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Calcium -dependent protein kinases are myristoylated and associated with different membranes in Arabidopsis thaliana

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CALCIUM-DEPENDENT PROTEIN KINASES ARE MYRISTOYLATED AND ASSOCIATED WITH DIFFERENT MEMBRANES IN *ARABIDOPSIS THALIANA*

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DEDICATION

I would like to dedicate this dissertation to my mother, Xi'e Lu, for giving me her endless love, encouraging me to follow my dreams and supporting me to complete my education. I also dedicate this dissertation to my husband Xiang Qu, the love of my life. I love you both and am thankful to have you in my life.

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ABSTRACT

MEMBRANE LOCALIZATION AND CHARACTERIZATION OF CALCIUM-DEPENDENT PROTEIN KINASES IN *ARABIDOPSIS THALIANA*

by

Sheen Xun Lu
University of New Hampshire, September, 2003

In plants, calcium-dependent protein kinases (CDPKs) are the predominant calcium-stimulated kinases and are known to be involved in many cellular processes. CDPK enzymatic activity previously has been detected in many locations in plant cells, including the membrane fraction. However, little is known about the subcellular locations of individual CDPKs or the mechanisms involved in targeting them to those locations. *Arabidopsis* contains 34 genes that are predicted to encode CDPKs and 28 of the predicted CDPK proteins have potential myristoylation motifs at their amino termini. Myristate is a 14-carbon saturated fatty acid that is attached co-translationally to the amino-terminal glycine of a nascent protein. Myristoylation can facilitate membrane binding and/or protein-protein interactions.

In these studies, *Arabidopsis* CDPK isoforms AtCPK1, AtCPK2, AtCPK5 and AtCPK6 have been investigated and the subcellular membrane location of each isoform, as well as the role that myristoylation plays in membrane association, have been addressed. An *in vitro* coupled transcription/translation system was used to demonstrate that AtCPK1, AtCPK2, AtCPK5 and AtCPK6 can be myristoylated and a mutation of the myristoylation site from glycine to alanine (G2A) in AtCPK2 and AtCPK5 prevented myristoylation *in vitro*. Subcellular localization studies were conducted using both

aqueous two-phase partitioning and sucrose density gradient fractionation of plant microsomes. AtCPK1 and AtCPK2 are associated with the endoplasmic reticulum and AtCPK5 and AtCPK6 are associated with the plasma membrane. Disruption of the myristoylation site (G2A mutation) decreased CDPK membrane binding. Using a translational fusion with the β-glucuronidase (GUS) reporter gene, I have demonstrated that the amino terminal region of AtCPK2 and AtCPK5 contains sufficient information for correct membrane localization.

My results indicate that myristoylation is important in membrane association of CDPKs, that different CDPK isoforms are targeted to different subcellular membrane locations and that the amino terminal region of CDPKs contains specific subcellular targeting information.

CHAPTER I

GENERAL INTRODUCTION

Arabidopsis thaliana

Arabidopsis thaliana (*L.*) Heynh. is a flowering plant in the mustard family (Brassicaceae), related to broccoli and cauliflower. In nature *Arabidopsis* grows throughout North America, Asia and Europe, and many different ecotypes are available for experimental analysis. Although *Arabidopsis* was described as having "no particular virtues or uses" by British botanist William Curtis in 1777 (The *Arabidopsis* Genome Initiative, 2000), it has become a model system for a wide range of studies in plant sciences for the past twenty years.

In 1873, Alexander Braun published the first *Arabidopsis* paper about a mutant plant found in the wild (Meyerowitz, 2001). *Arabidopsis* was first chosen as a genetic model organism for laboratory investigation by Friedrich Laibach in 1943. With the release of a detailed genetic map, *Arabidopsis* was widely adopted as a model plant in the 1980s (Meyerowitz, 2001). Its small size, rapid life cycle and prolific seed production made it ideal for laboratory studies. Further, its small genome, ability to be easily transformed, and availability of numerous mutants made it extremely powerful for genetic and molecular analysis. The 125 Mb genome of *Arabidopsis* is organized into five chromosomes and was completely sequenced in 2000 (The *Arabidopsis* Genome Initiative, 2000). For comparison, the genomes of maize and wheat are 2,500 Mb and

16,000 Mb, respectively. Despite its relatively small genome, *Arabidopsis* contains about 26,000 different genes which represent a complete set of genes for plant growth, including controlling developmental patterns, metabolism, disease resistance and stress responses. Information obtained from *Arabidopsis* not only provides the foundation for functional characterization of plant genes, but also extends our understanding of conserved cellular processes in all eukaryotes.

Calcium-Dependent Protein Kinases

Calcium plays important roles as a second messenger in eukaryotic signal transduction cascades. In plants, intracellular calcium levels are modulated by diverse processes, including responses to environmental signals such as light, to stress stimuli such as cold and drought stress, to developmental processes such as pollen tube growth, as well as to plant hormones (Bush, 1995; Sedbrook et al., 1996; Knight et al., 1998; Liu and Zhu, 1998; Plieth et al., 1999). Various calcium-binding proteins sense and respond to specific calcium signals and transduce them into downstream effects, including changes in metabolism and gene expression. More than 150 different calcium-modulated proteins have been characterized in eukaryotes and they all contain 2-8 copies of the EF-hand motif, which is a specialized helix-loop-helix calcium binding domain (Moncrief et al., 1990; Nakayama et al., 1992; Kawasaki et al., 1998). Due to the structural properties of the EF-hand, calcium-modulated proteins are able to bind calcium reversibly and calcium binding usually induces a conformational change resulting in a change in activity (Roberts and Harmon, 1992). In animals and fungi, the predominant calcium-modulated proteins include calcium/calmodulin-dependent protein kinases (CaMK) and calcium/phospholipid-dependent protein kinases (PKC). Both kinases require calcium

and an additional factor like calmodulin or phospholipids for full activation. In contrast, the predominant calcium-modulated kinase activity in plants is attributable to a unique class of enzymes known as calcium-dependent protein kinases (CDPKs) (Satterlee and Sussman, 1998).

Calcium-dependent protein kinase activity was first reported in pea extracts by Hetherington and Trewavas in 1982. After purification and characterization of the first CDPK from soybean (Putnam-Evans et al., 1990), CDPKs have been identified throughout the plant kingdom from green algae to higher plants, and also in apicomplexan protists, but not in animals, fungi and bacteria (Hrabak, 2000). CDPKs do not require either calmodulin or phospholipids for activation, which distinguishes them from both the CaMK and PKC families prevalent in animal and fungal cells. CDPKs bind and are activated by calcium directly because they contain a kinase catalytic domain and a calmodulin-like domain in the same polypeptide (Roberts and Harmon, 1992).

All known calcium-dependent protein kinases have a four-domain structure consisting of an N-terminal variable domain, a kinase catalytic domain, an autoregulatory domain and a C-terminal calmodulin-like domain (Fig. 1). The kinase domain (about 30 kDa) contains all twelve of the highly conserved subdomains of typical serine/threonine protein kinases (Hanks and Hunter, 1995). CDPKs are able to autophosphorylate and/or phosphorylate one or more classical kinase substrates in a calcium-dependent manner *in vitro*. The calmodulin-like domain (about 20 kDa) contains calcium-binding EF-hands that allow the protein to bind and be activated by calcium directly. Most CDPKs contain four EF-hands, but some have one, two or three. This diversity may differentiate CDPKs to respond to different concentrations of intracellular calcium (Hong et al., 1996).

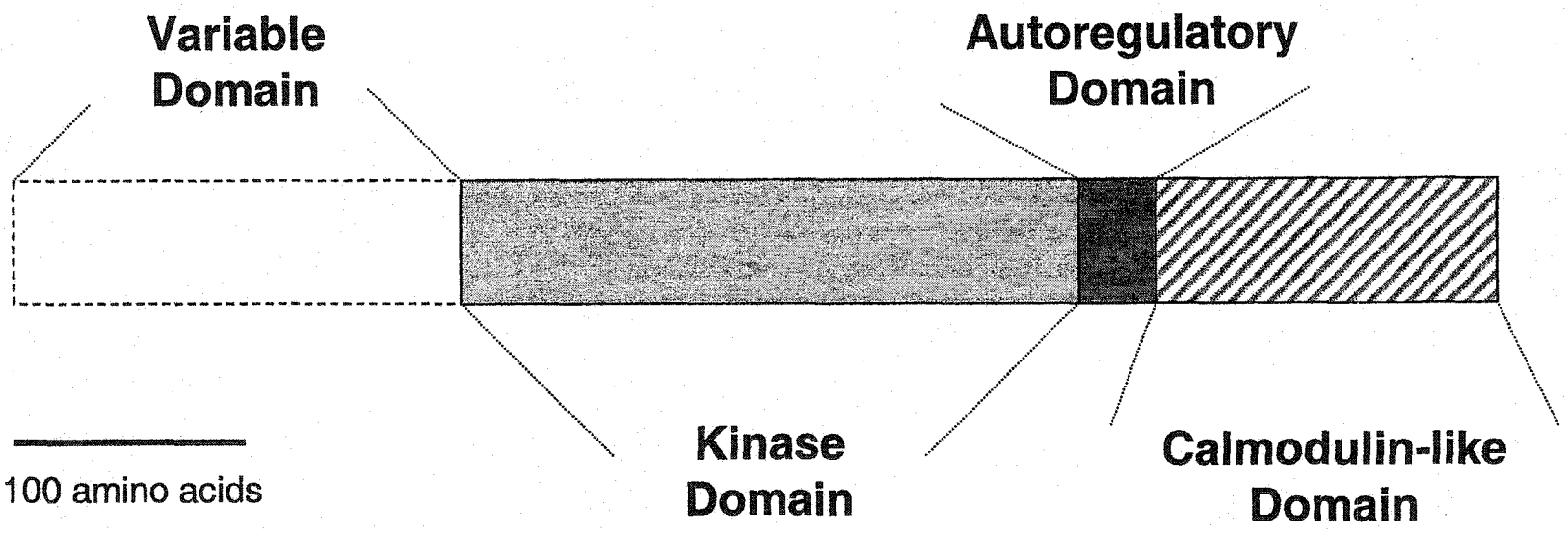

Figure 1. Schematic of the domain structure of calcium-dependent protein kinases.

Between the kinase and calmodulin-like domains lies a 30-35 amino acid autoregulatory domain which contains a pseudosubstrate site. CDPK activity is regulated via the autoregulatory and calmodulin-like domains of the protein. In the resting state where there is a low concentration of cytoplasmic free calcium, the pseudosubstrate site in the autoregulatory domain is bound by the kinase catalytic site and prevents phosphorylation of substrates. In response to stimuli, increased intracellular calcium binds to EF-hands in the calmodulin-like domain and induces the protein to undergo conformational changes that remove the pseudosubstrate from the catalytic site, activating the protein kinase activity (Harper et al., 1994). The kinase, calmodulin-like and autoregulatory domains are fairly well-conserved in all CDPKs. Phylogenetic analysis suggests that CDPKs may have arisen through the fusion of a CaMK-type gene and a calmodulin gene since the calmodulin-like domain of CDPKs resembles calmodulin and the kinase and autoregulatory domains of CDPKs share homology with the mammalian CaMK catalytic and regulatory domains, respectively (Harper et al., 1991; Suen and Choi, 1991; Zhang and Choi, 2001). While a high degree of sequence similarity resides in the kinase, autoregulatory and calmodulin-like domains, each CDPK has a very different N-terminal variable domain, which differs dramatically in length (20~200 amino acids) and amino acid sequence between CDPK isoforms (Hrabak, 2000; Cheng et al., 2002). The function of the variable domain is not well characterized but it has been proposed that this region may play a role in subcellular targeting and specifying functional diversity within the CDPK family.

Since the first complementary DNA (cDNA) clone SK5 encoding soybean CDPK was identified and sequenced in 1991 (Harper et al., 1991), cDNAs encoding CDPKs

have been cloned and characterized from carrot (*Daucus carota*; Suen and Choi, 1991), *Arabidopsis* (Harper et al., 1993; Urao et al., 1994; Hrabak et al., 1996), rice (*Oryza sativa*; Kawasaki et al., 1993), maize (*Zea mays*; Estruch et al., 1994) and numerous other plants as well as from protists, including *Plasmodium falciparum* (Zhao et al., 1993), *Eimeria maxima* (Dunn et al., 1996), and *Paramecium tetraurelia* (Kim et al., 1998). In all, at least 72 CDPK genes from 23 plants and protists have been cloned and sequenced (Hrabak, 2000). As novel calcium sensors, CDPKs have been implicated biochemically to play key roles in many cellular processes such as stomatal movement (Li et al., 1998), carbon and nitrogen metabolism (Huber et al., 1996; Huang and Huber, 2001), regulation of plant growth and development (Estruch et al., 1994; Anil et al., 2000), pathogen defense (Romeis et al., 2000), and responses to hormones and abiotic stress (Abo-el-Saad and Wu, 1995; Shinozaki and Yamaguchi-Shinozaki, 1997; Saijo et al., 2000).

CDPKs form a multigene family in plants and protists. There are 34 predicted genes encoding CDPKs in *Arabidopsis thaliana* whose genome has been completely sequenced (see sequence tree in Chapter IV), ten from *Zea mays*, and three from *Plasmodium falciparum*. Many other plant species, such as rice, soybean (*Glycine max*), and tobacco (*Nicotiana tabacum*) also contain multiple CDPK genes (Hrabak, 2000). Since most plant genomes are not completely sequenced, it is likely that multiple CDPK genes will be identified eventually in most plants and protists. The reason for the large number of CDPK isoforms in a given species is not known yet, but there are several possibilities. First, CDPKs may be essential for normal plant growth and development. Thus isoforms may have evolved to have partially redundant functions and be able to substitute for one another (Hrabak, 2000). Second, different CDPK genes may be

expressed in distinct cell types in a temporally and developmentally regulated manner (Estruch et al., 1994; Anil and Sankara Rao, 2001). Third, individual CDPKs may possess unique calcium-binding properties (Lee et al., 1998) or be targeted to specific subcellular locations in the cell (Martin and Busconi, 2000; Patharkar and Cushman, 2000; Lu and Hrabak, 2002), allowing interactions with different substrates during calcium-stimulated signal transduction. Finally, some isoforms contain specific motifs in the variable domain, such as a myristoylation motif or a PEST motif (Hrabak et al., 1996). These motifs suggest that CDPKs might undergo post-translational modification or rapid degradation (Rechsteiner, 1990; Han and Martinage, 1992).

To better understand the physiological function of CDPKs in plants, a variety of CDPK substrates have been identified which suggest that CDPKs play potential regulatory roles in metabolism, stress response, ion and water transport as well as the dynamics of the cytoskeleton. Potential CDPK substrates include both soluble proteins, such as nitrate reductase (Bachmann et al., 1996), sucrose phosphate synthase (Huber and Huber, 1996), sucrose synthase (Huber et al., 1996) and phosphoenolpyruvate carboxylase (Ogawa et al., 1992), and membrane proteins, such as nodulin-26 (Weaver and Roberts, 1992), the plasma membrane proton pump (Schaller and Sussman, 1988; Lino et al., 1998), a potassium channel in the guard cell plasma membrane (Li et al., 1998), an ER-localized Ca^{2+}-ATPase (Hong et al., 1999), and a vacuolar membrane-localized chloride channel (Pei et al., 1996). All of these proteins are known to be phosphorylated in a calcium dependent manner *in vitro* but much more information is needed before we draw a conclusion of the role for a particular CDPK in the regulation of the activity of these proteins *in planta*.

N-Myristoylation

Calcium-dependent protein kinase enzymatic activity has been identified in a number of cellular locations, including the cytosol (Putnam-Evans et al., 1990; Frylinck and Dubery, 1998), the nucleus (Li et al., 1991), the cytoskeleton (McCurdy and Harmon, 1992), and the plasma membrane (Schaller et al., 1992; Verhey et al., 1993; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998). In many other cases, CDPK activity has been reported in the microsomal fractions (Abo-el-Saad and Wu, 1995; Martin and Busconi, 2001), although the exact membrane location was not determined. No transmembrane domains have been predicted or reported in CDPKs, but the majority of CDPKs contain putative N-terminal myristoylation consensus sequences indicating that CDPKs might achieve the observed membrane association by attachment of a hydrophobic fatty acid group (Hrabak, 2000).

N-myristoylation, a lipid modification found in a variety of eukaryotic and viral proteins, is the covalent attachment of myristic acid (a 14-carbon saturated fatty acid) to the N-terminal glycine residue of a nascent polypeptide by an amide bond (Towler et al., 1988). Protein myristoylation is primarily a co-translational modification and considered to be irreversible, although there are a few examples of post-translational myristoylation (Nimchuk et al., 2000; Zha et al., 2000) and reversible myristoylation (Manenti et al., 1994). Myristoylation can promote protein-membrane binding, facilitate protein-protein interaction, and may be essential for protein function (Boutin, 1997).

All known N-myristoylated proteins begin with the sequence Met-Gly. During translation, the initial methionine residue is removed by an endogenous methionine aminopeptidase and then myristic acid is covalently linked to glycine-2 via an amide

bond by N-myristoyl transferase (NMT) (Towler et al., 1988; Utsumi et al., 2001). The requirement for a glycine residue at the newly exposed N-terminus is absolute. This glycine serves as the site of myristate attachment and myristoyl-CoA is highly favored as the myristate donor. NMT, which catalyzes the transfer of myristic acid from myristoyl-CoA to the N-terminal glycine residue has been purified and cloned from several organisms including *Arabidopsis* (Towler et al., 1987; Duronio et al., 1992; Lodge et al., 1994; Raju et al., 1996; Raju et al., 1997; Giang and Cravatt, 1998; Qi et al., 2000). The precise substrate specificity of NMT from both yeast and humans has been extensively characterized using purified enzyme and synthetic peptide substrates (Towler et al., 1988; Rocque et al., 1993). In general, the N-myristoylation consensus sequence can be expressed as $(M)^1$ G^2 N^3 X^4 X^5 S/T^6 X^7 R^8 R^9 where N=small, uncharged residues, X=almost any amino acids and R=basic residues (Fig. 2) (Towler et al., 1988). The consensus sequence helps us to understand the nature of the substrate recognized by NMT, although there are some important and unexpected exceptions such as several myristoylated proteins that have bulky, charged lysine or arginine residues at position 3 (Pullman and Bodmer, 1992). At position 6, Ser or Thr is neither sufficient nor critical for the recognition of the protein substrate by the NMT since other residues including lysine, aspartic acid, phenylalanine, proline or arginine have also been found at that position of some myristoylated proteins (Boutin, 1997). Since the consensus sequence was mainly deduced during studies on yeast NMT with some comparisons with NMT from rat liver cytosol and the human cloned enzyme (Towler et al., 1988; Rocque et al., 1993), plant NMT may have slightly different substrate specificity (Ellard-Ivey et al., 1999; Lu and Hrabak, 2002).

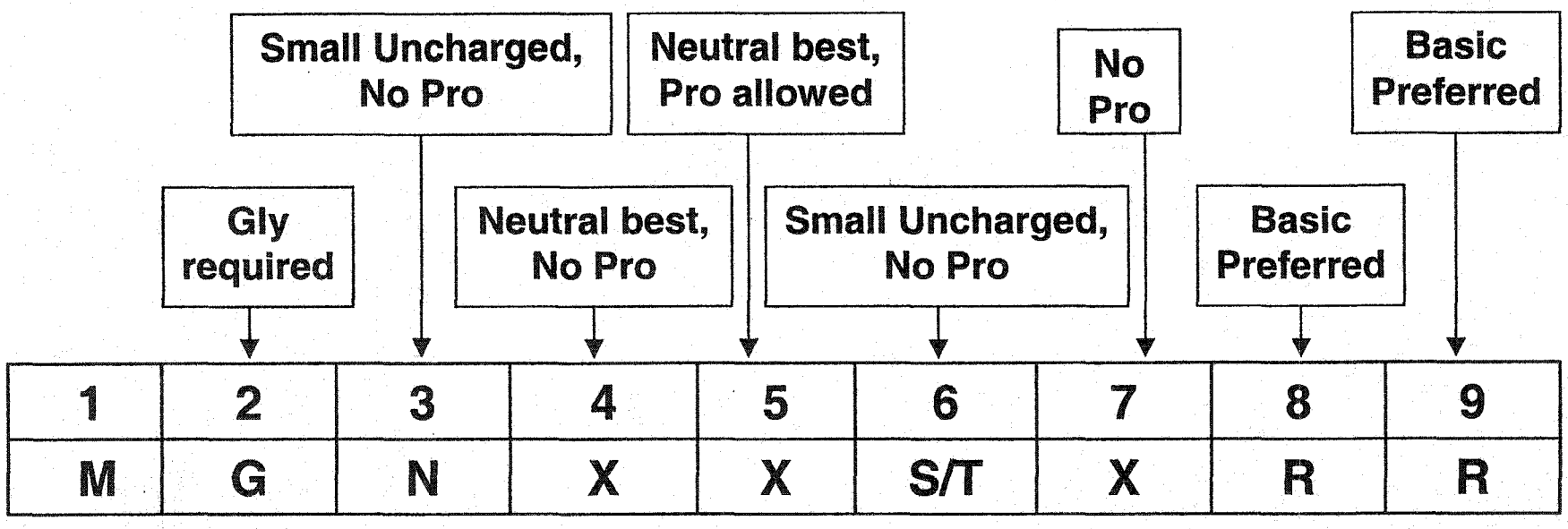

Figure 2. General requirement for myristoylation consensus sequence.

