Subcellular Targeting of Nine Calcium-Dependent Protein Kinase Isoforms from Arabidopsis

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Calcium-dependent protein kinases (CDPKs) are specific to plants and some protists. Their activation by calcium makes them important switches for the transduction of intracellular calcium signals. Here, we identify the subcellular targeting potentials for nine CDPK isoforms from Arabidopsis, as determined by expression of green fluorescent protein (GFP) fusions in transgenic plants. Subcellular locations were determined by fluorescence microscopy in cells near the root tip. Isoforms AtCPK3-GFP and AtCPK4-GFP showed a nuclear and cytosolic distribution similar to that of free GFP. Membrane fractionation experiments confirmed that these isoforms were primarily soluble. A membrane association was observed for AtCPKs 1, 7, 8, 9, 16, 21, and 28, based on imaging and membrane fractionation experiments. This correlates with the presence of potential N-terminal acylation sites, consistent with acylation as an important factor in membrane association. All but one of the membrane-associated isoforms targeted exclusively to the plasma membrane. The exception was AtCPK1-GFP, which targeted to peroxisomes, as determined by coin visualization with a peroxisome marker. Peroxisome targeting of AtCPK1-GFP was disrupted by a deletion of two potential N-terminal acylation sites. The observation of a peroxisome-located CDPK suggests a mechanism for calcium regulation of peroxisomal functions involved in oxidative stress and lipid metabolism.

Calcium-dependent protein kinases (CDPKs) are Ser/Thr protein kinases that are only found in plants and some protozoans. They are composed of a variable N-terminal domain, a catalytic domain, an auto-inhibitory region, and a calmodulin-like domain (Harper et al., 1991; Suen and Choi, 1991; Huang et al., 1996; Cheng et al., 2002; Hrabak et al., 2003). CDPKs are activated upon binding calcium to their calmodulin-like domain, which makes them effective switches for the transduction of calcium signals in plant cells.

Calcium signaling has emerged as a central mechanism to regulate responses to environmental stimuli such as cold, salt stress, and drought (for review, see Knight and Knight, 2001; Sanders et al., 2002). Research on CDPKs from several plant species has implicated these kinases in a variety of stimulus response pathways. In maize (Zea mays) leaf protoplasts, transient overexpression of constitutively active AtCPK10 or AtCPK30 caused the activation of the stress-inducible HVA1 promoter (Sheen, 1996). In rice (Oryza sativa), overexpression of OsCPK7 made the transgenic lines more tolerant to cold, drought, and salt stress (Saijo et al., 2000). In tomato (Lycopersicon esculentum) protoplasts, overexpression of AtCPK1 enhanced activity of NADPH oxidase and oxidative burst (Xing et al., 2001). In tobacco (Nicotiana tabacum), a virus-induced gene silencing method used to silence CDPKs reduced and delayed the hypersensitive response to fungal Avr9 elicitor (Romeis et al., 2001).

There are 34 CDPK genes in Arabidopsis. Although these kinases all have strongly conserved catalytic and calmodulin-like regulatory domains (Harmon et al., 2000, 2001; Hrabak, 2000), they are distinguished by their highly variable N-terminal domains, with lengths ranging from 21 to 185 amino acids and identities ranging from 15% to 91%. Twenty-seven of the 34 Arabidopsis isoforms contain predicted myristoylation sites with a Gly at position 2. Myristoylation has been shown to promote membrane association of OsCPK2 (Martin and Busconi, 2000), LeCPK1 (Rutschmann et al., 2002), and AtCPK2 (Lu and Hrabak, 2002). However, myristoylation by itself is normally not sufficient for membrane localization (Shahinian and Silvius, 1995). Stable anchoring to membranes requires a second feature like a polybasic domain with a cluster of positively charged amino acids.
acids, or an additional lipid group close to the myristoylation site (Resh, 1999). Twenty-nine Arabidopsis CDPKs have Cys residues near their N termini that are possible sites for the addition of palmitate. This reversible modification is crucial for the correct membrane localization of many proteins (McCabe and Berthiaume, 1999; for review, see Yalovsky et al., 1999). Only five AtCPKs have neither Gly nor Cys as potential acylation sites at their N termini. Taken together, lipid modifications are expected to play an important role in the localization of most CDPKs.

