits (Invitrogen). The sequence of all CKL inserts in these TOPO clones was confirmed before subcloning into the respective expression vectors.

### Transient Expression in BY-2 Cells and Plants

For localization of GFP and RFP fusion constructs, transient assays were performed by employing both biolistic DNA delivery and *Agrobacterium tumefaciens*-mediated transfection (agroinfiltration). Transient expression in BY-2 cells was as described (Lee et al., 2003), and bombardment

of *N. tabacum* or *Arabidopsis* leaf tissues was performed basically as for BY-2 cells, except for the use of rupture discs with lower strength (960 p.s.i.). Agroinfiltration in *N. benthamiana* was performed as described (Liu et al., 2002). After 16 to 48 h of incubation, at 25°C, cells expressing GFP or RFP fusion protein were observed by laser scanning confocal microscopy, as described below.

### Microscopic Imaging

Tobacco suspension cultured cells (BY-2) were imaged using a laser scanning confocal microscope (Leica LSM) as described (Lee et al., 2003). Confocal images of leaf cells were acquired on an Axiovert 200M inverted microscope equipped with a Zeiss LSM 510 NLO (Carl Zeiss) using a Zeiss 20× Plan-Apochromat lens (numerical aperture 0.75) or ×40 C-Apochromat (numerical aperture 1.2) objective lens. Data acquisition of EGFP was obtained using the 488-nm laser line of a 25-mW argon laser (LASOS) with a 505LP emission filter. Multichannel images of EGFP and DsRed2 were acquired in fastline-switch mode using the 488- and 543-nm helium neon laser lines (LASOS) with the 500–550 band-pass and 560 long-pass emission filters, respectively. Images were captured as single optical sections (two dimensional) or as a z-series of optical sections (three dimensional). For renderings, three-dimensional data sets were displayed as single maximum intensity projections generated using Zeiss LSM software v3.2.

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY943842 to AY943855.

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