Protein myristoylation is important for targeting of a variety of signaling molecules to membranes due to the partial insertion of the hydrophobic myristoyl hydrocarbon chain within the lipid bilayer. Such membrane localization has been shown to be critical for the biological function of some proteins such as retroviral Gag polyproteins, Src tyrosine kinases and heterotrimeric G protein α subunits (Johnson et al., 1994; Sankaram, 1994; Resh, 1996; Boutin, 1997). Abrogation of myristoylation by mutation of the N-terminal glycine to alanine (G2A) generally results in reduction or loss of membrane binding. For example, the G2A mutation of $PP60^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (RSV), not only prevents myristoylation but also abolishes binding to membranes. Non-myristoylated mutants lose the ability of cellular transformation although tyrosine kinase activity is unaffected (Kamps et al., 1985; Buss et al., 1986; Peitzsch and McLaughlin, 1993). Likewise, myristoylation is also required for plasma membrane binding and virion formation by retroviral and lentiviral Gag proteins. Mutation of Gly to Ala prevents myristoylation, inhibits membrane binding and blocks virus assembly (Schultz and Rein, 1989; Bryant and Ratner, 1990; Resh, 1999).

Membrane association of myristoylated proteins often appears reversible and dynamically regulated. Myristoylation can be necessary but not sufficient for membrane anchoring because myristate alone provide barely enough hydrophobic energy to associate with the lipid bilayer (Peitzsch and McLaughlin, 1993; Sankaram, 1994). It is not surprising that other factors including a nearby polybasic cluster, protein-protein interactions or additional lipid anchors, are often required for efficient membrane binding (Resh, 1999). Some proteins appear to possess a "myristoyl switch" which enables transient shifts between the membrane and other subcellular compartments in response to

either protein phosphorylation, binding of ligands or proteolysis. Myristoylated alanine-rich C kinase substrate (MARCKS) protein is a protein kinase C (PKC) substrate, whose membrane binding has been extensively studied for its "myristoyl-electrostatic switch". In addition to myristate, the MARCKS protein contains a cluster of basic amino acids which allows the binding of myristoylated nonphosphorylated MARCKS to membranes. Phosphorylation of serine residues within these basic clusters by PKC reduces the electrostatic interactions with acidic phospholipids in the membrane, resulting in the translocation of phosphorylated MARCKS protein from the membrane to the cytosol (Thelen et al., 1991; Kim et al., 1994; McLaughlin and Aderem, 1995; Resh, 1999). Two examples of a "myristoyl-ligand switch" are found in recoverin and ADP-ribosylation factor (ARF). Binding of ligands triggers a conformational change that regulates exposure of the myristate moiety. In recoverin, a calcium-binding protein in the retina, the myristate is buried in a hydrophobic pocket of the protein in the absence of calcium. Binding of calcium induces a conformational change within recoverin and the myristate is released to interact with the membranes (Ames et al., 1994; Tanaka et al., 1995). A similar switch mechanism operates for ARF, a small GTP binding protein, in which binding of GTP results in apparent exposure of the myristoyl group and protein translocation from cytosol to membrane (James and Olson, 1990; Amor et al., 1994; Boman and Kahn, 1995; Goldberg, 1998). The "myristoyl-proteolytic switch" is well characterized in HIV-Gag. Synthesized as a polyprotein precursor, Pr55gag binds to plasma membrane via a myristate plus basic residue cluster (Zhou et al., 1994). Cleavage of Pr55gag by HIV-1 protease produces an N-terminal cleavage product (p17MA) and

triggers a myristoyl switch in which myristate is sequestered within p17MA, triggering release p17MA from the membrane (Spearman et al., 1997).

In addition to the membrane-bound state, myristoylated proteins can be soluble or alternate between cytosol and membrane (Johnson et al., 1994; Bhatnagar et al., 1997). In many cases, myristoylation can be important for protein-protein interactions or protein stability (Yonemoto et al., 1993; Taniguchi, 1999). For example, myristoylation of α subunits of heterotrimeric G proteins increases their affinity for the β and γ subunits (Linder et al., 1991). In recoverin, the myristate moiety occupies a hydrophobic pocket within the protein and plays a structural role to stabilize the three-dimensional protein conformation (Tanaka et al., 1995). In still other proteins, the functional significance of myristoylation remains unclear because no obvious effects are seen when myristoylation is prevented (Loh et al., 1998).

In contrast to the growing body of knowledge concerning myristoylation in fungal and animal cell systems, until recently there were few examples of protein myristoylation in plants (Thompson and Okuyama, 2000). NMT activity has been described in both wheat germ extract and *Arabidopsis* with peptide substrates (Heuckeroth et al., 1988; Qi et al., 2000). Several plant proteins are known to be myristoylated and myristoylation is important for their correct function. Mutation of the potential myristoylation site of the *Fen* gene in tomato results in loss of its ability to confer sensitivity to the insecticide fenthion (Rommens et al., 1995) and the myristoylation of *Arabidopsis* SOS3 (salt overly sensitive 3) protein is required for SOS3 function in plant salt tolerance (Ishitani et al., 2000). The putative consensus sequence for N-myristoylation is present at the amino terminus of many CDPKs, including those from *Arabidopsis* (Harmon et al., 2000;

Hrabak, 2000; Cheng et al., 2002), rice (Kawasaki et al., 1993; Breviario et al., 1995), maize (Estruch et al., 1994; Saijo et al., 1997), mung bean (Botella et al., 1996), carrot (Suen and Choi, 1991; Lindzen and Choi, 1995) and zucchini (Ellard-Ivey et al., 1999). Several CDPKs have been shown to be myristoylated exclusively on glycine-2 in a wheat germ cell-free system (Ellard-Ivey et al., 1999; Lu and Hrabak, 2002) and myristoylation of both *Arabidopsis* and rice CDPKs is essential for their membrane binding (Martin and Busconi, 2000; Lu and Hrabak, 2002). These data suggest that plant proteins can be myristoylated, but more evidence is needed to confirm myristoylation *in planta* and determine the biological function of myristoylation of plant proteins.

Palmitoylation

Many myristoylated protein are also palmitoylated on cysteine residues found near the myristoylation site of the protein (Resh, 1994; Milligan et al., 1995). In contrast to myristoylation, palmitoylation is a dynamic, reversible modification involving post-translational attachment of palmitate, a 16-carbon saturated fatty acid to a cysteine residue via a thioester linkage (Wedegaertner and Bourne, 1994). No discrete recognition sequence for palmitoylation has been identified and the enzyme(s) involved in catalyzing palmitate addition is poorly understood, although there is evidence that enzymatic activity could be membrane-associated (Dunphy et al., 1996; Das et al., 1997; Roth et al., 2002). Recent studies demonstrate that palmitoyltransferase activity is enriched in the plasma membrane and palmitoylation can selectively target proteins to the plasma membrane (Resh, 1999; Thompson and Okuyama, 2000). It has been shown that most palmitoylated or dually acylated proteins are membrane bound because palmitate has about 10-fold greater membrane binding affinity than myristate (Resh, 1996). Reversible

palmitoylation/depalmitoylation cycles have been demonstrated for several proteins (Wedegaertner and Bourne, 1994; Steinert et al., 1996) and the turnover of palmitate has been reported to regulate membrane binding and/or function of proteins (McIlhinney, 1990). Several well-studied myristoylated proteins, such as heterotrimeric G protein α subunits and Src tyrosine kinases, are also modified by palmitoylation (Parenti et al., 1993; Resh, 1994; Milligan et al., 1995; Robbins et al., 1995). Interestingly, all CDPKs that contain a myristoylation consensus sequence also have at least one cysteine residue nearby, which could be a potential palmitoylation site (Hrabak, 2000). Palmitoylation in plants is largely unknown except that one Rab GTPase (Ara6) and one rice CDPK (OsCPK2) have been shown to be palmitoylated and myristoylated (Martin and Busconi, 2000; Ueda et al., 2001).

Rationale, Hypothesis and Significance

In plants, CDPKs are the major calcium-stimulated protein kinases and are known to be involved in diverse physiological and signaling processes (Estruch et al., 1994; Abo-el-Saad and Wu, 1995; Huber et al., 1996; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Li et al., 1998; Anil et al., 2000; Romeis et al., 2000; Saijo et al., 2000; Huang and Huber, 2001). Although the importance of CDPKs has been recognized for number of years, to date there is no direct evidence supporting their functional role. This is partly due to lack of relevant biochemical understanding and also due to the genetic complexity imposed by many CDPK isoforms with redundancy in function. Knowledge of subcellular localization of different CDPKs is likely to be one of the key determinants to dissect the specific functions of these enzymes.

Calcium often enters the cytoplasm through calcium channels in cellular membranes. Potential substrates for CDPKs include both soluble and membrane-bound proteins (Schaller and Sussman, 1988; Ogawa et al., 1992; Weaver and Roberts, 1992; Bachmann et al., 1996; Huber and Huber, 1996; Huber et al., 1996; Pei et al., 1996; Li et al., 1998; Lino et al., 1998; Hong et al., 1999). Therefore translocation of specific CDPK isoforms to the membrane could not only facilitate the interaction with membrane-bound substrates, but also allow for rapid activation of the enzyme in response to incoming calcium. In fact, CDPK enzymatic activity has often been detected in membrane fractions (Schaller et al., 1992; Verhey et al., 1993; Abo-el-Saad and Wu, 1995; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998; Martin and Busconi, 2001) although little is known about the subcellular locations of individual CDPKs.

The *Arabidopsis* genome is predicted to encode 34 different CDPKs. Although no transmembrane domains have been reported in CDPKs, most *Arabidopsis* CDPK isoforms contain potential myristoylation and palmitoylation sites at their amino termini (Hrabak, 2000). I hypothesize that acylation could be an important mechanism for the observed membrane association of CDPKs. I also hypothesize that different CDPK isoforms could be targeted to different membrane systems via subcellular targeting information located in the N-terminal variable domain of CDPKs. My long-term goal is to understand the mechanisms by which different CDPKs are targeted to different membrane systems. These studies will contribute to our overall understanding of CDPK function and increase our knowledge of membrane targeting mechanisms in plants.

CHAPTER II

A PLASMA MEMBRANE LOCALIZED CALCIUM-DEPENDENT PROTEIN KINASE IN *ARABIDOPSIS THALIANA*

(This chapter is based on a manuscript prepared for submission: Sheen X. Lu and Estelle M. Hrabak. A Plasma Membrane Localized Calcium-Dependent Protein Kinase in *Arabidopsis thaliana*.)

INTRODUCTION

In plants, calcium's role as a second messenger in signal transduction is well-established. In order for calcium to function as a second messenger, calcium is sequestered in intracellular stores including the vacuole, endoplasmic reticulum (ER), nuclear envelope, chloroplast and mitochondrion to maintain a low calcium concentration in the cytosol under resting conditions. In response to environmental or intrinsic stimuli, calcium can enter the cytosol through various differentially localized calcium channels, calcium-ATPases and calcium/proton antiporters (Bush, 1995; Harper, 2001; Huang and Huber, 2001; Cheng et al., 2002) and cause a transient increase in cytosolic calcium concentration. Unlike most other ions, calcium is not readily diffusible within cells and a calcium gradient is often formed within the cytosol (Trewavas, 1999). Translocation of calcium-stimulated proteins to positions on or near membranes could be important for protein function, by positioning proteins close to the source of incoming calcium, or

by allowing proteins to detect and respond rapidly to variations in cytosolic calcium levels (Hrabak, 2000).

In plants, the predominant calcium-stimulated protein kinase activities are attributed to calcium-dependent protein kinases (CDPKs), a novel class of calcium sensors. In many species, CDPK enzymatic activity has been detected not only in the cytoplasm (Putnam-Evans et al., 1990; Frylinck and Dubery, 1998) but also in microsomal fractions, such as plasma membrane (PM) (Schaller et al., 1992; Verhey et al., 1993; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998). Targeting CDPKs to the PM could contribute significantly to their function since many calcium-mediated signaling events are initiated by fluxes across the plasma membrane.

Arabidopsis contains 34 predicted genes that encode CDPKs. Based on sequence analysis, 28 of the 34 CDPKs in *Arabidopsis* were found to contain both a myristoylation consensus sequence and a potential palmitoylation site at their amino termini (Hrabak, 2000)). This indicates that most CDPKs could be dually acylated and associate with membranes. Acylated proteins are found associated with a wide variety of cellular membranes, including the nuclear envelope, Golgi, mitochondrion, plasma membrane and ER (James and Olson, 1990; Haun et al., 1993; Strittmatter et al., 1993; Fackler et al., 1997; Alsheimer et al., 2000; Zha et al., 2000). Many dually acylated proteins such as heterotrimeric G protein α subunits, Src tryrosine kinases and recoverin are associated with the plasma membrane. Recent studies demonstrated that they are localized to raft domains which are specific subdomains of the plasma membrane (Shenoy-Scaria et al., 1994; Galbiati et al., 1999; Brown and London, 2000; Moffett et al., 2000; Dunphy et al., 2001). Lipid rafts are low density, cholesterol and sphingolipid-enriched membrane

subdomains (Brown and London, 2000). They exist not only in the plasma membrane, but also in ER membranes and the Golgi network (Sevlever et al., 1999; Heino et al., 2000; Gkantiragas et al., 2001). Localization in lipid rafts could be important for protein conformation and function since rafts are believed to provide a mechanism for protein interaction by concentrating them in a unique membrane region (Brown and London, 2000; Galbiati et al., 2001). In animal cells, both dually acylated proteins and glycosyl phosphatidyl inositol (GPI)-anchored proteins have been shown to be enriched in lipid rafts (Brown and London, 2000; Zacharias et al., 2002). Although similar raft domains have been identified in the plant plasma membrane, very little is known about lipid rafts in plants (Peskan et al., 2000).

Although CDPK enzymatic activity has been identified in a variety of cellular locations in other plant species, the subcellular localization of *Arabidopsis* CDPKs is largely unknown except one isoform (AtCPK2) which has been shown to associate with the ER membrane (Lu and Hrabak, 2002). Knowledge of subcellular localization of different CDPKs has potentially important implications for studying the specific functions of these enzymes. Here I investigated the subcellular location of one *Arabidopsis* CDPK, AtCPK5, and the mechanisms involved in its membrane association.

MATERIALS AND METHODS

Growth of *Arabidopsis*

Arabidopsis thaliana was grown in liquid culture, on solid media in petri plates or in soil. For liquid culture, high sucrose MS medium containing 0.43% (w/v) Murashige & Skoog basal salt mixture with vitamins (Sigma, St. Louis, Cat. No. M-0404) and 1% (w/v) sucrose was adjusted to pH 5.6-5.8 with KOH and 50 ml media was autoclaved in 125-ml glass flasks capped with aluminum foil. If desired, kanamycin was added to a final concentration of 25 μg/ml before seeds were added. Seeds were sterilized by bleach treatment. Seeds were placed in a 1.5 ml microcentrifuge tube and incubated for one minute with 70% (v/v) ethanol. The ethanol was removed and the seeds were incubated for 12-15 min in a freshly-made solution of 30% (v/v) Clorox bleach containing 1% (v/v) Triton X-100. Under sterile conditions, the bleach was removed and seeds were washed at least 5 times with 1 ml sterile water, until the solution did not foam when shaken vigorously. Typically 20-50 seeds were used per flask and sterile seeds were added directly to the media. Flasks were placed on a platform shaker at about 75 rpm in a growth chamber (22°C, 18 hour light cycle).

For growth on petri plates, low sucrose MS medium containing 0.43% (w/v) Murashige & Skoog basal salt mixture with vitamins, 0.1% (w/v) sucrose and 0.8% (w/w) washed agar was used. After adjusting the pH and autoclaving, kanamycin was added to a final concentration of 50 μg/ml if necessary before pouring plates. Typically about 30 ml of medium was poured into each 100 x 25 mm petri plate and 50-2000 sterile seeds were plated out on each plate. Seeds were sterilized by ethanol treatment. In a 1.5 ml microcentrifuge tube seeds were incubated for 5 min in 70% (v/v) ethanol containing

one drop of 10% (v/v) Triton X-100. Under sterile conditions, the ethanol was removed and the seeds were incubated for 5 min in 100% ethanol containing one drop of 10% (v/v) Triton X-100. The ethanol was removed and the 100% ethanol treatment was repeated twice. Following the last ethanol incubation, seeds were poured on a piece of sterile filter paper and dried in the hood before transferring to the plates. In order to increase and coordinate seed germination, seeds on plates were stratified at 4°C in darkness for at least two days before being placed in a growth chamber (22°C, 18 hour light cycle).

For growth in soil, *Arabidopsis* seeds were sown typically at 1-10 seeds per 2 ¼ in. square pot. Plants were grown in a growth chamber (22°C, 18 hour light cycle) and watered as needed with commercial plant food.

Plasmid constructs

All constructs were made previously by E. Hrabak and are only briefly described here. The CPK5(16aa)-GUS transgene construct consisted of a fragment containing 1.6 kb of CPK5 genomic sequence upstream from the translational start site and the first 48 nucleotides of the coding sequence followed in-frame by the β-glucuronidase (GUS) gene and *nos* terminator (Fig. 3A). GUS is a reporter gene whose expression can be easily detected by fluorometric assay and the *nos* terminator is a common plant termination signal cassette. This construct was cloned into pBIN19, a binary vector for *Agrobacterium*, which contains the kanamycin resistance gene, for plant transformation. The CPK5(G2A)-GUS construct is identical to CPK5(16aa)-GUS except that site-directed mutagenesis of the glycine at position 2 to alanine was done (Fig. 4A). Similar constructs, CPK5(16aa)-GFP (Fig. 3B) and CPK5(G2A)-GFP (Fig. 4B), contained a

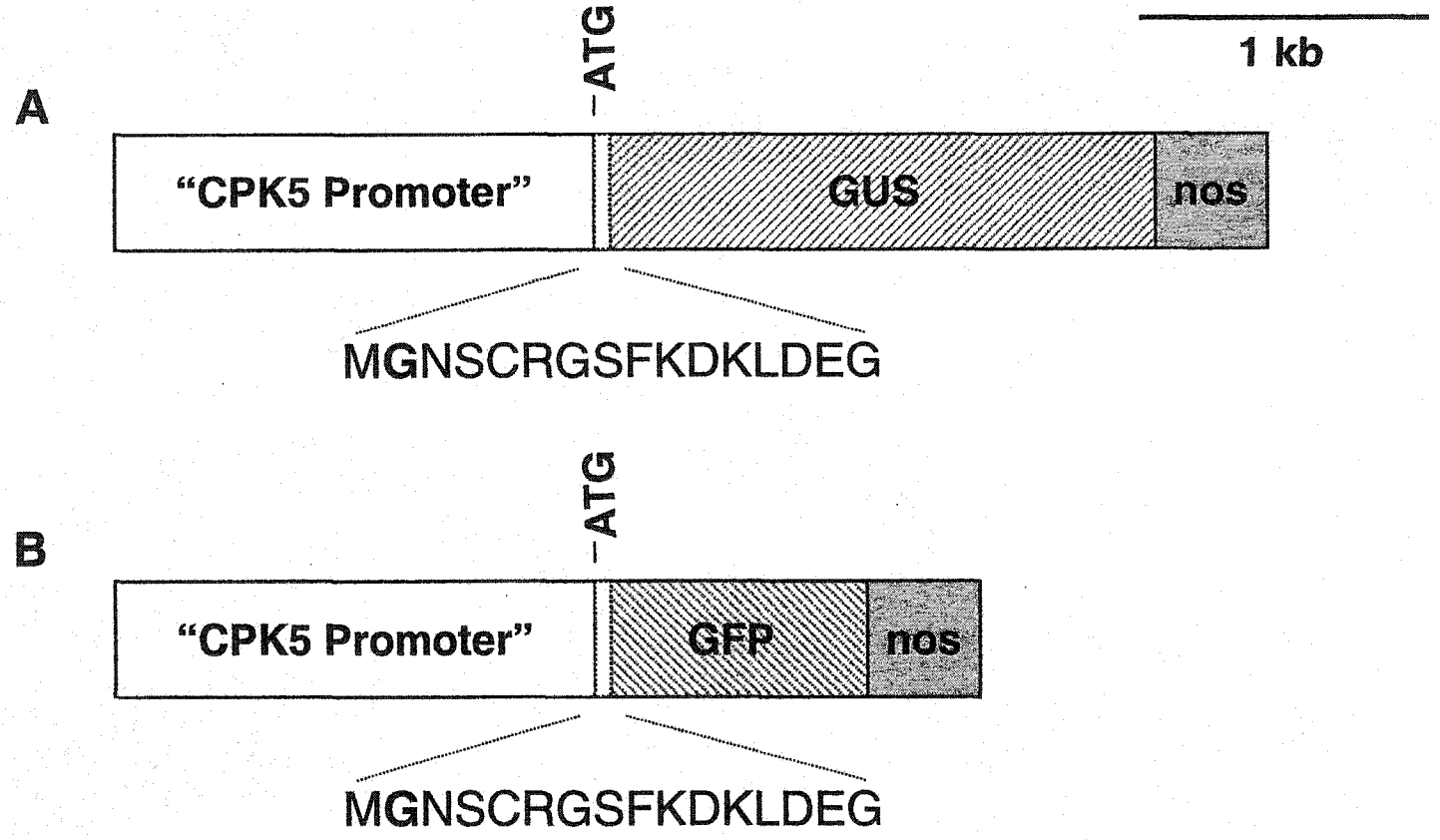

Figure 3. Schematic diagram of the CPK5(16aa)-GUS and CPK5(16aa)-GFP constructs. The amino acid sequence encoded by the first 48 nucleotides of the coding sequence is indicated. "CPK5 promoter" indicates the sequence upstream of the translational start site and includes both 5' untranslated leader and promoter sequences.