There is evidence for CDPKs in multiple subcellular locations, including the plasma membrane (e.g., Schaller et al., 1992; Verhey et al., 1993), endoplasmic reticulum (ER) membrane (Lu and Hrabak, 2002), endosperm storage vesicles (Anil et al., 2000), actin cytoskeletal system (Putnam-Evans et al., 1989), mitochondria (Pical et al., 1993), and nucleus (Patharkar and Cushman, 2000). However, in Arabidopsis, an isoform-specific subcellular location (ER) has been reported for only one of the 34 isoforms (AtCPK2; Lu and Hrabak, 2002).

Here, we provide evidence for the potential subcellular locations of nine additional Arabidopsis isoforms, based on localization of green fluorescent protein (GFP) fusions. Our results delineate three additional targeting patterns for the Arabidopsis CDPK family, with membrane association observed for all seven isoforms harboring potential N-terminal myristoylation sites. Interestingly, plasma membrane association was observed for six of these seven isoforms. The exception was AtCPK1-GFP, which was found associated with peroxisomes. These results demonstrate that in Arabidopsis, different CDPKs are targeted to specific subcellular locations, providing the potential for isoform-specific differences in regulating various cellular functions. In the case of AtCPK1, our results provide the first evidence for the potential role of a CDPK in regulating peroxisomal functions such as lipid metabolism and oxidative stress.

RESULTS

Subcellular Locations of AtCPK-GFP Fusion Proteins in Arabidopsis Root Tips

Potential subcellular locations for nine CDPKs were determined by expression of AtCPK-GFP fusions in stable transgenic plants. Isoforms AtCPKs 1, 3, 4, 7, 8, 16, 21, and 28 were chosen as a starting point for a complete survey of all 34 Arabidopsis isoforms, because these genes were available as full-length cDNAs at the initiation of the project. Each kinase was engineered with a C-terminal GFP and was expressed in plants under the control of a cauliflower mosaic virus 35S promoter. The nine isoforms revealed three different subcellular distribution patterns, as detected by fluorescence confocal microscopy of cells located near the root tip (Fig. 1).

The subcellular distribution of GFP alone is shown as a control in Figure 1A. These images provide a reference for cytosolic and nuclear localization patterns, as previously shown (e.g., Haseloff et al., 1997). For each CPK-GFP construct, multiple transgenic lines were examined, and images were analyzed for cells showing both high and low levels of expression. The patterns shown were observed consistently, even at the lower limits of detection in weakly fluorescent cells. This supports the contention that the observed localization patterns are not an artifact of extreme overexpression but rather reflect realistic targeting potentials for endogenous enzymes.

Imaging of isoforms AtCPK3-GFP and AtCPK4-GFP revealed distribution patterns similar to that of free GFP, including nuclear fluorescence in most cells (Fig. 1, C and D). This suggests that both isoforms are primarily soluble enzymes with the potential to target to the nucleus, although neither AtCPK3 nor AtCPK4 contains nuclear localization signals when analyzed by ProSite (Falquet et al., 2002). The presence of a CPK-GFP in the nucleus does not appear to be an artifact resulting from a proteolytic release of free GFP, because no degradation products the size of GFP were detected in a western-blot analysis of tissue extracts (not shown). Consistent with AtCPK3 and AtCPK4 as primarily cytosolic/nuclear enzymes, they partitioned as soluble proteins in a membrane fractionation analysis (Fig. 2).

The six GFP fusions for isoforms AtCPKs 7, 8, 9, 16, 21, and 28 were all observed in a thin layer at the periphery of the cells, consistent with a plasma membrane location. Moreover, all of these GFP fusions cofractionated with the microsomal membrane fraction (Fig. 2; AtCPK7-GFP is shown as a representative example).

AtCPK1-GFP was primarily seen in small, often spherical organelles approximately 0.5 to 1.5 μm in diameter, which is the typical size of mitochondria and peroxisomes (Fig. 1B). Consistent with an association with an organelar membrane, AtCPK1-GFP cofractionated with the microsomal membrane fraction, as shown by western-blot analysis in Figure 2.

AtCPK1-GFP Is Associated with Peroxisomes

To determine the identity of AtCPK1-GFP-associated organelles, we conducted covisualization experiments with markers for mitochondria and peroxisomes. For covisualization with mitochondria, we used the mitochondrion-specific fluorescent dye MitoTracker Red. The projected three-dimensional image in Figure 3A shows very little overlap between the MitoTracker and GFP signals. This result supports a non-mitochondrial location for AtCPK1-GFP.