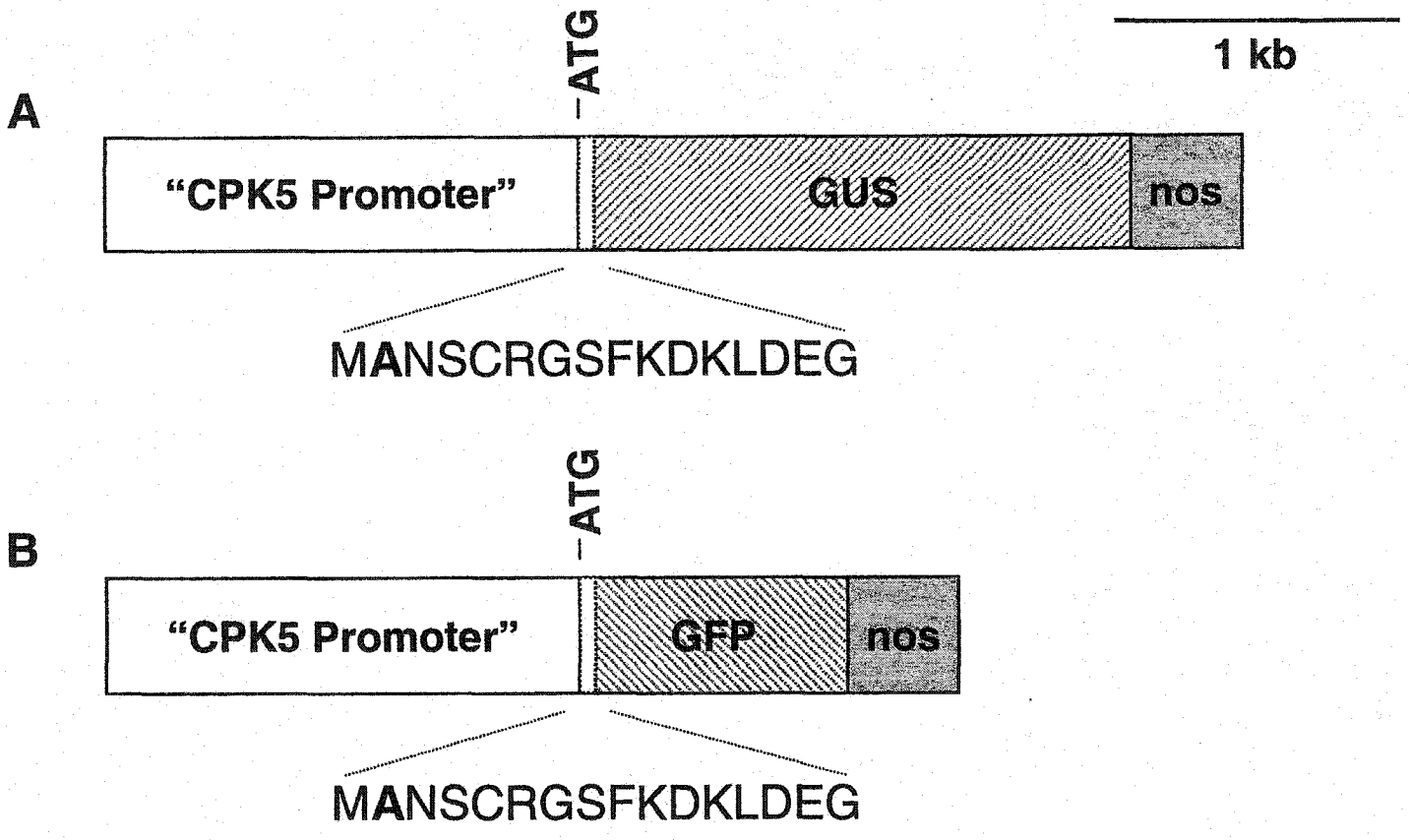

Figure 4. Schematic diagram of the CPK5(G2A)-GUS and CPK5(G2A)-GFP constructs. The amino acid sequence encoded by the first 48 nucleotides of the coding sequence is indicated. "CPK5 promoter" indicates the sequence upstream of the translational start site and includes both 5' untranslated leader and promoter sequences.

sequence encoding GFP instead of the GUS gene. GFP represents green fluorescent protein, a reporter gene whose expression can be directly detected by fluorescence microscopy in living cells.

Plant transformation and selection

All transgenic plants were generated by a floral dip method using *Arabidopsis thaliana* ecotype Columbia (Clough and Bent, 1998). About 10 wild type seeds per pot were grown in soil and infiltration was performed a few days after clipping primary bolts. A starter culture was prepared by inoculating *Agrobacterium tumefaciens* (strain GV3101) carrying the appropriate construct into 2-3 ml LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride] containing 50 μg/ml kanamycin. The starter culture was shaken at room temperature at 200-250 rpm for 1-2 days. The day before infiltration, 200-500 ml of LB medium containing 50 μg/ml kanamycin was inoculated with the starter culture and shaken overnight at the same speed at room temperature. Cells were harvested by centrifugation at 6,000*g* for 5 min at room temperature and resuspended in infiltration medium [5% (w/v) sucrose, 0.05% (v/v) silwet L-77] to an OD_{600} of approximately 0.8. Flower stalks were dipped in infiltration medium with gentle agitation for 10-20 seconds. The pots were laid on their sides in a flat covered with a plastic dome to maintain humidity overnight. The next day, plants and soil were rinsed thoroughly with water to remove any traces of resuspension medium. The infiltration procedure was repeated 5-7 days later to transform more young flower buds. After approximately 3-4 weeks growth, all seeds from one pot were harvested together and referred to as T_o seeds.

T_0 seeds were plated on low sucrose MS medium (described above) containing 50 mg/L kanamycin in petri plates and transformants could be easily identified as dark green plants with long roots and true leaves after 1-2 weeks. Kanamycin-resistant plants were further confirmed to contain the correct transgene using a rapid PCR method (Klimyuk et al., 1993). Transformed seedlings were transferred to soil and grown in a growth chamber (22°C, 18 hour light cycle) until seeds were ready to harvest. Seeds from transformants (T_1 plants) were referred to as T_1 seeds and experiments were usually conducted with T_2 plants.

Membrane Isolation

Microsomal membranes were prepared by modifying a procedure previously described by Schaller and DeWitt (1995). All homogenization and isolation procedures were conducted on ice or in a cold room with prechilled buffers and equipment. Two-week-old, liquid-grown *Arabidopsis* plants were homogenized in homogenization buffer [50 mM Tris-HCl (pH 8.5), 20% (v/v) glycerol] (1 ml/g starting material) with a mortar and pestle. Homogenates were filtered through Miracloth and then centrifuged at 5,000*g* for 5 min to remove nuclei, cell-wall debris and intact organelles. Microsomal membranes were separated from the soluble fraction by ultracentrifugation at 125,000*g* for 30 min, and then resuspended in a small volume (0.1 ml/g starting material) of resuspension buffer [25 mM Tris-HCl (pH 7.5), 10% (w/v) sucrose] using a ground glass homogenizer. All buffers contained protease inhibitor cocktail (Roche, Indianapolis). The supernatant after high speed centrifugation will be referred to as "soluble proteins" and the membrane pellet after resuspension will be referred to as "total microsomes".

Aqueous two-phase partitioning

An aqueous two-phase system (Larsson et al., 1987) was used to separate plasma membrane from intracellular membranes due to the differences in their surface properties. All manipulations were conducted at 4°C with prechilled solutions. Total microsomes were resuspended in SPK buffer (0.33 M sucrose, 5 mM KPO_4, and 3 mM KCl, pH 7.8) and then added to a 6.3% (w/w) DextranT500/PEG3350 phase mixture prepared in the same buffer. After thorough mixing by inverting, the phases were separated by centrifugation at 1,000*g* for 5-10 min. Both upper phase and lower phase were carefully removed and repartitioned two more times with fresh phases which were obtained from a bulk-phase system of identical composition prepared separately. The final upper and lower phases were diluted separately in 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, and 1 mM EGTA and centrifuged at 125,000*g* for 30 min. Phase pellets were resuspended in equal volumes of SPK buffer, separated by SDS-polyacrylamide gels (SDS-PAGE), and analyzed by immunoblotting. All buffers contained protease inhibitor cocktail tablets (Roche, Indianapolis).

Isolation of Triton X-100 insoluble membranes (lipid raft enrichment)

Triton X-100 insoluble membranes were prepared as previously described for tobacco with some modifications (Peskan et al., 2000). All steps were performed at 4°C with prechilled solutions. Total microsomal membranes from wild type (WT) *Arabidopsis* were resuspended in resuspension buffer [25 mM Tris-HCl (pH 7.5), 5 mM EDTA] with a ground glass homogenizer and centrifuged at 10,000*g* for 2 min to remove all membranes that were not well-resuspended. Triton X-100 was added to a final concentration of 1% (v/v) and the solution was incubated at 4°C for 30 min. Detergent

treated membranes were mixed with 70% (w/w) sucrose in resuspension buffer to a final concentration of 50% (w/w) sucrose and placed at the bottom of an ultracentrifuge tube. Membranes were overlayed with a continuous gradient of 20% to 48% (w/w) sucrose in resuspension buffer. After centrifugation in a swinging bucket rotor at 125,000*g* for 16 h at 4°C, 1-ml fractions were collected from the top of the gradient, separated by SDS-PAGE, and analyzed by immunoblotting as described below. All buffers contained protease inhibitor cocktail tablets (Roche, Indianapolis). The sucrose concentration of each fraction was measured with a refractometer (Fisher Scientific, Pittsburgh).

For some Triton X-100 insoluble membrane isolations, PM-enriched upper phases obtained by aqueous two-phase partitioning as described above were used as the starting material, treated with 1% (v/v) Triton X-100 and purified on a continuous sucrose gradient. Due to the small amounts of starting material, protein in these fractions had to be concentrated before SDS-PAGE. Fractions collected after ultracentrifugation were precipitated by adding 100% cold trichloroacetic acid (TCA) to a final concentration of 10% (v/v) and incubated at 4°C for 30 min. The precipitated protein was collected by centrifugation at 15,000*g* for 15 min, washed twice with cold 100% acetone, and resuspended in resuspension buffer, followed by SDS-PAGE and immunoblotting.

***In vitro* myristoylation assay**

Myristoylation assays were performed with the TNT Coupled Transcription /Translation Wheat Germ Extract System (Promega Corp., Madison) according to the manufacturer's instructions. Both WT and mutant AtCPK5 cDNAs were cloned into pBluescript to allow the production of a sense AtCPK5 transcript under control of the pBluescript T7 promoter. Site-directed mutagenesis was performed previously to create a

mutagenized derivative containing alanine instead of glycine at position 2, designated CPK5(G2A). One microgram of plasmid, linearized with *Eco*RI, was used as the template for transcription in the presence of either 10 μCi L-[^{35}S]methionine (1000 Ci/mmol; Amersham, Piscataway) to assess total protein synthesis or 50 μCi [9,10-^{3}H]myristic acid (54 Ci/mmol; Amersham, Piscataway) to detect myristoylated proteins. Control reactions contained no plasmid. Immediately before beginning the reaction, the [^{3}H]-labeled myristic acid was dried under nitrogen and resuspended by vortexing in DEPC-treated water at a concentration of 10 μCi/ml. Components of the *in vitro* transcription/translation mix are found in Table 1.

After 1.5 hours incubation at 30°C, the reaction was stopped by adding an equal volume of 2x Laemmli protein loading buffer [125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.01% (w/v) bromphenol blue] and reaction products were separated on 10% (w/v) SDS-PAGE at 4°C according to the method of Laemmli (1970). The gels were stained with Coomassie Blue [0.1% (w/v) Coomassie Blue, 40% (v/v) methanol and 10% (v/v) acetic acid] and destained with Coomassie destain solution [10% (v/v) methanol and 10% (v/v) acetic acid] for 30 min each at room temperature. After rinsing with water briefly, the gels were treated with Entensify Universal Autoradiography Enhancer (New England Nuclear, Boston) according to manufacturer's instruction and then dried between two sheets of cellophane before detection on Kodak X-ray film at -80°C. Typically, a 1-2 day exposure was used for [^{35}S]methionine samples and a 5-10 day exposure was used for [^{3}H]myristate samples.

Table 1. Components of *in vitro* transcription/translation reactions.

	For [^{35}S]methionine	For [^{3}H]myristic acid	For Immunoblot
Wheat germ extract*	12.5 µl	12.5 µl	12.5 µl
Reaction buffer*	1.0 µl	1.0 µl	1.0 µl
RNAsin (Promega, Cat. No. N251A)	0.5 µl	0.5 µl	0.5 µl
RNAse free H_2O	5.0 µl	1.0 µl	6.0 µl
A.A. mix (minus Met)*	0.5 µl	0.25 µl	0.25 µl
A.A. mix (minus Leu)*	0	0.25 µl	0.25 µl
Template DNA	4.0 µl	4.0 µl	4.0 µl
T7 DNA polymerase*	0.5 µl	0.5 µl	0.5 µl
[^{35}S]methionine	1.0 µl	0	0
[^{3}H]myristic acid	0	5.0 µl	0
Total	25.0 µl	25.0 µl	25.0 µl

(* = From Promega TNT Kit, Cat. No. L4140)

CDPK Dephosphorylation

A wheat germ *in vitro* transcription/translation system was used to produce both WT AtCPK5 protein and AtCPK5 protein containing a G2A mutation separately. About 10 µl of reaction products were incubated with 5 units of calf intestinal alkaline phosphatase for 15 min at 30°C in the absence or the presence of 1 µl of alkaline phosphatase inhibitor. After incubation, 10 µl of 2x Laemmli protein loading buffer was added to the reaction followed by boiling for 5 min to stop the reaction. Samples were separated by 10% SDS-PAGE and analyzed by immunoblotting with AtCPK5 specific antibody as described below.

Transient expression of AtCPK5/GFP fusion protein

Particle bombardment was used to introduce GFP fusion plasmids into leek epidermal cells with a Biolistic PDS-1000/He system (BioRad, Hercules). Gold particles were coated with the respective plasmid (Fig. 3B and 4B) and a helium pressure of 900 psi was employed. Leek epidermal cell layer peels were placed on low sucrose MS plates (described above) and 600 µg gold coated with 2 µg plasmid DNA was used per experiment. The target distance between the stop screen and leek tissue was set at 6 cm. After bombardment, tissues were kept in darkness at 28 °C for 15-24 h, allowing cell recovery and gene expression. Leek epidermal cells were transferred to glass slides, examined under a fluorescence microscope and images were collected using a SPOT camera (Diagnostic Instruments, Sterling Heights).

Western blots

Samples were mixed with 2x protein loading buffer and incubated at 37°C for 30 min, then separated by 10% (w/v) SDS-PAGE. After electrophoresis at 4°C, proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford). The transfer buffer contained 25 mM Tris and 192 mM glycine.

Blots were blocked overnight in blocking solution containing TBS-T [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.05% (v/v) Tween-20] and 2% (w/v) nonfat dry milk at 4°C. Primary antibodies were diluted in blocking solution. After 1 hour incubation with primary antibodies at room temperature, the membranes were washed three times with TBS-T for 5 min per wash. Secondary antibodies conjugated with horseradish peroxidase (Pierce, Rockford) were diluted in blocking solution and reacted with the blots for 45 min at room temperature. After three 5 minute washes in TBS-T and one 5 minute wash in TBS [20 mM Tris-HCl (pH 7.6), 137 mM NaCl], immunodecorated proteins were visualized by chemiluminescent reaction with SuperSignal Chemiluminescent Substrate (Pierce, Rockford) on X-ray film.

The blots could be stripped between detections by incubating blots with stripping buffer [62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS and 100 mM β-mercaptoethanol] at 50°C for 30 min with shaking. After two 10 minute washes in TBS-T, blots were blocked overnight in blocking solution and could be analyzed again with different antibodies.

GUS enzyme activity assay

β-glucuronidase (GUS) enzyme activity can be detected by a fluorometric assay with 4-methyl umbelliferyl glucuronide (MUG) as substrate. MUG is not fluorescent until cleaved by β-glucuronidase to release 4-methyl umbelliferone (MU) and this

fluorescence assay is intrinsically 100 to 1000 times more sensitive than colorimetric methods (Jefferson, 1987).

Fluorometric assay of GUS enzyme activity was conducted by modifying a procedure previously described by Jefferson (1987). In a 1.5 ml microcentrifuge tube, samples were mixed with 400 µl of assay buffer [50 mM $NaPO_4$ (pH 7.0), 10 mM EDTA, 0.1% (w/v) sarkosyl, 0.1% (v/v) Triton X-100, 10 mM β-mercaptoethanol and 1 mM MUG] and incubated at 37°C. At regular time intervals, 100 µl aliquots were removed into clean 1.5 ml microcentrifuge tubes containing 0.9 ml stop buffer (0.2 M sodium carbonate). Typically 5-10 minute intervals for high levels of GUS activity or 30-60 minute intervals for lower levels were used. A total of three points were done per sample. MU concentrations were determined with a fluorometer (Hoefer, San Francisco) by mixing the stopped samples with another 1 ml of stop buffer in a cuvette. The fluorometer was calibrated with 1 µM MU.

Lowry protein assay

The Lowry assay (Lowry et al., 1951) has often been used for determination of membrane protein concentration. Three stock reagents can be prepared in advance and stored indefinitely. Reagent A consists 2% (w/v) Na_2CO_3 in 0.1 M NaOH. Reagent B is 2% (w/v) NaK tartrate tetrahydrate. Reagent C is 1% (w/v) $CuSO_4 \cdot 5H_2O$. Just before the assay, reagent D was freshly prepared by mixing reagents A, B and C in a ratio of 100 : 1 : 1 (v:v:v). Reagent E was made fresh by diluting a stock solution of 2 M Folin and Ciocalteu's phenol reagent (Sigma, St. Louis) to 1 M in water. Protein samples were mixed with 250 µl of 0.4 % (w/v) deoxycholate in a 1.5 ml microcentrifuge tube and the assay was initiated by adding 750 µl of reagent D at timed intervals. After 10 min, 75 µl

of reagent E was added and the color was allowed to develop for about 45 min and absorbance was read at 710 nm. Bovine serum albumin was used in preparing a standard curve.

AtCPK5 specific antibody

AtCPK5 rabbit polyclonal antiserum was raised against a fusion protein, containing the first 50 amino acids of AtCPK5 fused with glutathione S-transferase (GST). The construct was made by Estelle Hrabak and the antiserum was prepared by Jeff Imbaro. Recombinant protein expressed in *E. coli* was purified on a glutathione-agarose matrix (Pharmacia, Piscataway) and injected into New Zealand White rabbits. Crude serum was precipitated with 50% ammonium sulfate and redissolved in phosphate-buffered saline (PBS) [137 mM NaCl, 4.3 mM $Na_2HPO_4.7H_2O$, 2.7 mM KCl and 1.5 mM KH_2PO_4]. After binding to a column coupled with AtCPK5-6His fusion proteins, AtCPK5 antibodies were eluted with 0.1 M glycine (pH 2.7) and immediately neutralized with 0.1 volume of 1 M Tris-HCl (pH 8.0), followed by dialysis against PBS. AtCPK5-6His columns were made using cyanogen bromide-activated Sepharose 4B (Sigma, St. Louis, Cat. No. C9142) according to manufacturer's instruction.

Membrane markers

Specific markers were used to detect each membrane. The Golgi membrane marker was analyzed by enzyme assay and a direct colorimetric assay was used to detect thylakoid membranes. Western blots were used for detection of vacuolar, mitochondrial and plasma membranes as well as endoplasmic reticulum membranes.

Golgi membrane: Latent UDPase activity is often used as a marker for Golgi membranes (Nagahashi and Kane, 1982). Triton-stimulated UDPase activity was assayed by adding samples to 100 µl of assay buffer [30 mM MES-Tris (pH 6.5), 3 mM $MnSO_4$ and 3 mM UDP] in the absence or presence of 0.03% (v/v) Triton X-100. Reactions were incubated at 37°C for 20 min and the Malachite Green method was used to quantitate the released phosphate. The Malachite Green method is a sensitive, standard assay for determining released phosphate by using molybdate/malachite green as the color regent (Lanzetta et al., 1979). Color reagent was prepared by mixing 3 parts 0.045% (w/v in water) malachite green/HCl with 1 part 4.2% (w/v in 4N HCl) ammonium molybdate. After filtering through two No. 1 Whatman filters, 0.04% (w/v) tergitol NP was added and color reagent could be stored in a light resistant polyethylene container at 4°C for up to 1 week. To determine released phosphate in the latent UDPase assay, 800 µl of color reagent was added to UDPase assay reaction and 1 minute later, 100 µl of 34% (w/v) sodium citrate was added to stop the reaction. Allowing 20 min for color development, absorbance was recorded at 630 nm. Phosphate standards were prepared containing 0 to 10 nmol phosphate from potassium phosphate. Triton-activated UDPase activity was calculated by subtracting the activity in the absence of detergent from that in the presence of detergent.

Thylakoid membrane: Chlorophyll a and b pigments reside specifically in thylakoid membranes, so measurements of their absorbances provide an excellent marker for thylakoid membranes. Chlorophyll a and b concentrations were measured spectrophotometrically by mixing a sample with 750 µl of 95% (v/v) ethanol, and then measuring both $A_{648.6}$ and $A_{664.2}$. Total chlorophyll a and b contents were then determined

by inserting the measured absorbance values into the equation $C_{a+b} = 5.24A_{664.2} + 22.24A_{648.6}$ (Lichtenthaler, 1987).

Mitochondrial membrane: Monoclonal antibodies against the β-subunit of the maize mitochondrial F_1-ATPase (β-ATPaseD) was used as a mitochondrial marker. β-ATPaseD antibodies can cross-react with the mitochondrial F_1-ATPase from a wild range of plant species (Luethy et al., 1993). Monoclonal antibodies against β-ATPaseD from Dr. T. Elthon (University of Nebraska, Lincoln) were used at a 1:100 dilution during western blotting.

Plasma membrane: One of the most frequently used markers for the plasma membrane is the H^+-ATPase. The H^+-ATPase is a proton pump that generates a proton gradient across the plasma membrane (DeWitt and Sussman, 1995). Antibodies directed against the plant H^+-ATPase offer a distinct marker for plasma membrane. H^+-ATPase polyclonal antibodies from Dr. M. Sussman (University of Wisconsin, Madison) were used at a 1:10,000 dilution during western blotting.

To identify the PM microdomains (lipid rafts), a PM-localized GPI-anchored protein (SKU5 protein) was used as a marker (Sedbrook et al., 2002). Anti-SKU5 polyclonal antibodies from Dr. C. Somerville (Carnegie Institution, Stanford) were used at a 1:1,000 dilution during western blotting.

Endoplasmic reticulum: The lumenal binding protein (BiP) is a commonly used endoplasmic reticulum (ER) marker. BiP is a key molecule in the protein maturation and transport machinery of the endomembrane system (Denecke et al., 1991). Polyclonal BIP antibodies from Dr. M. Chrispeels (University of California, San Diego) were used at a 1:1,000 dilution during western blotting.

Arabidopsis Ca^{2+}-ATPase isoform 2 (ACA2) is another ER marker. ACA2 is a ER-localized calmodulin-regulated Ca^{2+}-ATPase (Hong et al., 1999). Anti-ACA2 polyclonal antibodies from Dr. J. Harper (The Scripps Research Institute, La Jolla) were used at a 1:2,000 dilution during western blotting.

Vacuolar membrane: VM23, a 23 KDa vacuolar membrane protein, has become a useful marker for vacuolar membranes. VM23 is a hydrophobic, integral membrane protein in vacuoles and may act as a secondary transport system coupled with proton transport. In the sucrose density gradient, VM23 from radish co-sedimented with vacuolar H^+-pyrophosphatase and vacuolar H^+-ATPase (Maeshima, 1992). Polyclonal antibodies directed against VM23 from Dr. M. Maeshima (Hokkaido University, Japan) were used at a 1:3,000 dilution during western blotting.

RESULTS

AtCPK5 is associated with the plasma membrane

In many cases, calcium-dependent protein kinase enzymatic activity has been found associated with the plasma membrane, such as in zucchini (Verhey et al., 1993), tobacco (Iwata et al., 1998), oat (Schaller et al., 1992) and red beet (Baizabal-Aguirre and de la Vara, 1997). To examine whether AtCPK5 is associated with the plasma membrane, an aqueous two-phase system was used to analyze membranes from wild type plants. This technique is excellent for distinguishing plasma membrane from intracellular membranes. Due to the differences in surface properties of the membranes, plasma membrane is enriched in the upper phase and other cellular membranes are enriched in the lower phase. After two-phase separation of total membranes from wild type *Arabidopsis* plants, both phases were analyzed by immunoblotting to locate endogenous AtCPK5 protein, as well as various membrane markers. PM marker (H^+-ATPase) was enriched in the upper phase as expected and all the intracellular membrane markers tested were enriched in the lower phase (Fig. 5). AtCPK5 was also highly enriched in the upper phase, comparable to the PM marker (Fig. 5). These data suggest that AtCPK5 is associated with the PM.

AtCPK5 is not associated with plasma membrane lipid rafts

Some plasma membrane localized proteins such as GPI-anchored proteins and dually acylated proteins have been shown to associate with plasma membrane subdomains called raft domains. To further investigate whether AtCPK5 is associated with detergent-insoluble lipid rafts, total microsomes were treated with 1% Triton X-100 and then separated in a continuous sucrose gradient. Triton treatment was previously used

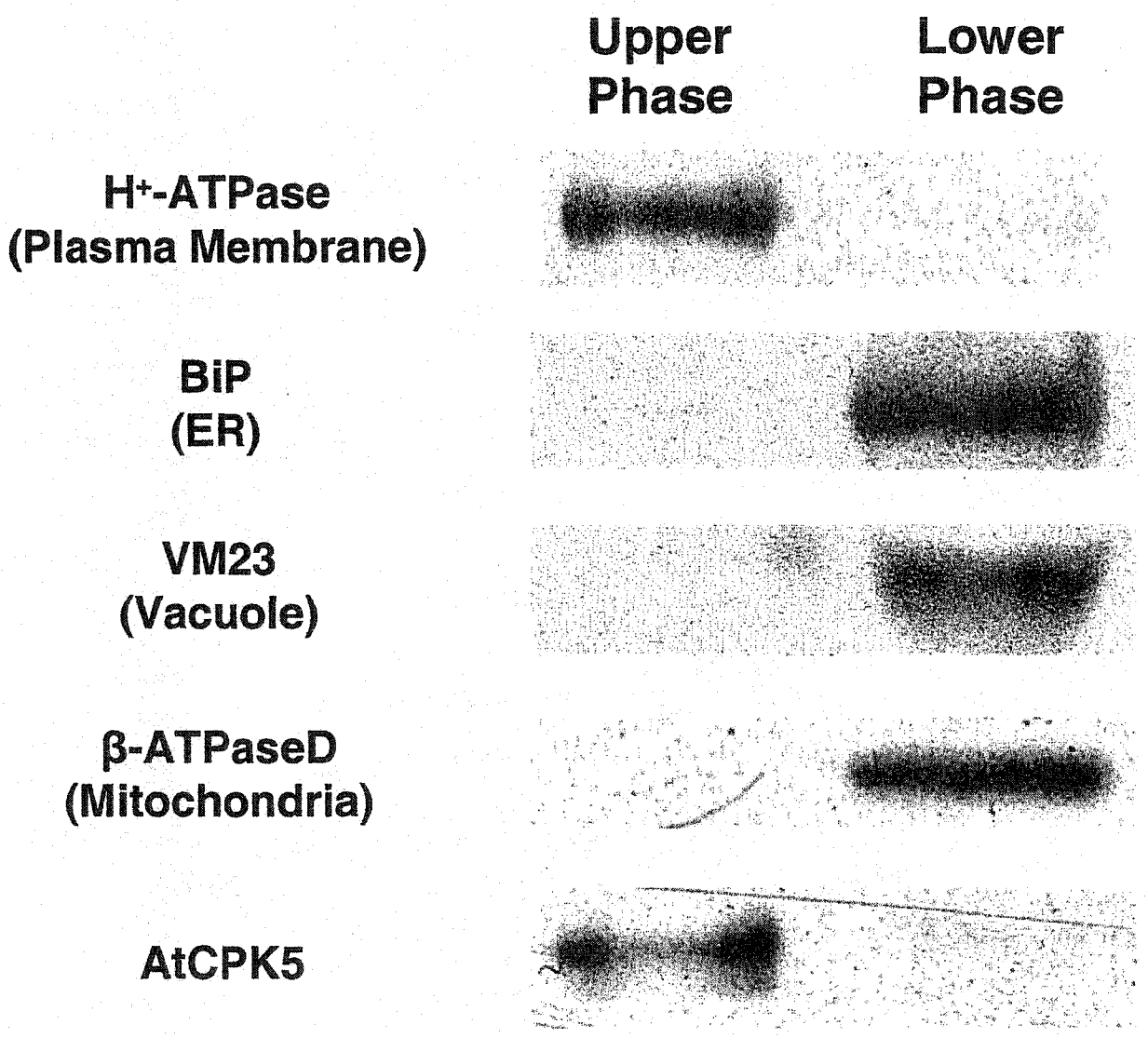

Figure 5. Aqueous two-phase partitioning of microsomal membranes from wild type *Arabidopsis* plants. AtCPK5 protein is enriched in the upper phase comparable to the PM marker. Equal proportions of the upper and lower phases were separated by SDS-PAGE and assayed by immunoblotting with specific antibodies.

to isolate PM microdomains as a floating fraction by sucrose gradient centrifugation in tobacco (Peskan et al., 2000). After sucrose gradient fractionation, equal volumes of each fraction were separated by SDS-PAGE and analyzed by immunoblotting. Since several GPI-anchored proteins have been shown to be associated with the lipid rafts (Brown and London, 2000), a GPI-anchored SKU5 protein was used as a positive control. SKU5 protein was abundant in the fractions of 27%-32% sucrose (Fig. 6), which is similar to the buoyant densities reported for lipid rafts (Peskan et al., 2000). SKU5 protein detected at the bottom of the gradient (50%-53% sucrose) could represent protein which does not associate with lipid rafts or protein dissociated from the lipid rafts during the procedure. In contrast, all AtCPK5 protein was detected at the bottom of the gradient, indicating that AtCPK5 protein is not tightly associated with lipid rafts (Fig. 6). Interestingly, all other membrane markers floated up through the gradient to some extent (Fig. 6). One possible explanation is that 1% Triton X-100 is not sufficient to disrupt the membranes, such that membrane vesicles containing integral membrane proteins remain intact. Due to the lack of data on plant lipid rafts and their contents, I can not rule out the possibility that those marker proteins are associated with lipid rafts.

To avoid potential interference from intracellular membranes during the reverse flotation, PM-enriched upper phases were collected, treated with 1% Triton X-100 and separated by reverse sucrose gradient fractionation as described above. Under these conditions, SKU5 protein spread out in the fractions from 35%-47% sucrose, but both AtCPK5 protein and the PM marker were detected only at the bottom of the gradient (45%-47%) (Fig. 7). Since very little is known about lipid rafts in *Arabidopsis*, the reason why SKU5 protein did not peak at the expected fractions of lipid rafts could be

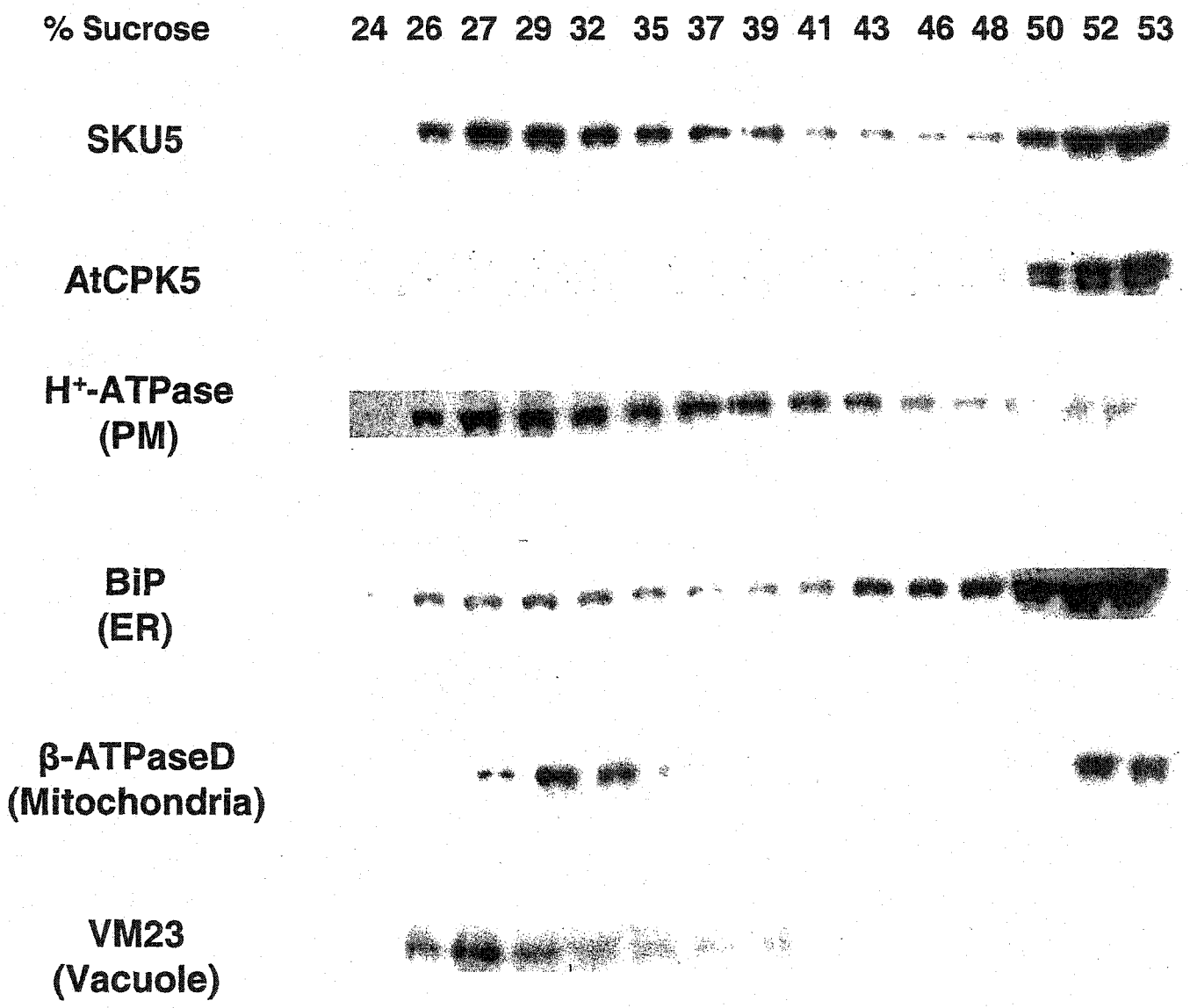

Figure 6. Sucrose gradient flotation of microsomal membranes from wild type *Arabidopsis* plants. Microsomal membranes were treated with 1% Triton at 4°C for 30 min, adjusted to 53% sucrose and overlayed with a sucrose gradient (22-50% w/w). After centrifugation, one ml fractions were collected starting from the top of the gradient, and analyzed by SDS-PAGE and immunoblotting. Lipid rafts are expected to be located at a sucrose density of 28-32%.

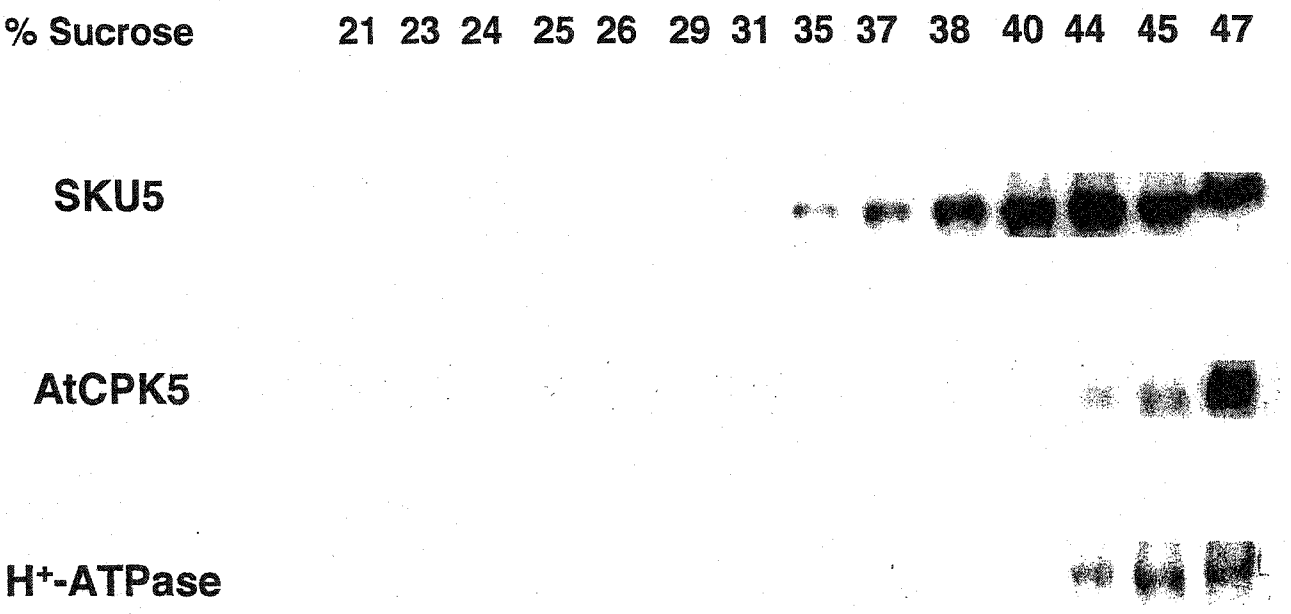

Figure 7. Sucrose gradient flotation of PM-enriched upper fraction after two-phase partitioning. PM-enriched fractions obtained by aqueous two-phase partitioning as described in Materials and Methods were treated with 1% Triton and subjected to sucrose gradient flotation. One ml fractions were collected from top of the gradient and analyzed by SDS-PAGE and immunoblotting.

complicated. One possibility is due to the differences in the complexity of the starting materials, 1% Triton X-100 treatment may have different effects on the distribution of lipid rafts in sucrose gradients. These preliminary data suggest that PM-localized AtCPK5 may not associate with lipid rafts.

AtCPK5 is myristoylated *in vitro*

The AtCPK5 protein has no predicted transmembrane domains that could account for its observed plasma membrane localization, but it contains a potential N-terminal myristoylation consensus sequence. To determine whether AtCPK5 protein can be myristoylated, a coupled transcription/translation system from wheat germ which has been shown to contain NMT activity (Heuckeroth et al., 1988; Ellard-Ivey et al., 1999; Lu and Hrabak, 2002) was used to produce AtCPK5 mRNA from the T7 promoter in a linearized pBluescript construct. Trancription was coupled to translation in the presence of either [^{35}S]methionine or [^{3}H] myristic acid. Figure 8 (lane 2) shows that AtCPK5 protein of the predicted size (~60 kDa) was synthesized *in vitro* in the presence of [^{35}S]methionine. Figure 8 (lane 5) shows that radiolabel was incorporated into the protein when AtCPK5 was synthesized in the [^{3}H] myristic acid-containing reaction. The identity of AtCPK5 protein was confirmed by immunoblotting with AtCPK5 antibody (Fig. 8, lane 8). These results indicate that AtCPK5 is myristoylated *in vitro*.