To test whether AtCPK1-GFP was colocalized with
peroxisomes, we first compared plant cells expressing either AtCPK1-GFP or a peroxisome targeted GFP. A modified GFP was targeted to peroxisomes by the addition of a C-terminal sequence KSRM (GFPrs-KSRM; Trelease et al., 1995). The organelles visualized with both AtCPK1-GFP and GFPrs-KSRM were very similar in size, morphology, and numbers (Fig. 3, B and C). Equivalent images were also observed with Arabidopsis leaf cells and tobacco cv Bright Yellow 2 cells (data not shown). Together, these results support a peroxisome location for AtCPK1-GFP.

Figure 1. Three different patterns of AtCPK-GFP localization in Arabidopsis roots. Confocal images of transgenic Arabidopsis roots that express either GFP alone (GFP) or AtCPK-GFP fusion proteins (AtCPKs 1, 3, 4, 7, 8, 9, 16, 21, and 28) under the control of the 35S promoter. The pictures on the left show an overview; the pictures on the right represent enlargements of a selected region. Plant lines were SS#156 (AtCPK1), SS#175 (AtCPK3), SS#284 (AtCPK4), SS#150 (AtCPK7), SS#286 (AtCPK8), SS#153 (AtCPK9), SS#288 (AtCPK16), SS#290 (AtCPK21), and SS#292 (AtCPK28). Plant lines for Figure 4 were SS#156 (AtCPK1) and SS#294 (AtCPK1-ΔM).
To further verify a peroxisomal location, we conducted ccowisualization experiments in which peroxi-
somes and AtCPK1 were labeled with two spectrally
distinct GFPs. For the experiment shown in Figure 3,
D through F, AtCPK1 was labeled with a “shar-
green” GFP (GFPrs) with an excitation spectrum
peaking at 398 nm. The peroxisome-targeted GFPrs-
KSRM was constructed with a “red-shifted” GFP
(GFPs) with an excitation peak at 488 nm (similar to
EGFP, which was used for all other CDPK-GFP fu-
sions). Both GFPs retained the same emission peak
at 511 nm. Controls showed that each type of GFP could
be uniquely visualized using the excitation filters and
exposure times used in this study. When AtCPK1-
GFPrs and GFPrs-KSRM were co-expressed in stably
transformed plants, their fluorescence images overlapped
with each other (Fig. 3, D–F). Equivalent re-
sults were obtained with a second set of constructs in
which the GFPs and GFPs tags were switched with
respect to the labeling of CPK1 and a peroxisome-
targeted GFP (not shown). This colocalization anal-
ysis strongly supports a peroxisomal location for
AtCPK1-GFP.

A final line of evidence for a peroxisomal location of
AtCPK1-GFP is the distinct “torus” morphology that
could be observed in high-magnification images,
such as Figure 3D. This torus or “doughnut” mor-
phology has previously been noted for images of plant peroxisomes (e.g. Cutler et al., 2000;

Removal of Potential Acylation Sites Prevents
Peroxisome Association of AtCPK1-GFP

As predicted for many CDPKs, AtCPK1 has two
potential acylation sites at its N-terminal end. To
investigate whether these acylation sites have a role
in peroxisome targeting, they were removed by
swapping the seven N-terminal amino acid residues
(MGNTCVG-P8) of AtCPK1 with the four N-terminal
residues from AtCPK12 (MANK-P5). The rationale
was to swap N-terminal extensions defined by the
sequences located upstream from the first Pro in each
CDPK (positions 8 and 5, respectively). This se-
quence exchange removed all potential acylation
sites, namely the Gly in position 2 (for myristoyla-
tion) and the Cys in position 5 (for palmitoylation).
The resulting mutant construct, referred to as
AtCPK1-ΔM-GFP (ΔM for deletion of myristoyla-
tion), failed to localize to peroxisomes (Fig. 4B).
Instead, this acylation-deficient mutant protein was
mainly present in the cytosol. Equivalent results
were observed in Arabidopsis leaves and tobacco cv
Bright Yellow 2 cells (data not shown).