The 50 kDa band recognized by AtCPK5 antibodies represents non-specific binding to a wheat germ protein since it was detected in the mock reaction that contained no plasmid template (Fig. 8, lane 7). I also observed that AtCPK5 routinely resolved itself by SDS-PAGE as a doublet of 60 and 62 kDa species. The form of higher molecular weight (AtCPK5*) represents a phosphorylated form of the polypeptide as

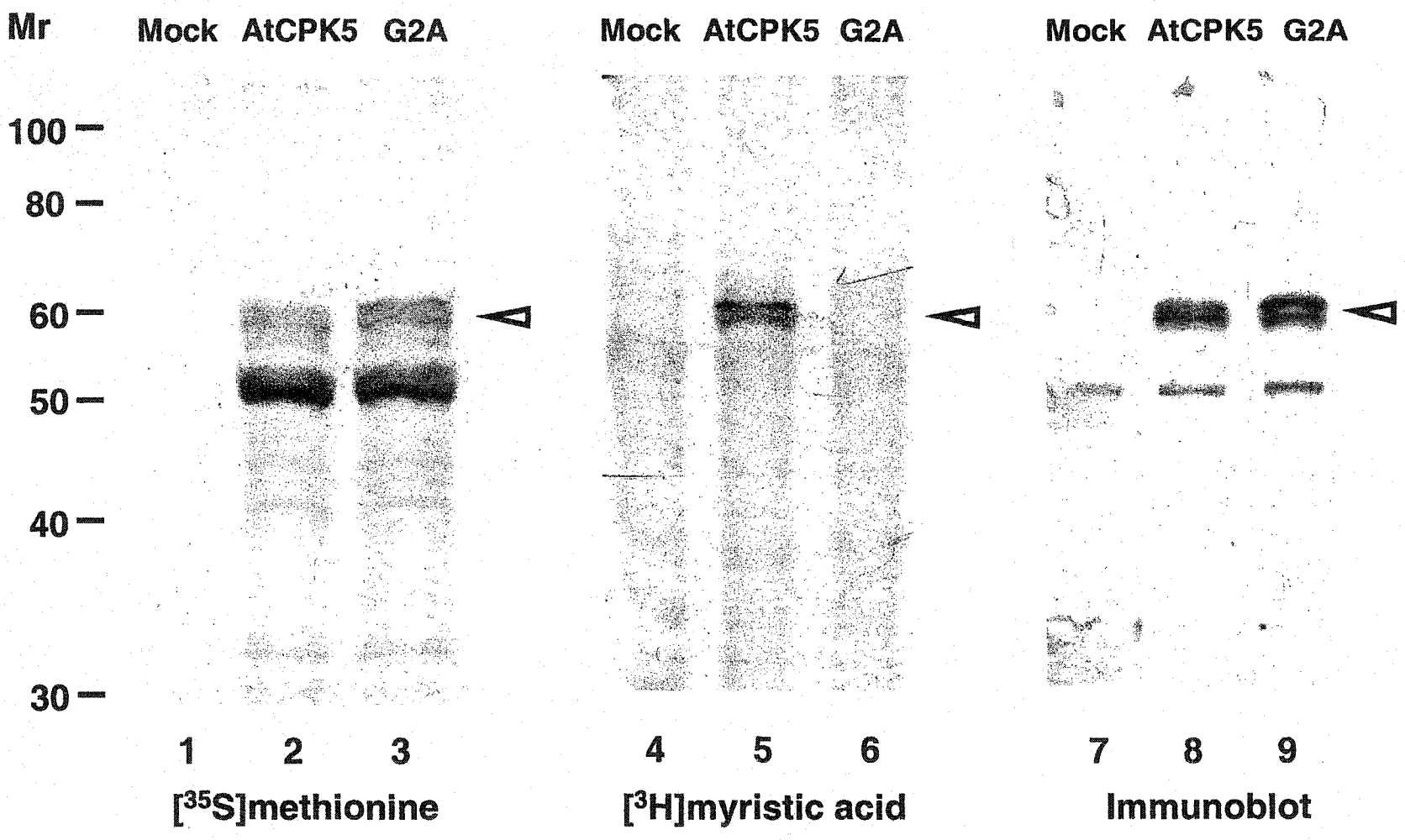

Figure 8. Wheat germ *in vitro* transcription/translation showing AtCPK5 is myristoylated at glycine-2. CPK5 cDNA (lane 2, 5 and 8) was expressed in the presence of either [^{35}S]methionine (lanes 1-3), or [^{3}H]myristic acid (lanes 4-6), or no radiolabel (lanes 7-9), followed by SDS-PAGE and fluorography or immunoblotting. Parallel experiments were performed after site-directed mutagenesis to change the glycine at position 2 of AtCPK5 to an alanine (lanes 3, 6 and 9). Mock reactions (lane 1, 4 and 7) contained no plasmid. Arrow indicates AtCPK5 protein.

discussed below. The smaller proteins routinely observed in [^{35}S]methionine-labeled reactions possibly corresponded to an abortive translation product or an AtCPK5 degradation product (Fig. 8, lanes 2 and 3).

A G2A mutation prevents myristoylation of AtCPK5 in vitro

To confirm that the N-terminal glycine residue was the site of myristic acid attachment, site-directed mutagenesis was used to create a second position glycine to alanine (G2A) mutation and plasmid CPK5(G2A) was tested in the wheat germ cell-free system in the presence of either [^{35}S]methionine or [^{3}H]myristic acid. In the [^{35}S]methionine reaction, AtCPK5 protein synthesis was not affected by the G2A mutation (Fig. 8, lane 3), but the myristoylation of AtCPK5 protein was abolished (Fig. 8, lane 6). [^{3}H]myristate label was only incorporated into the wild type AtCPK5 protein (Fig. 8, lane 5), but not the mutated AtCPK5 protein (Fig. 8, lane 6) despite the presence of significant protein production as determined by the control reaction with [^{35}S]methionine (Fig. 8, lane 3). Since the N-terminal glycine residue is absolutely required by N-myristoyl transferase (NMT) (Towler et al., 1988; Johnson et al., 1994), these results demonstrate that the AtCPK5 protein is a specific substrate for wheat germ NMT *in vitro* and that the glycine at position 2 of AtCPK5 protein is the site of myristoylation.

AtCPK5* is a phosphorylated form of AtCPK5

With the plasmids expressing both WT AtCPK5 and the G2A mutant, AtCPK5 protein was detected as a doublet of 60 and 62 kDa species in the wheat germ cell-free system suggesting that the doublet does not represent myristoylated and non-

myristoylated AtCPK5. CDPK enzymatic activity is known to be modified by reversible phosphorylation (Harmon et al., 2000; Cheng et al., 2002). To examine whether the 62 kDa AtCPK5* protein is a phosphorylated form of AtCPK5 (60 kDa), *in vitro* synthesized AtCPK5 protein was treated with alkaline phosphatase (AP) in the absence or presence of alkaline phosphatase inhibitor (API). Alkaline phosphatase treatment resulted in the disappearance of the 62 kDa AtCPK5* protein (Fig. 9, lane 2 and 5). Alkaline phosphatase inhibitor specifically prevented the loss of the doublet (Fig. 9, lane 3 and 6) indicating that AtCPK5* represents a phosphorylated form of AtCPK5.

The first 16 amino acids of AtCPK5 are sufficient for PM localization

In several well-studied acylated proteins, it has been demonstrated that correct subcellular targeting information resides in a short N-terminal region (Sigal et al., 1994; Borgese et al., 1996; Alsheimer et al., 2000). In addition, the first 10 amino acids of AtCPK2 are known to be sufficient for its ER targeting (Lu and Hrabak, 2002). To determine whether the amino terminal region of AtCPK5 contains the PM-targeting information, transgenic plants containing the CPK5(16aa)-GUS construct (Fig. 3) were used for analysis of soluble and microsomal fractions. About 73% $\pm$ 7% (n=6) of GUS enzyme activity, representing the CPK5(16aa)-GUS fusion protein, was found in the microsomal fraction (Fig. 10). Aqueous two-phase partitioning experiments were conducted for CPK5(16aa)-GUS plants and GUS enzyme activity was highly enriched in the upper phase comparable to the wild type AtCPK5 protein and PM marker (Fig. 11). These results suggest that the first 16 amino acids of AtCPK5 are sufficient to target a soluble GUS protein to the PM.

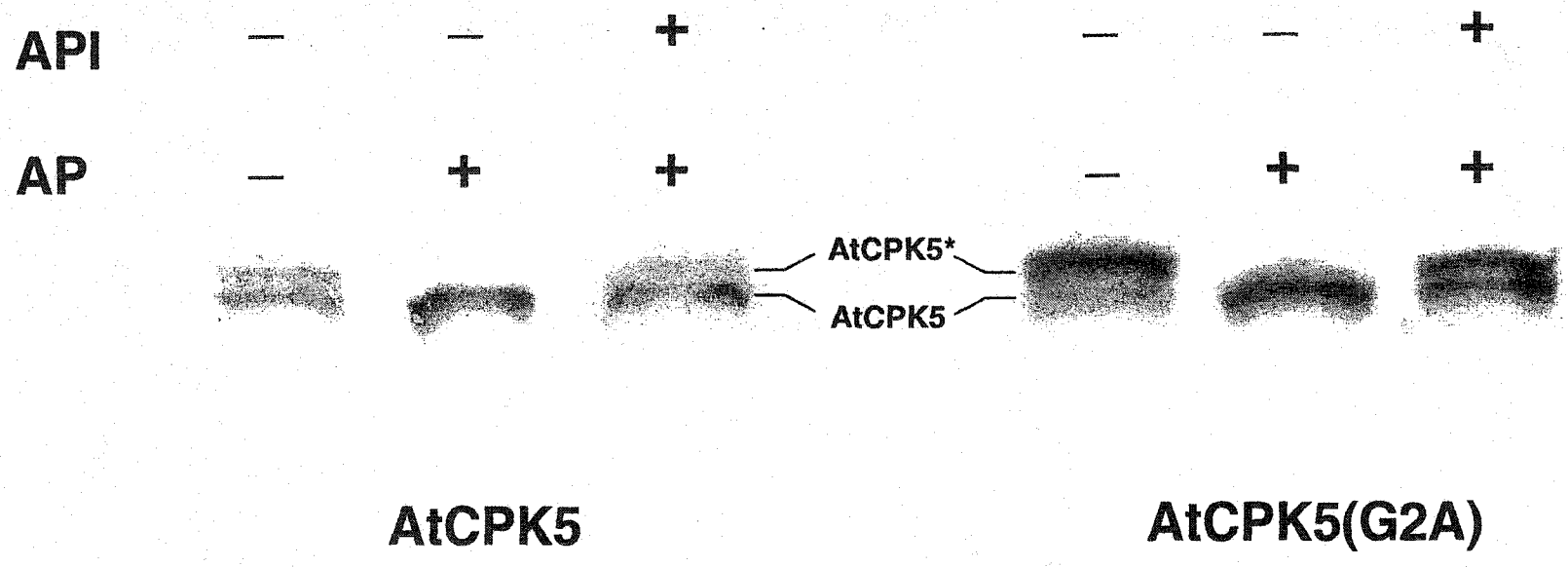

Figure 9. Phosphatase treatment converted AtCPK5* into the AtCPK5 form. A cell-free wheat germ extract was used to express both AtCPK5 wild type and G2A mutant cDNA. Extract (10 µl) was incubated with 5 units of calf intestinal alkaline phosphatase (AP) for 15 min at 30°C in the absence (-) or the presence (+) of alkaline phosphatase inhibitor (API). The reaction products were analyzed by SDS-PAGE and immunoblotting with AtCPK5 specific antibody.

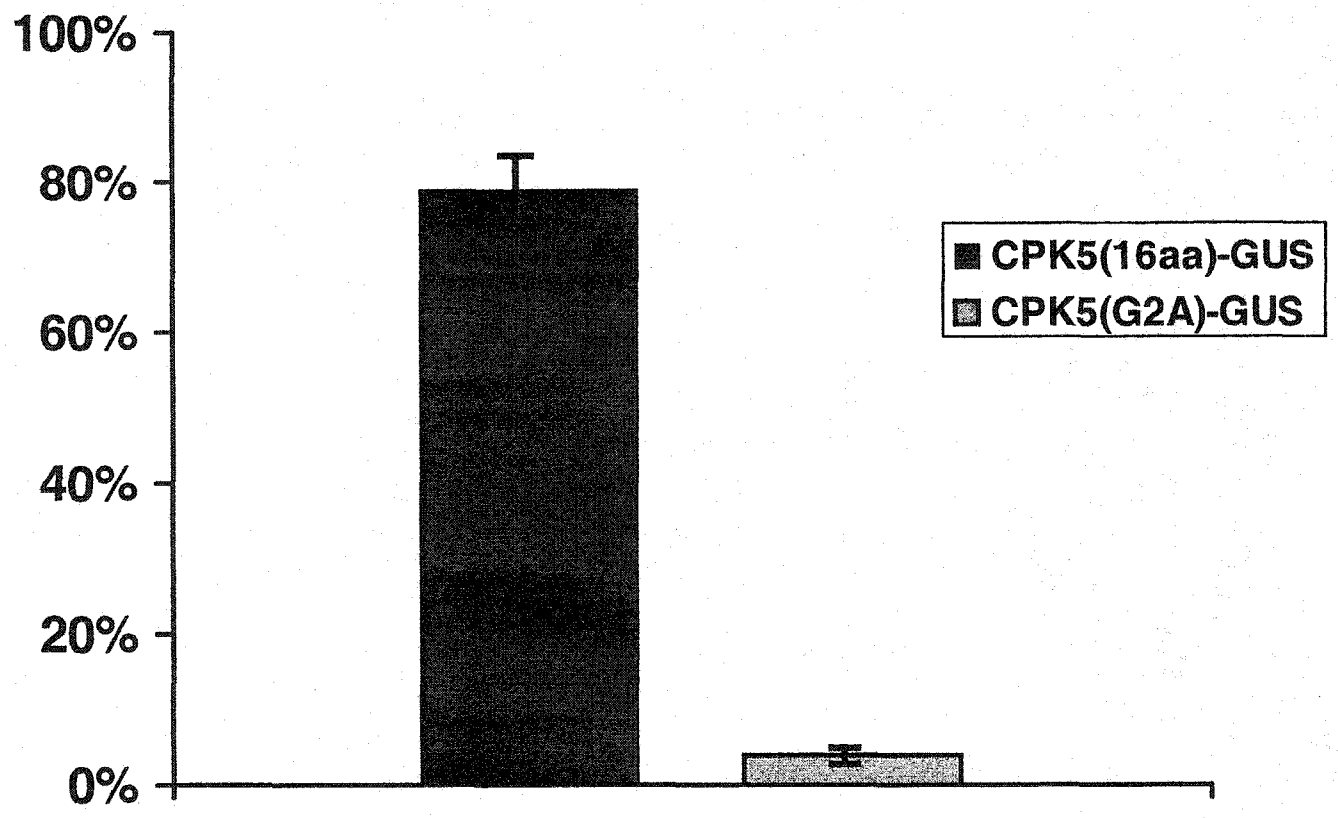

Figure 10. Percentage of membrane-associated GUS activity from CPK5(16aa)-GUS plants and CPK5(G2A)-GUS plants. Soluble proteins and total microsomes were separated by ultracentrifugation and GUS activity of both soluble and microsomal fractions was assayed. In 3 independent transgenic lines, total membrane-bound GUS activity (nmol min^{-1} mg^{-1}) varied from $1.1x10^4$ to $1.8\ x10^4$ in CPK5(16aa)-GUS plants and from $1.0x10^3$ to $2.4x10^3$ in CPK5(G2A)-GUS plants.

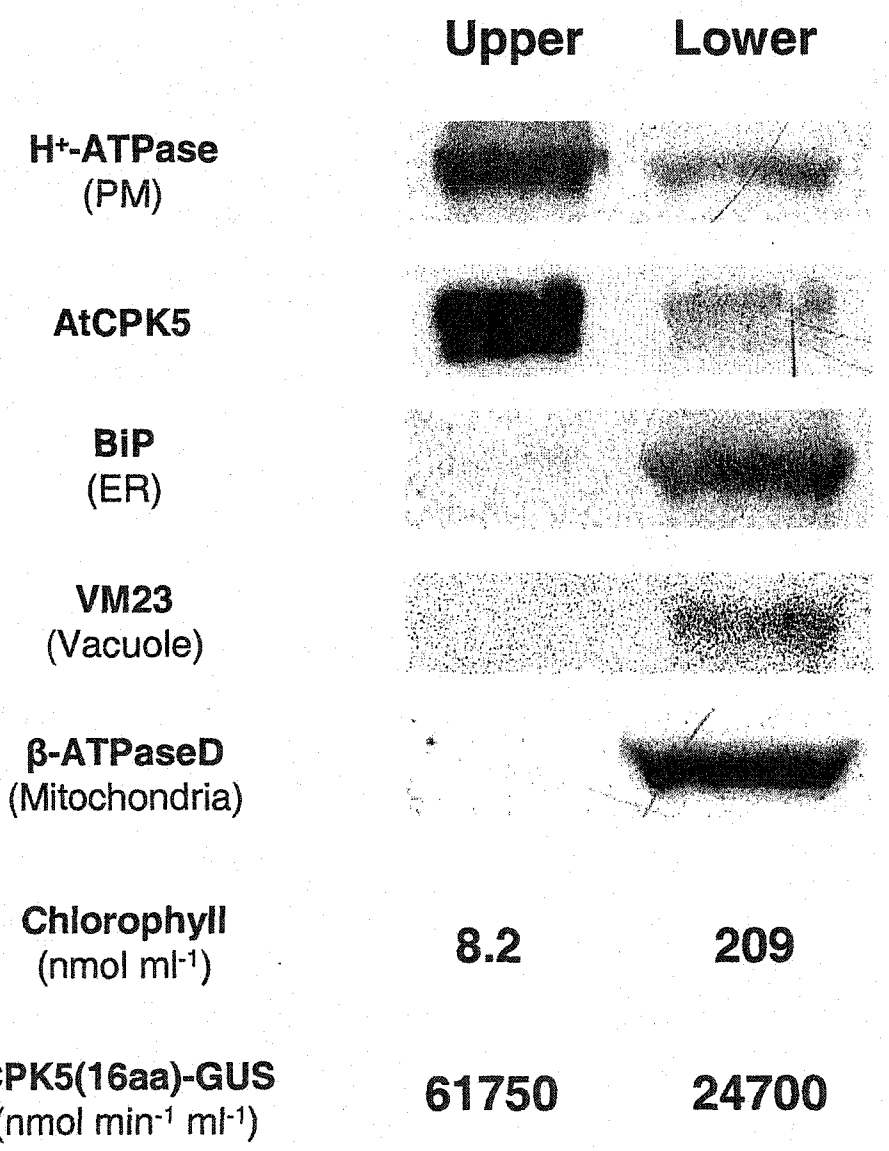

Figure 11. Aqueous two-phase partitioning of microsomal membranes from CPK5(16aa)-GUS transgenic plants. CPK5(16aa)-GUS fusion protein, native AtCPK5 protein, as well as the PM marker accumulated in the upper phase. Equal proportions of the upper and lower phases were separated by SDS-PAGE and assayed by immunoblotting with specific antibodies. Chlorophyll absorbance (thylakoid marker) was measured spectrophotometrically and AtCPK5(16aa)-GUS fusion protein was assayed fluorometrically for GUS enzyme activity.

To confirm that the N-terminal region of AtCPK5 contains PM-targeting information, we analyzed the subcellular localization of CPK5(16aa)-GFP fusion protein by fluorescence microscopy using a transient expression system. When expressed in leek epidermal cells under control of the AtCPK5 native promoter, the CPK5(16aa)-GFP fusion protein was targeted to the cell periphery, likely the plasma membrane, although some fluorescence was also observed in the nucleus and cytosol (Fig. 12). In fact, fluorescence was observed in the nucleus for all transgenic plants tested (Fig. 12). Free GFP, CPK5(16aa)-GFP and CPK5(G2A)-GFP should be able to freely enter the nucleus due to the small size of the proteins ($\leq$ 28 kDa), which is below the exclusion limit of the nuclear pore complex (40 kDa) and is unlikely to represent specific targeting. Fluorescence not associated with the membrane probably represents the non-membrane-bound form of the CPK5(16aa)-GFP fusion protein. Results from laser confocal microscopy of CPK5-GFP *Arabidopsis*, which is larger than 40 kDa, also support a dual PM and cytosol localization for AtCPK5 (N. Etheridge, personal communication). All of these data suggest that the first 16 amino acids of AtCPK5 are sufficient for PM localization.

G2A mutation abolishes AtCPK5 membrane association in plants

As already shown, a G2A mutation prevents myristoylation of AtCPK5 *in vitro* (Fig. 8). To address the role of myristoylation in AtCPK5 membrane binding, we examined the effect of a G2A mutation in *Arabidopsis*. Transgenic plants containing the CPK5(G2A)-GUS construct (Fig. 4) were fractionated into soluble and microsomal fractions by ultracentrifugation. GUS enzyme assays of both fractions indicated that only 3% of the GUS activity was detected in the membrane fraction compared with 73% in

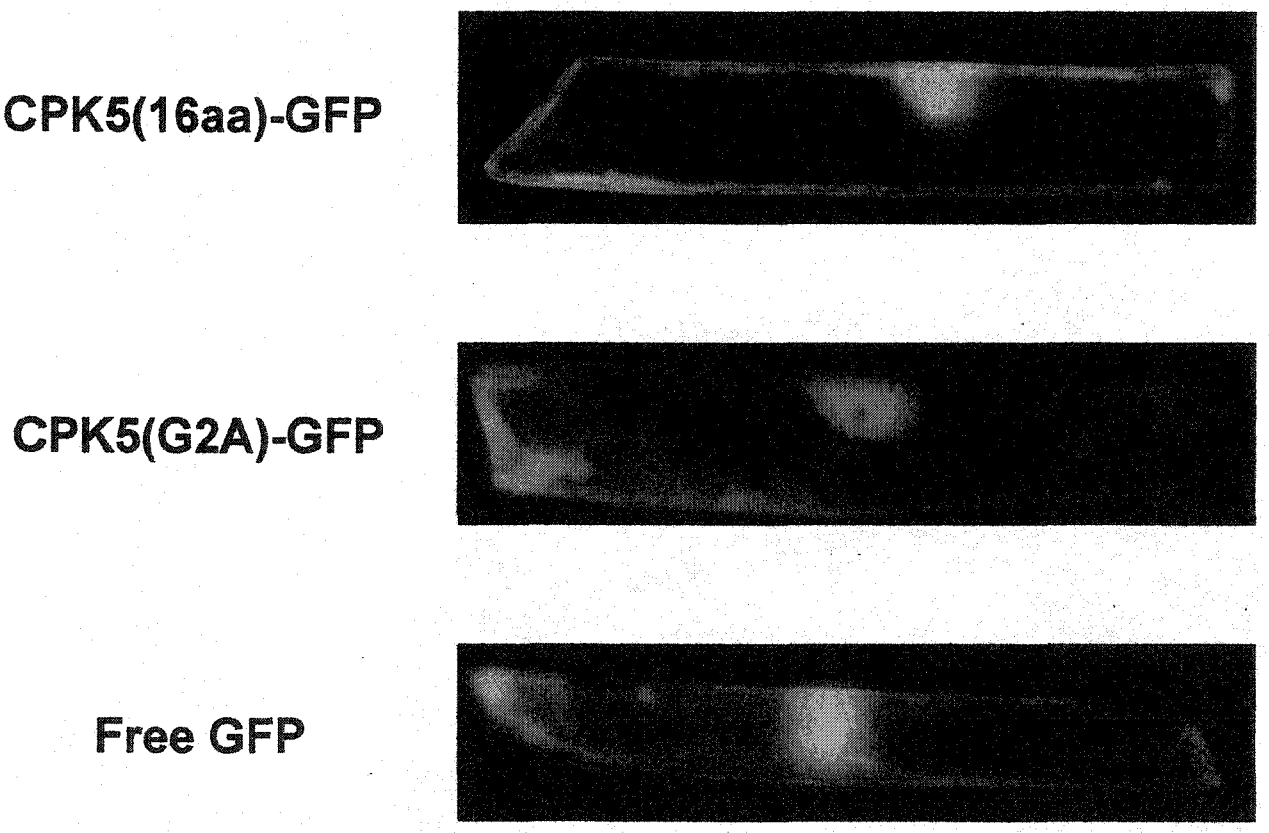

Figure 12. Fluorescence microscopy of leek epidermal cells after biolistic bombardment. The CPK5(16aa)-GFP fusion protein was observed at the cell periphery and in the nucleus. The CPK5(G2A)-GFP fusion protein was no longer abundant at the cell periphery but was localized in the cytosol and nucleus. Fluorescence found in the nucleus probably represents non-membrane-bound CPK5-GFP fusion protein which is about 28 kDa and below the exclusion limit of the nuclear pore complex. GFP protein alone was localized in the nucleus and cytosol.

plants expressing CPK5(16aa)-GUS (Fig. 10). Membrane-associated GUS activity in these CPK5(G2A)-GUS plants is similar to that found in the microsomal fraction of transgenic control plants expressing the GUS protein alone (~2%) (data not shown). Therefore, the 3% membrane-bound GUS in the CPK5(G2A)-GUS plants most likely represents protein non-specifically bound to membranes. These data demonstrate that a G2A mutation of AtCPK5 abolishes its membrane binding. Similar results were observed when CPK5(G2A)-GFP (Fig. 4) was expressed in leek epidermal cells. CPK5(G2A)-GFP fusion protein was mainly localized in the cytosol and nucleus comparable to free GFP (Fig. 12). All of these results suggest that myristoylation is involved in membrane association of AtCPK5 in plants.

DISCUSSION

CDPKs are known to play a key role in many cellular processes such as carbon and nitrogen metabolism (Huber et al., 1996; Huang and Huber, 2001), stomatal movement (Li et al., 1998), plant growth and development (Estruch et al., 1994; Anil et al., 2000), plant defense (Romeis et al., 2000) and response to stress (Abo-el-Saad and Wu, 1995; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Saijo et al., 2000). The large number of CDPKs in a particular plant species suggests that individual CDPKs may perform a specific function in different cells and/or in specific subcellular locations (Harmon et al., 2000; Hrabak, 2000; Cheng et al., 2002). Localization of proteins to the correct cellular location is often required for proper function. Here I have investigated the subcellular localization of AtCPK5 and the mechanisms involved in its membrane association.

Since CDPK enzymatic activity was often found to associate with the PM (Schaller et al., 1992; Verhey et al., 1993; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998), aqueous two-phase partitioning was conducted to separate PM from intracellular membranes. AtCPK5 protein accumulated in the PM-enriched upper phase suggesting that AtCPK5 was localized to the PM. Many well-studied acylated animal proteins such as most members of the Src family of kinases and Gα subunits are targeted to the plasma membrane and have recently been found to localize to specific PM microdomains. To further investigate whether AtCPK5 is associated with lipid rafts, Triton X-100 treatment followed by sucrose gradient fractionation was used to isolate detergent-insoluble lipid rafts. In contrast to the lipid rafts which showed flotation to a sucrose density of 30%-35%, AtCPK5 always remained at the bottom of gradient (47%-

50% sucrose) indicating that AtCPK5 was not associated with PM raft domains. The SKU5 gene encodes a glycoprotein that is related to the multiple-copper oxidases (Sedbrook et al., 2002). In both animal and plant cells, several GPI-anchored proteins have been demonstrated to be enriched in raft domains (Brown and London, 2000), so it is very possible that SKU5 protein localizes to lipid rafts although there is no published experimental evidence to support it. Since very little is known about lipid rafts in plants, a known raft-localized protein such as the heterotrimeric G protein α subunit should be tested to confirm lipid raft location when antibody is available.

To determine which region of AtCPK5 is required for PM targeting, the amino terminal region of AtCPK5 was fused with either GUS or GFP protein. Aqueous two-phase partitioning of transgenic plants expressing the CPK5(16aa)-GUS fusion protein demonstrated that the first 16 amino acids of AtCPK5 was not only sufficient for membrane binding, but also contained PM-targeting information. Fluorescence microscopy of transient expression of CPK5(16aa)-GFP in leek epidermal cells also demonstrated that the amino terminal region of AtCPK5 retained the PM-targeting ability and may be useful for targeting other proteins to the PM.

Although no transmembrane domains have been reported in any CDPKs, AtCPK5 contains an N-myristoylation consensus sequence at its amino terminus. Since myristoylation is known to promote protein-membrane association, this may be part of the mechanism by which AtCPK5 associates with the PM. In an *in vitro* wheat germ coupled transcription/ translation system, I demonstrated that the protein encoded by AtCPK5 is a substrate for plant N-myristoyl transferase (NMT). By converting the second position glycine residue, which is the proposed myristoylation site, into an alanine

residue, I created a CPK5(G2A) mutant that failed to incorporate myristate *in vitro*. This strongly suggests that the second position glycine residue is the site of myristic acid attachment. Although I did not prove that AtCPK5 is myristoylated *in planta*, the observation that the G2A mutation abolished membrane binding of AtCPK5 *in planta* indicates that N-myristoylation of AtCPK5 contributes to its membrane binding in plants. With the current tools, I can not rule out the possibility that the glycine itself is important for interaction with membrane receptor.

AtCPK5 protein was detected as a doublet of 60 and 62 kDa species by isoform specific antibodies. Alkaline phosphatase treatment eliminated the upper band indicating that the 62 kDa species could represent the phosphorylated form of AtCPK5 (AtCPK5*) and the 60 kDa species could represent the non-phosphorylated form of AtCPK5. No evidence suggested that different phosphorylated forms of AtCPK5 have different subcellular locations. One tobacco CDPK (NtCDPK2) has been shown to be autophosphorylated and phosphorylation is required for full activation (Romeis et al., 2001). How this phosphorylation event affects NtCDPK2 activation is not known, but there are some evidence to support that NtCDPK2 could be activated through phosphorylation by an upstream protein kinase (Romeis et al., 2001). Whether AtCPK5* results from autophosphorylation or phosphorylation by other protein kinases and whether this phosphorylation event affects AtCPK5 function will be further investigated.

Myristoylated proteins have been reported in many cellular membranes (James and Olson, 1990; Fackler et al., 1997; Alsheimer et al., 2000), so it is unlikely that myristic acid alone is sufficient to target proteins to specific subcellular membranes. Since I have demonstrated that the first 16 amino acids of AtCPK5 are sufficient to direct

the soluble GUS or GFP protein to the PM, any targeting information that contributes to membrane specificity must be retained in the amino terminal region of AtCPK5. AtCPK5 contains a cysteine residue at position 5 nearby the myristoylation site, which is a potential palmitoylation site (Milligan et al., 1995; Hrabak, 2000). Palmitoylation is a post-translational and reversible modification catalyzed by palmitoyltransferase, an enzyme which is probably associated with the PM (Dunphy et al., 1996). Many myristoylated proteins are also palmitoylated. In addition, mutation of the N-terminal glycine to alanine usually not only prevents myristoylation but also decreases or completely blocks palmitoylation. It is hypothesized that prior myristoylation may promote palmitoylation by increasing hydrophobicity of the protein and thereby increasing its affinity for the PM, the probable site of palmitoyltransferase action (Degtyarev et al., 1994; Hallak et al., 1994; Mumby et al., 1994; Wilson and Bourne, 1995).

Most previously described dually acylated proteins are localized in the PM (Shenoy-Scaria et al., 1994; Galbiati et al., 1999; Brown and London, 2000; Moffett et al., 2000; Dunphy et al., 2001) and it appears that palmitoylation can selectively target proteins to the PM (Dunphy et al., 1996). A palmitoylation site mutant of the heterotrimeric G protein α subunit is no longer targeted to the PM although the protein is still myristoylated and able to bind membranes (Morales et al., 1998). Both myristoylation and palmitoylation have been shown to occur in the membrane-bound rice OsCPK2, although the subcellular location of OsCPK2 has not been determined (Martin and Busconi, 2000). I have demonstrated that a G2A mutation totally abolishes AtCPK5 membrane association, but whether the effect is due to the lack of myristoylation or the

lack of both myristoylation and palmitoylation is not clear. Further investigation is required to determine the role of palmitoylation in AtCPK5 membrane association and PM targeting. I also can not rule out the possibility that another protein may interact with the first 16 amino acids of AtCPK5 and facilitate AtCPK5 PM association.

In summary, my results demonstrate that *Arabidopsis* CDPK isoform AtCPK5 is associated with the plasma membrane in plants, but is likely not associated with PM raft domains. AtCPK5 can be myristoylated *in vitro* and mutation of the myristoylation site not only prevents myristoylation, but also abolishes membrane binding in plants indicating that myristoylation is at least part of the mechanism by which AtCPK5 associates with the PM. The amino terminus of the AtCPK5 protein contains the information necessary for PM localization, indicating that this region maybe useful for protein targeting applications. These studies provide the basic information for understanding the specific function of AtCPK5 and contribute to our understanding of myristoylation in plants.

CHAPTER III

AN ENDOPLASMIC RETICULUM LOCALIZED CALCIUM-DEPENDENT PROTEIN KINASE IN *ARABIDOPSIS THALIANA*

(This chapter is based in part on a manuscript published in *Plant Physiology*, March 2002, Vol. 128, pp. 1008-1021: Sheen X. Lu and Estelle M. Hrabak. An *Arabidopsis* Calcium-Dependent Protein Kinase Is Associated with the Endoplasmic Reticulum.)

INTRODUCTION

CDPK enzymatic activity has been identified in both soluble and membrane fractions. In some studies, it was reported to associate with the plasma membrane (PM) (Schaller et al., 1992; Verhey et al., 1993; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998), but in many cases, the specific membrane was not determined (Abo-el-Saad and Wu, 1995; Martin and Busconi, 2001). Therefore, it is possible that CDPKs are associated with cellular membranes other than the PM. In fact, potential CDPK substrates include a vacuolar membrane-localized chloride channel (Pei et al., 1996) and an ER-localized calcium pump (Hong et al., 1999) in addition to proteins localized in the cytosol or PM (Schaller and Sussman, 1988; Ogawa et al., 1992; Weaver and Roberts, 1992; Bachmann et al., 1996; Huber and Huber, 1996; Huber et al., 1996; Li et al., 1998; Lino et al., 1998).

In this study, I focused on *Arabidopsis* CDPK isoform AtCPK2. With a predicted molecular mass of 72 kDa, AtCPK2 is the largest of the 34 *Arabidopsis* CDPKs. In the variable domain, it has 185 amino acids and contains an N-myristoylation consensus sequence. Some myristoylated proteins remain soluble (Johnson et al., 1994; Bhatnagar et al., 1997), some are membrane associated (James and Olson, 1990; Haun et al., 1993; Strittmatter et al., 1993; Fackler et al., 1997; Alsheimer et al., 2000; Zha et al., 2000), and some exist in an equilibrium between soluble and membrane-bound states (Resh, 1999; Romeis et al., 2000). In animal and yeast studies, myristoylated proteins are found associated with many cellular membranes and often the short amino terminus of the proteins are able to specify association with the correct membranes (Pellman et al., 1985; Sigal et al., 1994; Borgese et al., 1996; Alsheimer et al., 2000).

Here I investigated the subcellular localization of AtCPK2 and the mechanisms involved in its membrane association as well as the function of its amino terminal region in membrane targeting.

MATERIALS AND METHODS

The materials and methods described in Chapter II are not repeated here.

Plasmid constructs

These constructs were made previously by E. Hrabak and are only briefly described here. The CPK2-GUS transgene construct consisted of the GUS (β-glucuronidase) reporter gene inserted in-frame into the CPK2 genomic sequence at a location 30 nucleotides upstream of the stop codon (Fig. 13A). This CPK2 genomic clone contained 1.6 kb of sequence upstream from the translation start site and 0.6 kb of sequence downstream from the stop codon as well as all introns and exons. This construct was cloned into pBIN19 which contains the kanamycin resistance gene for plant transformation. The CPK2(G2A)-GUS construct is identical to CPK2-GUS except that site-directed mutagenesis of the glycine at position 2 to alanine was done (Fig. 13B). A fragment containing 1.6 kb of CPK2 genomic sequence upstream from the translational start site and including the first 30 nucleotides of the coding sequence was cloned into pBI101 (Clontech, Palo Alto) upstream of and in-frame with the GUS reporter gene and followed by *nos* terminator. The *nos* terminator is a plant termination signal cassette. This construct was referred to as CPK2(10aa)-GUS (Fig. 13E).

Site-directed mutagenesis

Site-directed mutagenesis was performed with the QuickChange kit (Stratagene, La Jolla) according to manufacturer's instructions using CPK2-GUS as the template DNA. The C5A mutant was generated using the primers (5' GATGGGTAATGCTGCTG TTGGACCAAAC) and (5' GTTTGGTCCAACAGCAGCATTACCCATC). The G2A/C5A double mutant was generated using the primers (5' GATATTTTGATGGCTA

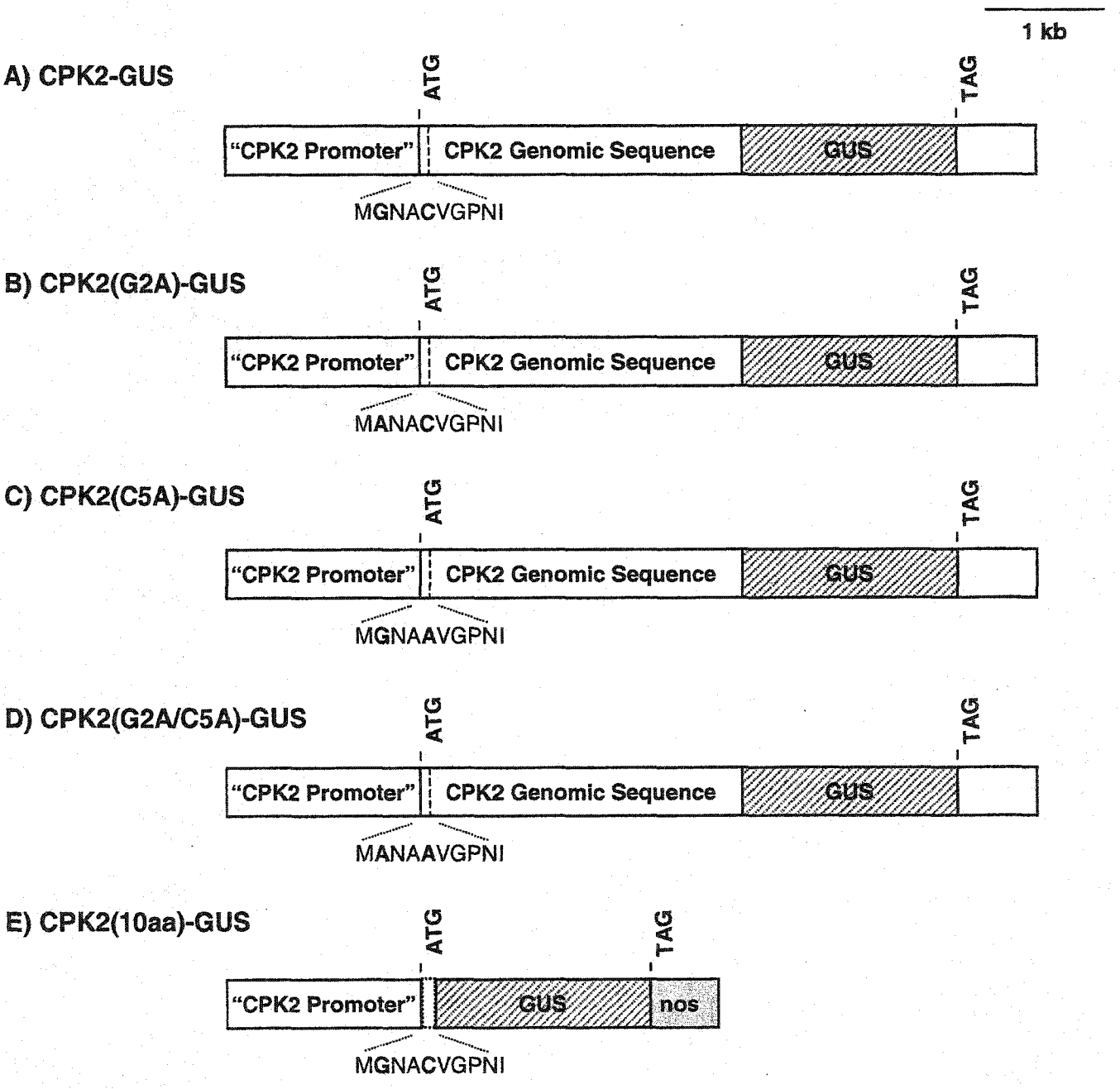

Figure 13. AtCPK2 constructs used for plant transformation. The amino acid sequence encoded by the first 30 nucleotides of the coding sequence is indicated. "CPK2 promoter" indicates the sequence upstream of the translational start site and includes both 5' untranslated leader and promoter sequences. Striped boxes are the coding region of GUS. *nos* is a plant termination signal cassette.

ATGCTGCTGTTGGACCAAAC) and (5' GTTTGGTCCAACAGCAGCATTAGCCA TCAAAATATC). The presence of the desired mutations in all the PCR-generated fragments was confirmed by sequencing. The mutated *AtCPK2* gene was subcloned into the vector pBIN19 and named CPK2(C5A)-GUS (Fig. 13C) or CPK2(G2AC5A)-GUS (Fig. 13D).

Membrane-binding assay

Microsomal membranes were resuspended at 0.5 mg/ml in resuspension buffer [25 mM Tris-HCl (pH 7.5), 10% (w/v) sucrose] containing protease inhibitor cocktail tablets (Roche, Indianapolis) using a ground glass homogenizer. Resuspended membranes were incubated with a final concentration of one of the following: 10 mM EDTA, 1 M NaCl, 1% (v/v) Triton X-100, or 0.1% (w/v) SDS. After incubation at 4°C for 30 min, treated samples were centrifuged at 125,000*g* for 30 min at 4°C. The supernatants were collected and pellets were resuspended in resuspension buffer. Both the supernatants and pellets were analyzed by fluorometric GUS enzyme assay.

Sucrose Gradient Fractionation

Membrane vesicles derived from various organelles have distinct densities due to their protein/lipid ratio and lipid composition. This characteristic allows membrane vesicles to be separated by their buoyant densities using equilibrium density centrifugation. All manipulations were conducted at 4°C with prechilled solutions and all buffers contained protease inhibitor cocktail tablets (Roche, Indianapolis). After separating total microsomes from soluble proteins, resuspended membranes were layered onto linear gradients of 20% to 50% [w/w] sucrose in centrifugation buffer (10 mM Tris-

HCl, pH 7.5), which were prepared with a gradient maker (Hoefer, San Francisco). For all the "+Mg^{2+}" sucrose gradients, 2 mM EDTA and 5 mM $MgCl_2$ were added to the homogenization, resuspension and centrifugation buffers. For all the "–Mg^{2+}" preparations, 5 mM EDTA was added to the homogenization and resuspension buffers, whereas 2 mM EDTA was used in centrifugation buffer. Gradients were centrifuged in a swinging bucket rotor at 125,000*g* for 16 hours at 4°C and one ml fractions were collected from the bottom of the tube. These fractions could be frozen at –80°C or immediately used for marker assays. Sucrose concentration of each fraction was measured with a refractometer.

AtCPK2 specific antibody

AtCPK2 rabbit polyclonal antiserum was raised previously against a fusion protein containing the first 90 amino acids of AtCPK2 fused with glutathione S-transferase (GST). The construct was made by Estelle Hrabak and the antiserum was prepared by Jeff Imbaro. Recombinant protein expressed in *E. coli* was purified on a glutathione-agarose matrix (Pharmacia, Piscataway) and used for immunization of New Zealand White rabbits by standard protocols. The antibody did not cross-react with AtCPK1, the *Arabidopsis* CDPK isoform most closely related to AtCPK2, or with AtCPK5 or AtCPK6 protein (data not shown).

RESULTS

AtCPK2 exists in a membrane associated form

Due to the low expression level of AtCPK2 protein in wild-type *Arabidopsis* (E. Hrabak, personal communication), it was difficult to consistently detect AtCPK2 protein using AtCPK2 specific antibody. In initial experiments, transgenic plants containing the CPK2-GUS construct (Fig. 13A) were used to determine whether AtCPK2 was membrane associated. After the isolation of membranes, both the supernatant (soluble proteins) and the pellet (total microsomes) were assayed for GUS activity using the standard MUG fluorometric assay. As controls, non-transformed WT plants and transgenic plants expressing the GUS protein alone were tested. No detectable GUS activity was found in WT plants. In transgenic plants expressing the GUS protein alone, about 2% of the GUS activity was detected in the microsomal fraction (data not shown), which probably represents protein nonspecifically bound to the membranes or trapped in the membrane vesicles during the procedure. In plants expressing the CPK2-GUS fusion protein, about 40% of the GUS activity was detected in the microsomal fraction (Fig. 14). These results indicate that a portion of the AtCPK2 protein exists in a membrane associated form.