To confirm the disruption of peroxisome localiza-
tion, we fractionated membranes and soluble pro-
teins and performed a western-blot analysis. The
wild-type AtCPK1-GFP was more abundant in the
membrane fraction, whereas the mutant AtCPK1-
ΔM-GFP was almost exclusively found in the soluble
fraction (Fig. 4C). There was no evidence for prote-
oysis of either AtCPK1-GFP or AtCPK1-ΔM-GFP in
the transgenic lines, indicating that the observed dif-
ference in localization was not an artifact caused by
the proteolytic release of GFP.

DISCUSSION

CDPKs Are Located in Many Subcellular Locations

To understand the isoform-specific functions of all
34 Arabidopsis CDPKs, it will be necessary to deline-
eate their subcellular locations, tissue specificity, sub-
strate specificities, and biochemistry. Here, we pro-
vide a comparison of subcellular targeting properties
for nine AtCPK isoforms. Our results provide evi-
dence for: (a) a cytosolic and nuclear location for
isoforms AtCPKs 3 and 4 (Fig. 1, C and D), (b) a
plasma membrane location for isoforms AtCPKs 7, 8,
9, 16, 21, and 28 (Fig. 1, E–J), and (c) a peroxisome
membrane location for isoform AtCPK1 (Figs. 1B and
4). Together with evidence for an ER location of
AtCPK2 (Lu and Hrabak, 2002), there is now support
for at least four distinct isoform-specific localization patterns for Arabidopsis CDPKs. In studies on other
plants, there is also evidence for CDPKs associated
with endosperm storage bodies (Anil et al., 2000),
mitochondria (Pical et al., 1993), plasma membranes,
and nuclei (Patharkar and Cushman, 2000; Ruth-
schmann et al., 2002). Thus, CDPKs are clearly lo-
eated in many subcellular locations, some of which
are unique to specialized cell types (e.g. endosperm
storage bodies; Anil et al., 2000).

This survey includes at least two CDPKs from each
of the four subfamilies (Hrabak et. al., 2003): I, CPK1
and 4; II, CPK3, 9, and 21; III, CPK7 and 8; and IV,
CPK16, 28. The analysis of the CDPK-GFP fusions
shows clearly that members of the same subfamily do
not necessarily share the same localization. CPK1 and
CPK4 both belong to subfamily I and are 64.5% iden-
tical, but their distribution in the cell is very different.
CPK1 and CPK2 not only belong to the same subfam-
ily but are 83.4% identical. However, these two CD-
PKs are localized to different endomembranes. On
the other hand, CPK16 from subfamily IV and CPK8

![Figure 2: AtCPK3 and AtCPK4 fractionate as soluble proteins, whereas all others are membrane-associated. Western-blot analysis of proteins from transgenic Arabidopsis roots probed with anti-GFP antiserum. Equal amounts of protein (5 μg) were loaded in each lane. S, Soluble protein fraction; M, Microsomal membrane fraction. Results showing membrane association for AtCPK7 are representative for AtCPKs 8, 9, 16, 21, and 28 (not shown).](http://deepgreen.stanford.edu)
from subfamily III localize to the plasma membrane despite being only 36% identical. Thus, the relatedness of CDPKs does not provide a means to predict the possible localization.

Although our survey clearly identifies isoform-specific differences in targeting potentials, we did not verify the actual locations of endogenous (untagged) kinases. Our approach was limited to a survey of GFP-tagged isoforms, expressed using a 35S promoter, and imaged in root tip cells. Potential differences between locations of GFP-tagged and endogenous CDPKs could result from several factors, including the following: (a) The addition of a C-terminal GFP-tag may interfere with proper targeting, for example, by disrupting a targeting signal or a protein-protein interaction. (b) The imaging of isoexpress either AtCPK1-GFP or the peroxisome-targeted GFPs-KSRM fusion protein (C). D through F, Wide-field images of an Arabidopsis root expressing both AtCPK1-GFP and GFPs-KSRM. D, AtCPK1-GFP image (530-nm emission, 800 ms) obtained from fluorescence at 405-nm excitation (shown in green). E, GFPs-KSRM peroxisome image (530-nm emission, 200 ms) obtained from fluorescence at 490-nm excitation (shown in red). In control experiments with AtCPK1-GFP and GFPs-KSRM expressed independently, we confirmed that the images for each channel were specific for only one GFP fusion within a 4-fold range of exposure time (data not shown). “Bleed-through” from GFPs-KSRM at 405-nm excitation and AtCPK1-GFP at 490-nm excitation was insignificant using the described settings. F, Merged images from D and E. Yellow color indicates the extensive overlap of AtCPK1-GFP and GFPs-KSRM signals.

Figure 4. Removal of potential acylation sites from AtCPK1-GFP results in loss of peroxisomal localization. Confocal images of epidermal and cortical cells of an Arabidopsis root expressing AtCPK1-GFP (A) and an Arabidopsis root expressing AtCPK1-ΔM-GFP (B). In each picture, the approximate boundary of one cell is indicated by brackets. C, A western blot with proteins extracted from roots of transgenic plants probed with anti-GFP antibody. Equal amounts of protein (5 μg) were loaded in each lane. S, Soluble protein fraction; M, microsomal membrane fraction.
forms ectopically expressed in root tip cells may fail to reveal targeting potentials unique to the specific cell type, such as a guard cell or pollen tube. (c) The environmental conditions under which the roots were grown and imaged may have failed to reveal a conditional targeting potential, such as seen for Mc-CPK1, which appears to translocate from the plasma membrane to the nuclei of ice plant (Mesembryanthemum crystallinum) epidermal cells only after exposure to salt stress (Patharkar and Cushman, 2000). (d) Overexpression of a tagged isoform may result in aberrant targeting, for example by overwhelming the capacity of a membrane-sorting pathway. Although we cannot exclude any of the preceding caveats for a given isoform, we have conducted immunodetection of CDPKs in an aqueous two-phase membrane fractionation experiment (data not shown). These analyses show that some CDPKs are present in the soluble fraction, others are enriched in the endomembrane fraction, and still others are enriched in the plasma membrane fraction. Thus, the isoform-specific targeting potentials reported here are consistent with expected locations of endogenous isoforms based on simple membrane fractionations.

AtCPK1-GFP Is Associated with Peroxisomes

Of the seven membrane-associated CDPKs examined here, AtCPK1-GFP was the only one that was not associated with the plasma membrane. Two lines of evidence support the contention that AtCPK1-GFP is associated with peroxisomes. First, imaging of GFP-tagged AtCPK1 indicated that AtCPK1-GFP is associated with 0.5- to 1.5-μm organelles, a size most consistent with peroxisomes and mitochondria. These organelles were shown not to be mitochondria by colocalization experiments with a mitochondrion-specific dye (Fig. 3A). In contrast, colocalization experiments with a peroxisomal marker showed a high degree of overlap (Fig. 3, B–F). Second, when AtCPK1-GFP fluorescence was examined at high magnification, the spherical bodies were frequently observed to have a doughnut morphology. This same morphology was observed with a marker (GFPps-KSRM) targeted to the lumen of peroxisomes. A similar morphology has also been noted for other peroxisome-targeted proteins (http://deepgreen.stanford.edu), including a common peroxisomal marker (catalase) tagged with GFP.

Although we have not excluded the possibility that AtCPK1-GFP is located within the peroxisome, occurrence at the cytosolic surface is considered more likely. This is based on our observation that peroxisome localization was disrupted by removal of the N-terminal acylation sites of AtCPK1 (Fig. 4). To our knowledge, there is no precedent for the involvement of an N-terminal myristoyl or palmitoyl group in peroxisomal import. Thus, the simplest explanation is that acylation potentiates an AtCPK1 peroxisome membrane interaction analogous to the membrane interactions of CDPKs localized to the ER or plasma membrane (Martin and Busconi, 2000; Lu and Hrabak, 2002).

Mechanism of Membrane-Specific Targeting

Most CDPKs have predicted N-terminal acylation sites that could promote membrane association. This prediction has been experimentally confirmed for three isoforms: OsCPK2, LeCPK1, and AtCPK2 (Martin and Busconi, 2000; Lu and Hrabak, 2002; Rutschmann et al., 2002). Here, we provide additional support by showing that two CDPKs without putative acylation sites fractionated as soluble enzymes (AtCPKs 3 and 4), whereas all seven CDPKs harboring potential acylation sites were found to be membrane-associated (AtCPKs 1, 7, 8, 9, 16, 21, and 28; Fig. 1). These seven CDPKs harbored sites for both myristoylation and palmitoylation (e.g. in AtCPK1, MGNTCVGP). When both acylation sites were disrupted in AtCPK1, we observed a nearly complete loss of membrane association, similar to that observed for OsCPK2 (Martin and Busconi, 2000). In contrast, a disruption of only one acylation site (penultimate G) in AtCPK2 was reported to result in only a partial loss (50%) of membrane association (Lu and Hrabak, 2002). Thus, for some CDPKs, multiple acylation events may be important for membrane interactions. This is consistent with results on other acylated proteins. For example, stable plasma membrane association of the protein Tyr kinase p59fyn and the G-protein subunit Gaα, only occurs if the proteins are myristoylated and palmitoylated (Alland et al., 1994; Morales et al., 1998).