To investigate the type of interaction of AtCPK2 with membranes as well as the strength of the interaction, isolated total microsomes were resuspended and further treated with either 10 mM EDTA, 1 M NaCl, 1% Triton X-100, 0.1% SDS or buffer alone as a control. Treated samples were recentrifuged and the GUS activity in both supernatant and pellet was measured. About 75% membrane-associated GUS activity remained in the pellet after treatment with buffer alone (Fig. 15). Similar results were

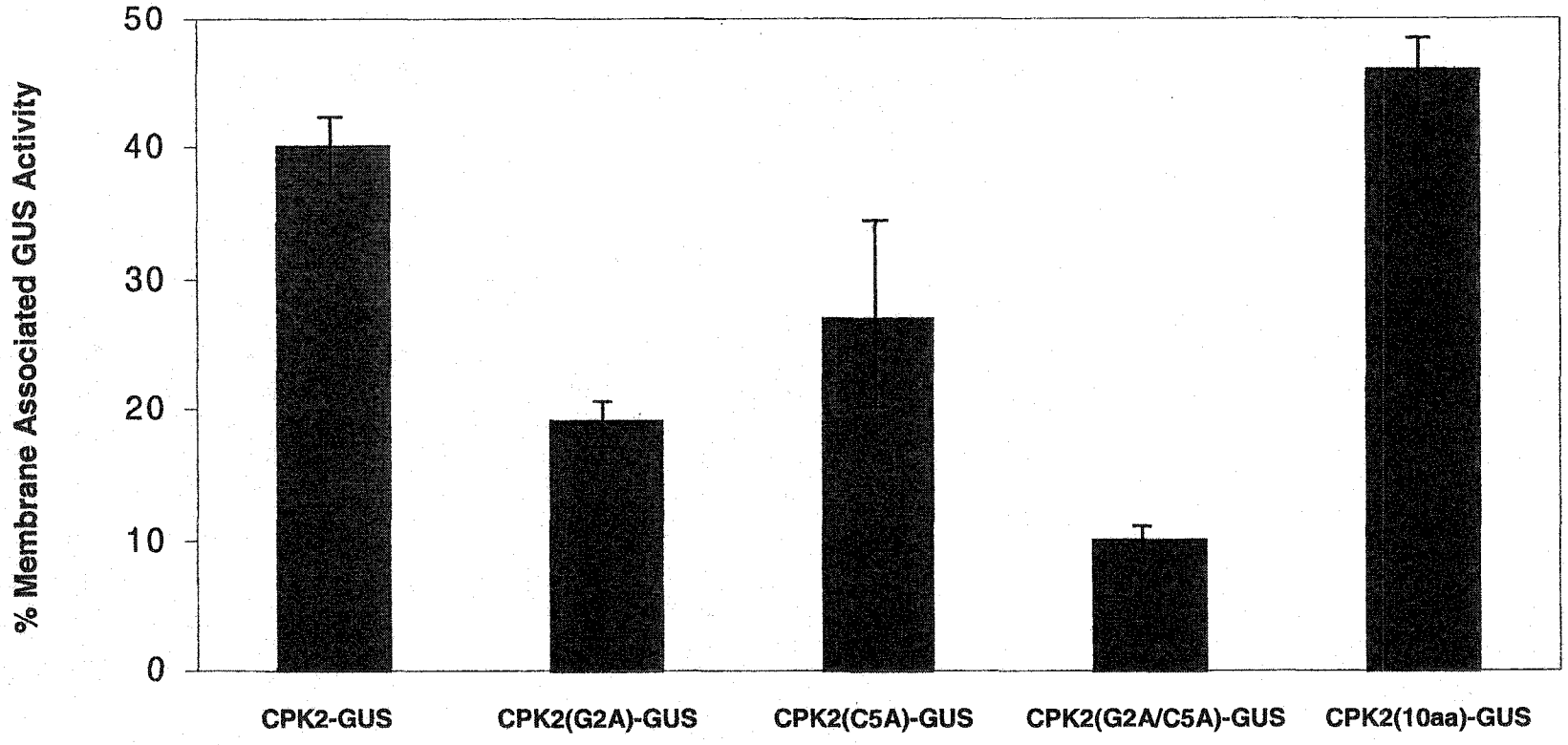

Figure 14. Membrane association of GUS-tagged AtCPK2 protein in transgenic plants. Soluble proteins and total microsomes were separated by ultracentrifugation and GUS activity of both soluble and membrane protein fractions were assayed. In 2-3 independent transgenic lines, total membrane-bound GUS activity (nmol min^{-1} mg^{-1}) was 67.1-154.2 in CPK2-GUS plants, 37.5-76.6 in CPK2(G2A)-GUS plants, 87.3-123.6 in CPK2(C5A)-GUS plants, 17.2-38.3 in CPK2(G2AC5A)-GUS plants and 5.2-8.3 in CPK2(10aa)-GUS plants.

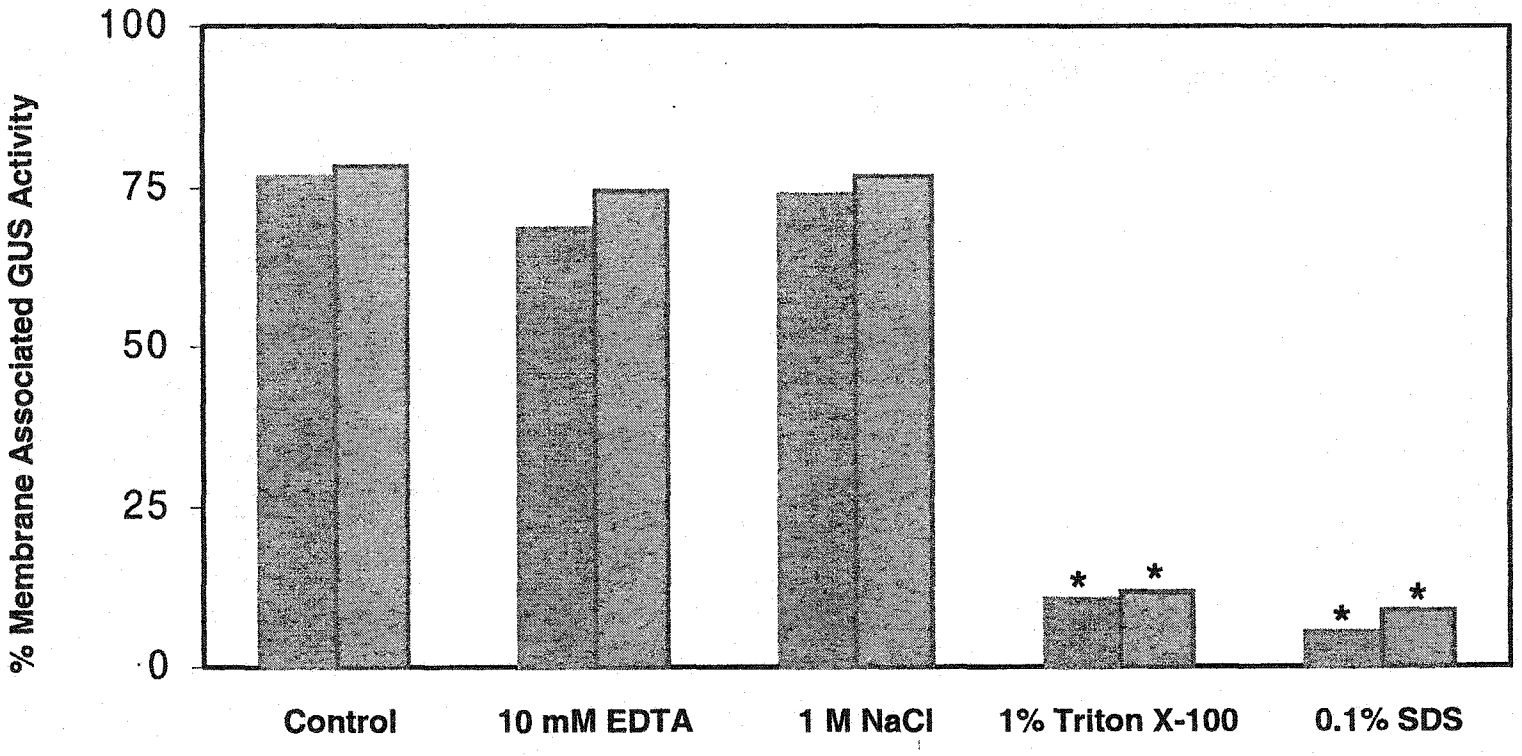

Figure 15. AtCPK2 associated with the membrane fraction after various treatments. Microsomal membranes were isolated from transgenic plants expressing CPK2-GUS. Membrane pellets after ultracentrifugation were homogenized in resuspension buffer alone (control) or resuspension buffer containing EDTA, NaCl, Triton X-100 or SDS and incubated at 4°C for 30 min before repelleting. The resulting supernatant and pellet were assayed for GUS activity to determine the effect of the treatment on AtCPK2 membrane binding. Results from two independent experiments are shown. Asterisks indicate values that were significantly different from the buffer control ($P \leq 0.05$) using one-way ANOVA.

obtained with the treatment with either a chelating agent (EDTA) or high ionic strength (NaCl) indicating that ionic or electrostatic interactions are not important for AtCPK2 membrane binding. As expected, treatment with an ionic detergent (SDS) which disrupts the membrane, efficiently released almost all the GUS activity from the membranes (Fig. 15). Similar results were obtained after treatment with a nonionic detergent (Triton X-100), which could disrupt most types of hydrophobic interactions, but not the detergent-resistant membranes such as lipid rafts (Moffett 2000, Peskan 2000). Since some, but not all, AtCPK2 protein could be removed from membranes by treatment with buffer alone, these results suggest that AtCPK2 may exist in an equilibrium between soluble and membrane-bound forms. Since Triton treatment could release most of the GUS activity from the membrane, AtCPK2 is unlikely to be associated with lipid rafts.

AtCPK2 is associated with the ER membrane

To further define the subcellular location of AtCPK2, an aqueous two-phase system was used initially to determine whether AtCPK2 is associated with plasma membrane (PM). Total microsomes from WT plants were separated into upper phase and lower phase based on the surface properties of the membranes. Equal proportions of both phases were analyzed by SDS-PAGE and immunoblotting to locate AtCPK2 protein, as well as various membrane markers. As expected, the PM marker was enriched in the upper phase and intracellular markers such as ER and mitochondrial markers were enriched in the lower phase (Fig. 16). Golgi, thylakoid and vacuolar membrane markers were also found in the lower phase (data not shown). Using AtCPK2 specific antibody, AtCPK2 protein was mainly detected in the lower phase comparable to the intracellular markers (Fig. 16) indicating that AtCPK2 is not associated with the plasma membrane.

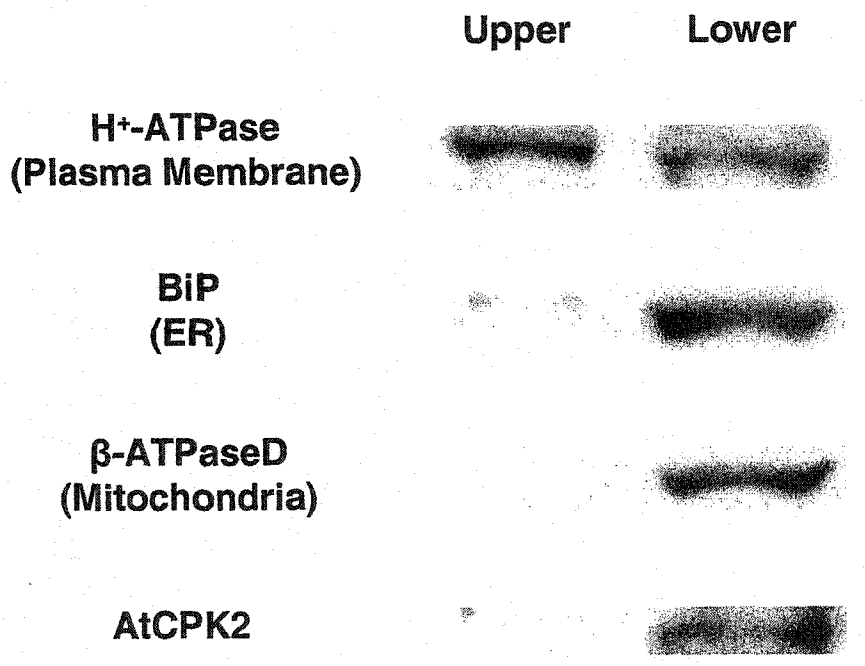

Figure 16. Aqueous two-phase partitioning of microsomal membranes from WT plants showing that AtCPK2 is not associated with the plasma membrane. The PM marker was enriched in the upper phase, whereas AtCPK2 protein accumulated in the lower phase comparable to the ER and mitochondrial markers. Equal proportions of the upper and lower phases were separated by SDS-PAGE and assayed by immunoblotting with specific antibodies.

To localize AtCPK2 to a specific intracellular membrane, total microsomes from WT plants were fractionated based on their buoyant density on sucrose gradients and analyzed by western blots and marker enzyme assays. Since a large Mg^{2+}-dependent density shift is a characteristic of ER membranes, EDTA shifting experiments were conducted during sucrose gradient fractionation. Microsomes and sucrose gradients were prepared in buffers containing EDTA alone ($-Mg^{2+}$) or EDTA plus excess $MgCl_2$ ($+Mg^{2+}$). In "$+Mg^{2+}$" gradients, the presence of Mg^{2+} stabilizes ribosomes on the ER membranes causing them migrate to a sucrose density of 40% to 46%. In "$-Mg^{2+}$" gradients, chelation of Mg^{2+} by EDTA dissociates ribosomes from the ER and causes the ER membrane to sediment at a slightly lower sucrose density (Lord, 1987).

In the presence of Mg^{2+}, AtCPK2 was most abundant at a sucrose density of 41%-46% and in the absence of Mg^{2+}, the AtCPK2 peak shifted to a lighter sucrose density of 33%-37% (Fig. 17). Similar results were only detected with the ER markers BiP (an ER lumen chaperone) and ACA2 (*Arabidopsis* Ca^{2+}-ATPase, isoform 2 protein). Five other marker enzymes were also detected, PM (H^+-ATPase marker at 35%-41% sucrose), vacuole (VM23 marker at 33%-39% sucrose), mitochondria (β-ATPaseD marker at 41%-46% sucrose), Golgi (latent UDPase marker at 28%-32% sucrose), and thylakoid membrane (chlorophyll marker at 41%-46% sucrose). None of these marker proteins showed a significant shift comparable to AtCPK2 or the ER markers in the absence of Mg^{2+}, although broadened sedimentation profiles were observed for some of the membrane marker proteins in the absence of Mg^{2+} (Fig. 17). All these data indicate that CPK2 is associated with the ER membrane.

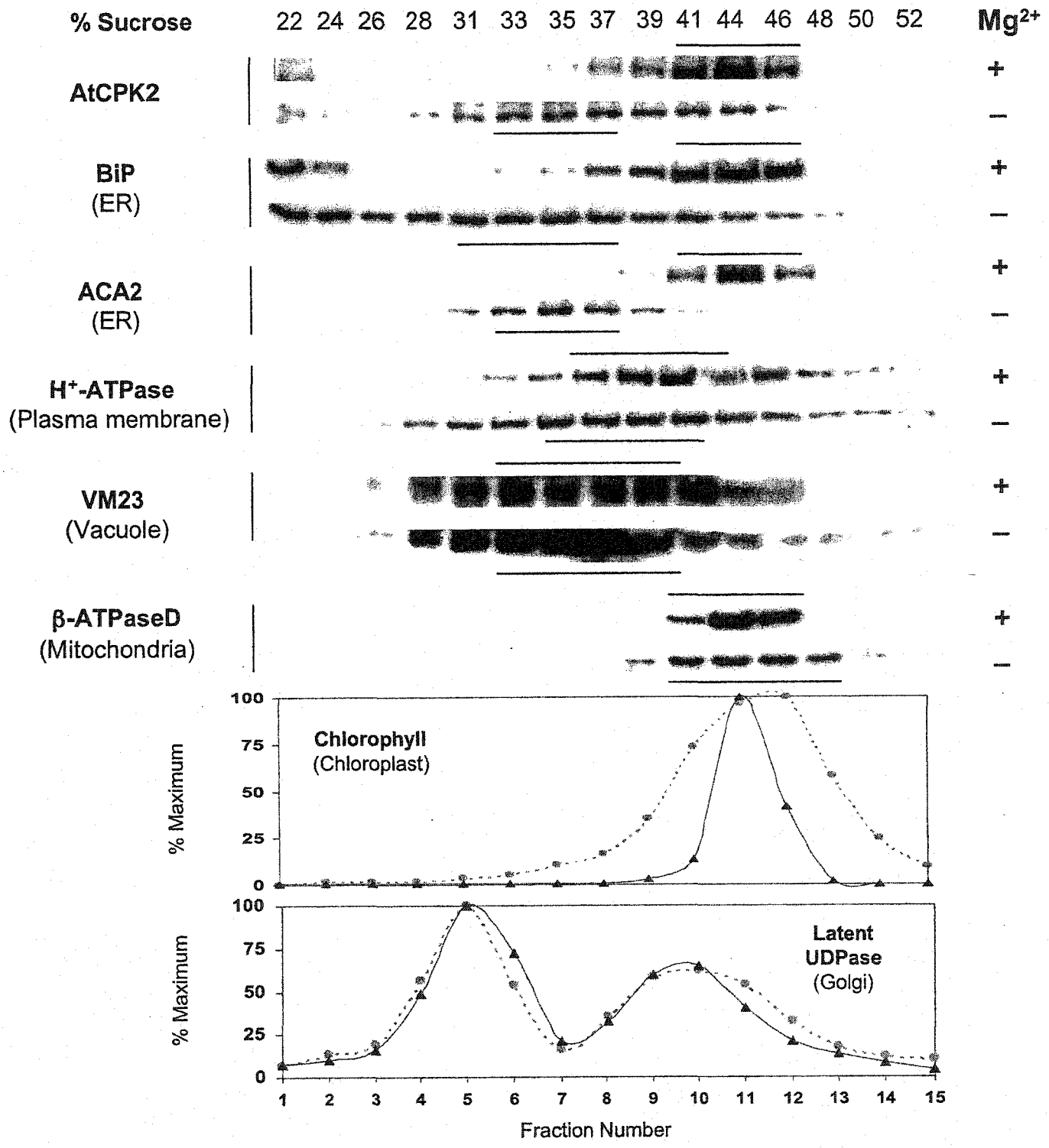

Figure 17. Sucrose gradient fractionation of microsomal membranes from WT plants showing co-fractionation of AtCPK2 with ER markers. Microsomal membranes were fractionated on 20% to 50% (w/w) sucrose gradients. Fractions from parallel gradients, with and without Mg^{2+}, were separated by SDS-PAGE and assayed by immunoblotting with specific antibodies . Horizontal bars indicate the peak fractions. Graphs show chlorophyll absorbance (chloroplast marker) and enzyme analysis for latent UDPase (Golgi marker). ▲, + Mg^{2+} gradients; ●, – Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100%.

The AtCPK2 protein detected near the top of the gradients (22%-24% sucrose) could represent AtCPK2 that dissociated from the membranes during resuspension and centrifugation (Fig. 17). This observation is consistent with our previous results that some AtCPK2 protein could be solubilized by treatment with resuspension buffer alone (Fig. 15). Similar results were observed for the ER membrane marker BiP, which is an ER lumenal binding protein without any transmembrane domains, but not for all other membrane marker proteins, which are integral membrane proteins.

AtCPK2 protein tagged with GUS localizes to the ER

To determine whether a GUS tag would affect the localization of AtCPK2, similar sucrose gradient fractionation combined with EDTA shifting was performed on microsomal membranes from transgenic plants containing the CPK2-GUS construct (Fig. 13A). As expected, the ER membrane marker ACA2 was most abundant at a sucrose density of 41%-46% in the presence of Mg^{2+} and shifted to lower sucrose density of 30%-37% when the Mg^{2+} was chelated by EDTA (Fig. 18). Similar shifting results were obtained for endogenous AtCPK2 protein when detected by immunoblotting with AtCPK2 specific antibody (Fig. 18). No significant shifts were observed for PM marker protein (Fig. 18) or any other membrane markers (data not shown). To locate the CPK2-GUS fusion protein, GUS enzyme activity was detected by the standard fluorometric assay. The distribution of GUS activity was most similar to the distribution of the ER marker ACA2 and the endogenous AtCPK2 protein (Fig. 18) indicating that the GUS tag did not interfere with the localization of AtCPK2. These results suggest that transgenic plants expressing the CPK2-GUS fusion protein could be used interchangeably with WT plants to specifically monitor the location of AtCPK2.