At present, the majority of membrane-associated CDPKs have been found localized to the plasma membrane (e.g. AtCPKs 7, 8, 9, 16, 21, 28, LeCPK1, and OsCPK2). However, two of the Arabidopsis isoforms are now known to associate with endomembranes, with AtCPK1 associated with peroxisomes (this study) and AtCPK2 with ER (Lu and Hrabak, 2002). Thus, the presence of acylation sites is not predictive of which membrane a CDPK will target to. Other targeting information may include additional protein-protein interactions and protein modifications such as phosphorylation.

In the context of isoform-specific targeting of CDPKs, it is noteworthy that the N-terminal acylation sites are located in highly variable N-terminal domains. Because acylation is only one of several features contributing to membrane association, we speculate that other sequences within the N-terminal domains may provide the basis for isoform-specific protein-protein interactions. Even among the isoforms known to target to the plasma membrane, there is no obvious conserved sequence that suggests a common mechanism for plasma membrane localization. Thus, it is possible that many of the plasma
membrane isoforms are actually integrated into different isoform-specific regulatory complexes.

**Isotypic-Specific Functions for CDPKs**

Subcellular targeting information presented here provides a starting point for understanding the isotypic-specific functions for nine of the 34 AtCDPKs. For AtCDPKs 3 and 4, which may translocate between the cytosol and nucleus, potential substrates include ion pumps and channels (e.g., Li et al., 1998; Hwang et al., 2000). This finding provides a mechanism to dedicate a unique calcium-sensor module to the regulation of a given complex. Although Arabidopsis CDPKs in the above locations were expected based on other plant studies, our results with AtCDPK1 provide the first evidence for a peroxisome-associated CDPK. This finding provides a potential mechanism for calcium regulation of various metabolic pathways, including lipid metabolism and oxidative stress, or the movement of peroxisomes through the cytoplasm.

**MATERIALS AND METHODS**

**Plant Material and Transformation**

All transgenic plants were generated using Arabidopsis ecotype Columbia. The following CDPK-GFP fusion constructs were transformed into plants with *Agrobacterium tumefaciens* (GV3101; Koncz and Schell, 1986) using the floral dip method (Clough and Bent, 1998):

- AtCPK1-ΔM (PS#602), AtCPK1 (PS#601), AICPK3 (PS#284), AICPK4 (PS#364), AICPK5 (PS#605), AICPK21 (PS#506), and AICPK28 (PS#507). Three other constructs GFPs-KSRM (PS#949), AtCPK1-GFPsg (PS#998), and GFPs-KSRM/AtCPK1-GFPsg (PS#600) were transformed via spraying *A. tumefaciens* (GV101) supported in 0.2% (w/v) Silwet L-77 (Lethle Seeds, Round Rock, TX) onto developing flowers (Weigel et al., 2000). Transgenic lines were selected on 30 mg L⁻¹ kanamycin plates.

**DNA Constructs**

CDPK cDNAs were amplified by PCR with the following gene-specific primers (the first and last codon of the cDNA is in bold, restriction sites are underlined): AtCPK1-5' (867A): 5'-CAGTACGCTAAAACATGGGATGACT-TGGTT3' and CPK1-3' (867B): 5'-CTGGCCGTCCTCATAGGATTTTTTCT-TCAC-3'; AtCPK3-5' (255A): 5'-AGGCTCGAC-AATGGGCGACAGAC-AGGAACTAACAAATC-3' and CPK3-3' (255B): 5'-TCAATACTGCGCC-CCATTCTGCGTCGGTTTGGCAC-3'; AtCPK4-5' (256A): 5'-AAGCTCGAC-AATGGGCGACAGAC-AGGAACTAACAAATC-3' and CPK4-3' (256B): 5'-TCAATACTGCGCC-CCATTCTGCGTCGGTTTGGCAC-3'; AtCPK21-5' (734A): 5'-AAGCTCGAC-AATGGGCGACAGAC-AGGAACTAACAAATC-3' and CPK21-3' (734B): 5'-CGGGTCTAGACCGCCACCCTGGAATCCGGAC-3'.

All CDPK clones derived from PCR were sequenced to verify the absence of mistakes. Fragments were cloned into the XhoI/SpeI site in vector p35S-GFP-JFH1 (Hong et al., 1999), a pmG19 vector derived with a kanamycin resistance marker for *A. tumefaciens* and plants (Frech et al., 1995). The resulting constructs consist of a 35S promoter, a CDPK cDNA, an EGFP protein, a 6His tag, and a NOS terminator. AICPK1-ΔM-GFP was generated by amplifying a cDNA of AtCDPK1 with primers CDPK1-ΔM5' (869): 5'-CTGGCCGTCCTCATAGGATTTTTTCT-TCAC-3' and CDPK1-3' (870): 5'-TCAATACTGCGCC-CCATTCTGCGTCGGTTTGGCAC-3'. The new 5' end of the gene has the sequence ctcgagggccctgaagcggccgagaattgcgctctgggccgagc-3'.

The GFP constructs for Figure 3, B and D to F, are based on Monsanto plant expression vector pMON10998 (Klee et al., 1991). To build GFP fusion constructs, vectors with only GFP were made first. GFP obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus; http://aims.cps.msu.edu/aims/) GenBank accession no. U70495 was mutated to make GFP with shifted excitation spectra. A GFP variant was made by mutating Ser-65 to Thr using primers G65T-5' (870A), 5'-CAGTACTCTCTGATGTGTGCGCCGGT-3' and G65T-3' (870B), 5'-ACACCATATGTGAAGTCGCGCGCCG-3'. (Heim et al., 1995). A GFPs variant was made by mutating Ser-202 to Phe and Thr-203 to Ile using primers G202F-5' (871A), 5'-CATCTCTCTCACAATCTCGGC-3' and G202F-3' (871B), 5'-GCGAGTGTGTAACAGGTGAATG-3'. (Heim et al., 1994). Primes G-fuses5' (872A), 5'-CTGGAGATCGTCGGTTTGGCACGAGAAATCGGATGGAAGCAGTT-3'. A GFPsg variant was made by mutating Ser-202 to Phe and Thr-203 to Ile using primers G202F-5' (871A), 5'-CATCTCTCTCACAATCTCGGC-3' and G202F-3' (871B), 5'-GCGAGTGTGTAACAGGTGAATG-3'. (Heim et al., 1994). The mutated GFPs were cloned into the EcoRI site of a modified pMON10998 vector. The resulting construct (pMGr.ps; PS#998) has GFPs with the amino acids KSRM added to the C-terminal end, the 35S promoter driving the open reading frame followed by GFP with a S6NB and Ascl site at the 5' end for cloning in frame with GFP, a hinge to separate GFP from the cloned sequence and an E9 terminator. GFP targeted to peroxisomes (seen in Fig. 3C) was accomplished by adding the plant peroxisome-targeting signal KSRM to the beginning of GFPs. Primers oGFP12 (873A), 5'-GAGTACTCTCTGATGTGTGCGCCGGT-3' and oGFP-CRM (873B), 5'-GCGAGTGTGTAACAGGTGAATG-3'. (Heim et al., 1994). The resulting construct (pMGr.ps; PS#998) has GFPs with the amino acids KSRM added to the C-terminal end, the 35S promoter driving the open reading frame, and an E9 terminator.

The construct with two GFPs (used in Fig. 3, D-F) was prepared by first building AtCDPK1 fused to GFPsg, AtCDPK1 was amplified and cloned into pMGr.ps. The resulting construct, pMGr.AICPK1-5' (PS#999), was combined with pMGr.dpx.r (PS#957) to make a double GFP construct (pMGr.dpx.r, is similar to pMGr.ps.r, except that it has an additional SfiI site for tandem cloning). The resulting construct pMGr.ps.AICPK1 (PS#600) has both GFPs-KSRM and AtCDPK1-GFPsg expressed in tandem on the same plasmid.

**Imaging**

Seeds from transgenic plants were selected on Murashige and Skoog plates (one-half-strength Murashige and Skoog salts and 0.5% [w/v] Suc, pH 5.7) containing 30 mg L⁻¹ kanamycin. After 10 d, plants were transferred to Murashige and Skoog plates without antibiotics and were grown at 23°C with constant light. After 1 week, plants were used for imaging. Plants in Figure 4 were treated overnight with 10 μM dexamethasone before imaging.
Images of CDPK-GFP fusion proteins in Figures 1, 3, B and C, and 4 were captured with a confocal laser scanning microscope (IX70 [Olympus, Tokyo/with Fluoview software]). Excitation and emission filter peaks were 488 and 525 nm, respectively.

For covisualization of MitoTracker Red (Molecular Probes, Eugene, OR) and AtCPK1-GFP, the image stack in Figure 3A was captured with a computational optical sectioning microscope system with the 20X lens and the filters described by Gens et al. (1996). Use of a Gaussian filter and preparation of the stereo view was accomplished with the software and parameters also described by Gens et al. (1996). As MitoTracker Red easily crosses cell walls and membranes, no special loading procedure was necessary. Before imaging, the transgenic AtCPK1-GFP roots were simply incubated in 0.2 μM MitoTracker Red for 7 min on ice.

Localization of AtCPK1-GFP and KSRM-GFP fusion proteins in Figure 3, D through F, was assessed with a wide-field fluorescence microscope fitted with controls and Slidebook software by Intelligent Imaging Innovations (Santa Monica, CA). Excitation filters were 405 and 490 nm, respectively, with a bandpass of 20 nm; the emission filter was 530 nm with a bandpass of 20 nm. All micrographs were obtained with a bandpass of 20 nm.

Localization of GFP and GPPs was determined using a Gaussian filter and the filters described by Gens et al. (1996). Use of a Gaussian filter and preparation of the stereo view was accomplished with the software and parameters also described by Gens et al. (1996). As MitoTracker Red easily crosses cell walls and membranes, no special loading procedure was necessary. Before imaging, the transgenic AtCPK1-GFP roots were simply incubated in 0.2 μM MitoTracker Red for 7 min on ice.

Microsomal Membrane Preparation

Two-week-old transgenic Arabidopsis plants were further cultivated in liquid Murashige and Skoog medium (one-half-strength Murashige and Skoog salts, 2% [w/v] Suc, and vitamin solution [Sigma, St. Louis], pH 5.7) at 22°C for three weeks in the dark on a rotary shaker. Roots were drained and frozen in liquid nitrogen. Samples were ground in a mortar and mixed with homogenization buffer (100 mM Tris-HCl, pH 7.5, 30 mM Suc, 10 mM EDTA, and 2 mM EGTA). To remove cell debris, the slurry was first passed through cheesecloth and then centrifuged for 15 min at 2,000 rpm. For covisualization of MitoTracker Red (Molecular Probes, Eugene, OR) and AtCPK1-GFP, the image stack in Figure 3A was captured with a computational optical sectioning microscope system with the 20X lens and the filters described by Gens et al. (1996). Use of a Gaussian filter and preparation of the stereo view was accomplished with the software and parameters also described by Gens et al. (1996). As MitoTracker Red easily crosses cell walls and membranes, no special loading procedure was necessary. Before imaging, the transgenic AtCPK1-GFP roots were simply incubated in 0.2 μM MitoTracker Red for 7 min on ice. localization of AtCPK1-GFP and KSRM-GFP fusion proteins in Figure 3, D through F, was assessed with a wide-field fluorescence microscope fitted with controls and Slidebook software by Intelligent Imaging Innovations (Santa Monica, CA). Excitation filters were 405 and 490 nm, respectively, with a bandpass of 20 nm; the emission filter was 530 nm with a bandpass of 20 nm. All micrographs were obtained with a bandpass of 20 nm. localization of GFP and GPPs was determined using a Gaussian filter and the filters described by Gens et al. (1996). Use of a Gaussian filter and preparation of the stereo view was accomplished with the software and parameters also described by Gens et al. (1996). As MitoTracker Red easily crosses cell walls and membranes, no special loading procedure was necessary. Before imaging, the transgenic AtCPK1-GFP roots were simply incubated in 0.2 μM MitoTracker Red for 7 min on ice.