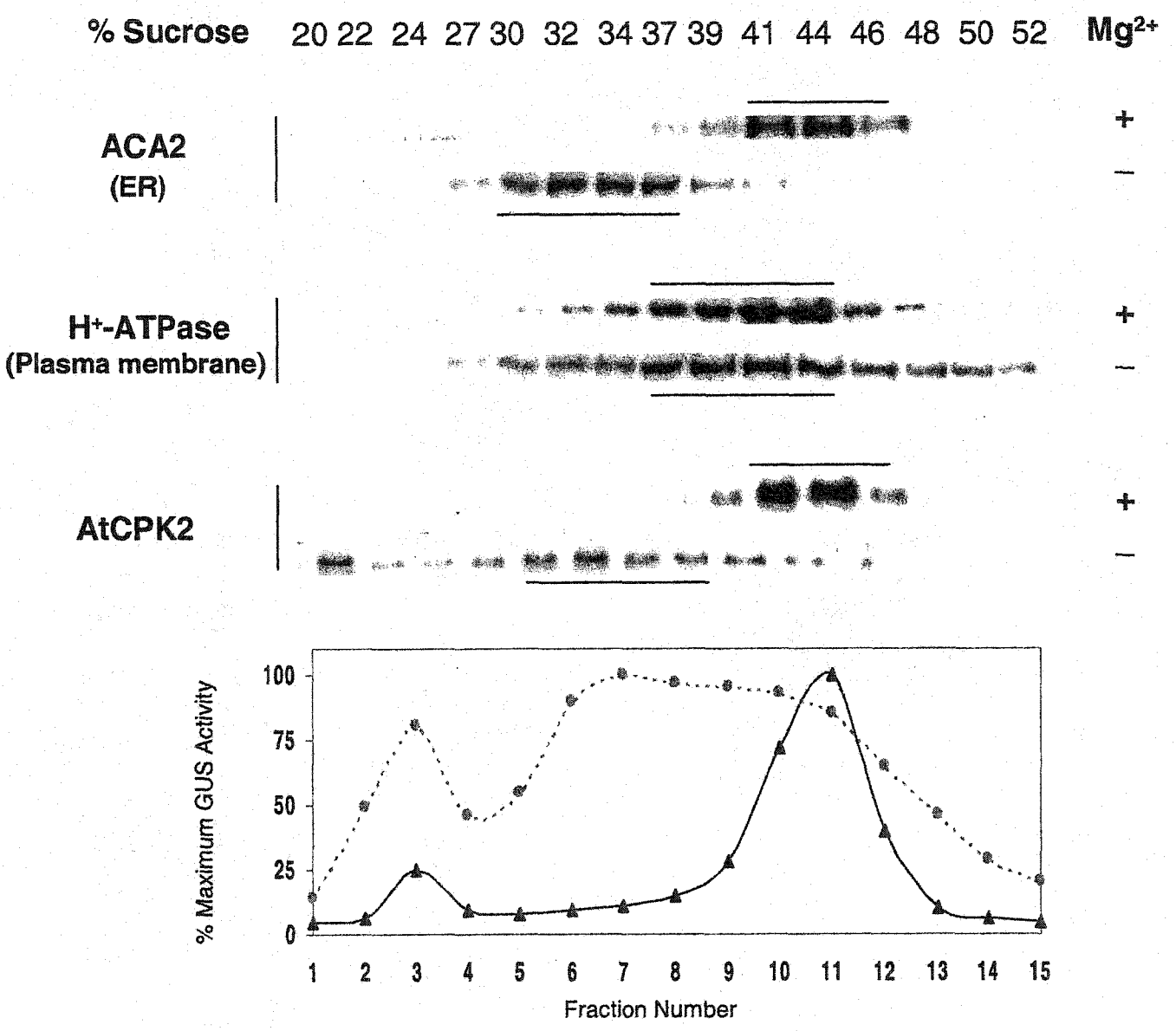

Figure 18. Sucrose gradient fractionation of microsomal membranes from plants expressing CPK2-GUS. CPK2-GUS co-fractionated with the ER marker. Microsomal membranes were fractionated and analyzed as described in Figure 17. Western blot analysis of membrane fractions analyzed with anti-H^+-ATPase, anti-ACA2 and anti-CPK2 antibodies is shown. Horizontal bars indicate the peak fractions. AtCPK2-GUS fusion protein was assayed fluorometrically for GUS enzyme activity. ▲, + Mg^{2+} gradients; ●, – Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100%.

The first 10 amino acids of AtCPK2 are sufficient for ER localization

To determine whether the amino terminal region of AtCPK2 was sufficient for membrane association, transgenic plants containing the CPK2(10aa)-GUS construct (Fig. 13E) were used to isolate microsomal membranes. Both soluble and membrane fractions were analyzed for GUS enzyme activity which represents the CPK2(10aa)-GUS fusion protein. About 46% of GUS activity was detected in the membrane fraction, which closely resembled the results from plants expressing CPK2-GUS, the full-length CPK2 protein tagged with GUS (Fig. 14). These data suggest that the first 10 amino acids of AtCPK2 retained the membrane binding characteristics of the intact protein and were able to direct a soluble GUS protein to the membranes.

To further examine whether the first 10 amino acids of AtCPK2 contained sufficient information to specify ER targeting, total microsomes from CPK2(10aa)-GUS plants were fractionated on sucrose gradients. The distribution of membrane-bound GUS activity closely resembled the distribution of the ER marker ACA2 and the endogenous AtCPK2 protein (Fig. 19), comparable to the previous results from transgenic plants expressing CPK2-GUS (Fig. 18). These results demonstrate that the first 10 amino acids of AtCPK2 are sufficient to direct a soluble GUS protein to the ER membrane, indicating that the amino terminus of AtCPK2 contains sufficient information not only for membrane binding but also for specific ER targeting.

AtCPK2 is myristoylated *in vitro* at the second position glycine

Although there is no functional transmembrane domain, AtCPK2 contains an N-myristoylation consensus sequence in its amino terminal region. This same region has been shown to contain sufficient information for ER targeting. To determine whether

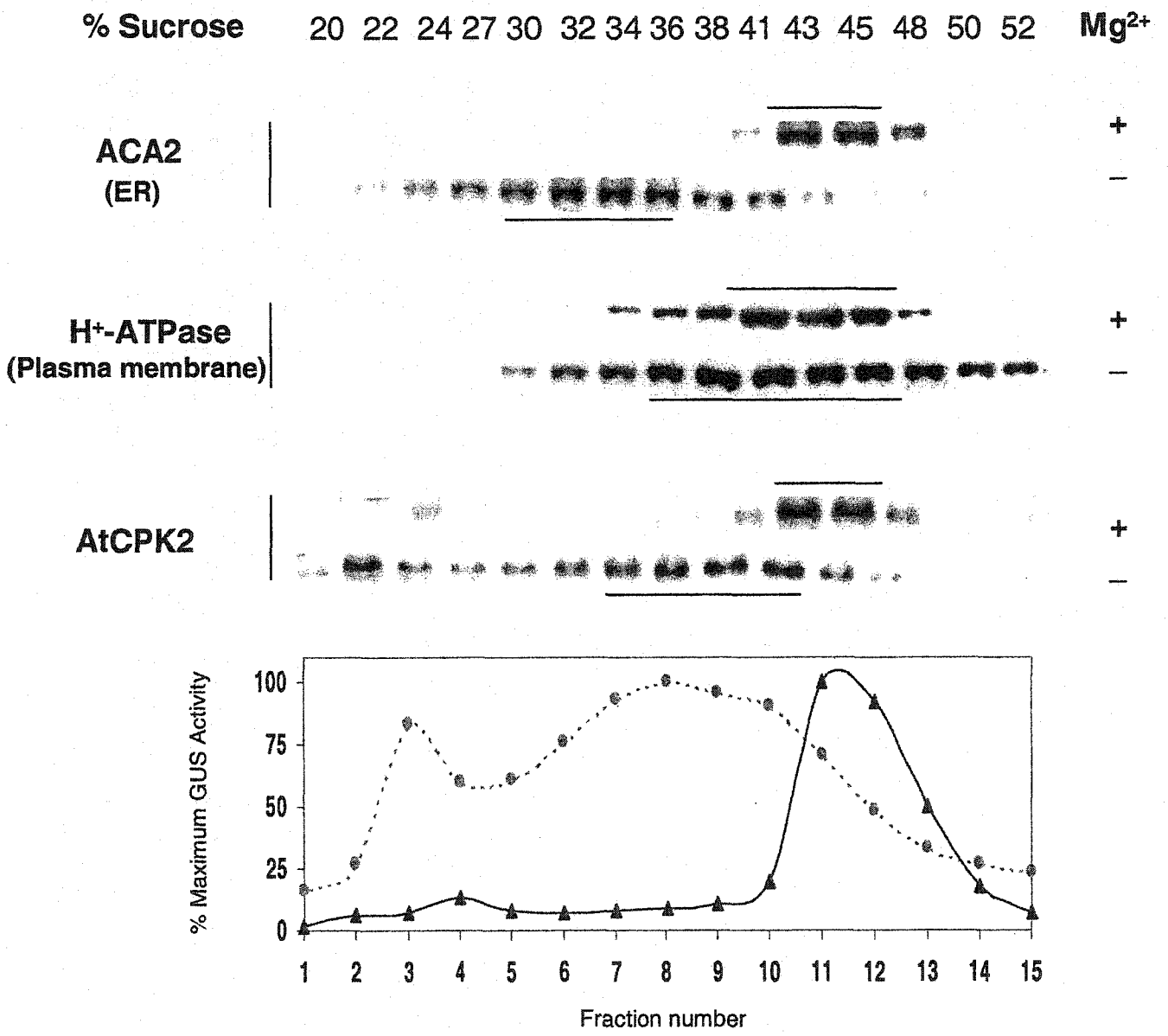

Figure 19. Sucrose gradient fractionation of microsomal membranes from plants expressing CPK2(10aa)-GUS. CPK2(10aa)-GUS co-fractionated with the ER marker. Microsomal membranes were fractionated and analyzed as described in Figure 17. Western blot analysis of membrane fractions analyzed with anti-H^+-ATPase, anti-ACA2 and anti-CPK2 antibodies are shown. Horizontal bars indicate the peak fractions. AtCPK2(10aa)-GUS fusion protein was assayed fluorometrically for GUS enzyme activity. ▲, + Mg^{2+} gradients; ●, – Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100%.

AtCPK2 could be myristoylated, an *in vitro* transcription/translation system was used to produce CPK2 mRNA from the T7 promoter in a linearized pBluescript construct. Transcription was coupled to translation in the presence of either [^{35}S]methionine for detection of total protein synthesis or [^{3}H]myristic acid to detect myristoylated proteins. Both the wild-type AtCPK2 cDNA and the N-terminal glycine to alanine mutant construct CPK2(G2A) were tested in the cell-free system from wheat germ which has been shown to contain N-myristoyltransferase (NMT) activity (Heuckeroth et al., 1988; Ellard-Ivey et al., 1999). An N-terminal glycine to alanine mutation prevents myristoylation because the N-terminal glycine residue is the site of myristate attachment (Ellard-Ivey et al., 1999; Martin and Busconi, 2000).

With both of the plasmids, a prominent protein of approximately 80 kDa was synthesized in the presence of [^{35}S]methionine (Fig. 20A), but [^{3}H]myristate label was incorporated into the CPK2 protein only when the wild type CPK2 construct was expressed (Fig. 20B). The G2A mutation did not affect the synthesis of the CPK2 protein (Fig. 20A), but prevented the addition of [^{3}H]myristate (Fig. 20B). The identity of the CPK2 protein was confirmed by immunoblotting with AtCPK2 specific antibody (Fig. 20C). No proteins were detected in mock reactions that contained no plasmid template and the smaller proteins routinely observed in [^{35}S]methionine-labeled reactions may correspond to abortive translation products or AtCPK2 degradation products (Fig. 20A). All these data demonstrate that the AtCPK2 protein can be myristoylated *in vitro* and the second position glycine is essential for AtCPK2 myristoylation.

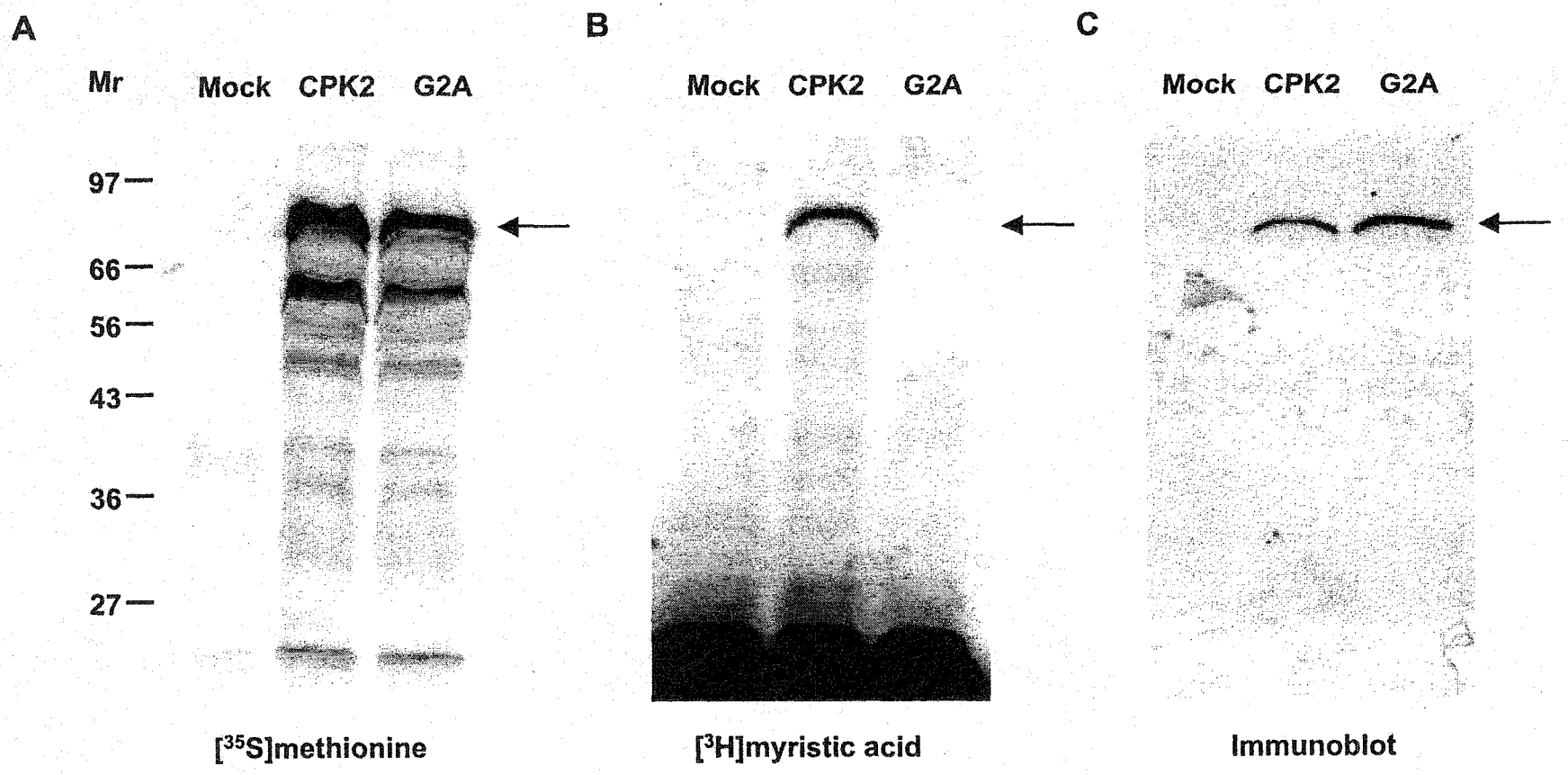

Figure 20. *In vitro* transcription/translation showing AtCPK2 is myristoylated at glycine-2. Both WT CPK2 and CPK2(G2A) mutant plasmids were expressed in a cell-free wheat germ system. A, Proteins synthesized in the presence of [^{35}S]methionine. B, Proteins synthesized in the presence of [^{3}H]myristic acid. C, Proteins detected by immunoblotting with AtCPK2 specific antibody. Mock reactions contained no plasmid. Arrow indicates AtCPK2 protein.

A G2A mutation affects membrane association of AtCPK2 in plants

To determine whether myristoylation is important for AtCPK2 membrane binding, the effect of a G2A mutation in *Arabidopsis* was examined. Transgenic plants containing the CPK2(G2A)-GUS construct (Fig. 13B) were fractionated into soluble and microsomal fractions and GUS enzyme activity for both fractions was analyzed. The amount of membrane-bound GUS activity was reduced to 19%, compared to 40% in plants expressing CPK2-GUS (Fig. 14), but it was significantly higher than background. As a control, transgenic plants expressing the GUS protein alone were tested and 2% of the GUS activity was found in the membrane fraction. All these results suggest that the G2A mutation reduces AtCPK2 membrane association, but does not abolish it.

Effects of C5A and G2A/C5A mutations on AtCPK2 membrane association

Since mutation of the myristoylation site (G2A) did not abolish membrane binding, it is likely that other factors also contribute to the membrane association of AtCPK2. To determine whether the cysteine residue at position 5, a potential palmitoylation site, is important for AtCPK2 membrane binding, site-directed mutagenesis was used to create a cysteine to alanine (C5A) mutation and a G2A/C5A double mutation. Membrane-bound GUS activity was determined for transgenic plants containing either the CPK2(C5A)-GUS (Fig. 13C) or CPK2(G2A/C5A)-GUS constructs (Fig. 13D). The amount of GUS activity in the membrane fraction was reduced to 27% in CPK2(C5A)-GUS plants, compared to 40% in CPK2-GUS plants and 10% in CPK2(G2A/C5A)-GUS plants, compared to 19% in CPK2(G2A)-GUS plants (Fig. 14). Since a mutation on cysteine-5 alone reduced membrane association of AtCPK2, the

cysteine residue at position 5 is also important for AtCPK2 membrane binding, indicating a possible role for palmitoylation.

DISCUSSION

As novel calcium sensors, CDPKs have been shown to be involved in many signaling pathways (Cheng et al., 2002). However, the precise biological functions of most CDPKs are largely unknown due to the large number of isoforms with partially redundant functions in a given species. To allocate defined biological functions to specific CDPKs, it is necessary to understand their distribution in various plant cells.

Here I investigated the membrane localization of *Arabidopsis* CDPK isoform AtCPK2. Due to the limited expression of AtCPK2 in plant cells (E. Hrabak, unpublished data), transgenic plants expressing CPK2-GUS were used initially to assess the membrane localization of AtCPK2 by detecting GUS enzyme activity. GUS is a common reporter gene whose expression can be detected by a sensitive fluorometric assay, allowing the specific and quantitative detection of AtCPK2 in the presence of other members of the CDPK family. After separating total microsomes from the soluble fraction, about 40% of the GUS activity was detected in the microsomal membrane fraction. Further membrane binding assays demonstrated that some AtCPK2 was removed from the membrane fraction by treatment with buffer alone. The removal was not increased in the presence of EDTA or NaCl, indicating that ionic or electrostatic interaction are not essential for binding. Similar results have been reported for myristoylated Src protein (Resh 1989) and a CDPK from tobacco (Romeis 2000), suggesting that AtCPK2 may exist in an equilibrium between soluble and membrane-bound forms. Triton X-100 was able to release AtCPK2 from membrane, suggesting that AtCPK2 is unlikely to be associated with detergent-insoluble lipid rafts.

To further investigate the subcellular location of AtCPK2, both aqueous two-phase partitioning and sucrose gradient fractionation were conducted on WT *Arabidopsis*. The phase partitioning experiments demonstrated that AtCPK2 protein accumulated in the lower phase but not the PM-enriched upper phase suggesting that AtCPK2 is not associated with the plasma membrane. Sucrose gradient fractionation combined with EDTA shifting experiments demonstrated that endogenous AtCPK2 and the tagged isoform CPK2-GUS co-fractionated with two ER markers, BiP (ER lumenal binding protein) and ACA2 (ER-type calcium pump). These results also indicate that the GUS tag did not interfere with localization of AtCPK2 protein to the ER membrane. This is not only the first report of an ER-localized CDPK but also the first example of a myristoylated protein localized to the endoplasmic reticulum (ER) in plants. ER localization could be important for CDPK function in calcium signal transduction since the ER lumen is a major storage site for calcium (Trewavas, 1999). To determine whether the amino terminal region of AtCPK2 was sufficient for ER targeting, transgenic plants expressing CPK2(10aa)-GUS were fractionated on sucrose gradients. The distribution of the CPK2(10aa)-GUS fusion protein most closely resembled the distribution of endogenous AtCPK2 as well as an ER marker protein, indicating that the first 10 amino acids of AtCPK2 retained ER targeting ability and may be useful for targeting other proteins to the ER membrane.

Most CDPKs, including AtCPK2, contain potential myristoylation and palmitoylation sites in their amino terminal region (Hrabak 2000). A cell-free wheat germ system was used to transcribe and translate the AtCPK2 protein *in vitro*. The results demonstrated that AtCPK2 was a substrate for plant NMT. Mutation of the myristate

attachment site (G2A) not only prevented the myristoylation of AtCPK2, but also reduced the membrane association of AtCPK2 in plants, indicating that myristoylation contributes to membrane association of AtCPK2. The fact that a significant portion of AtCPK2 is still associated with the membrane fraction in the G2A mutants suggests that other factors must be involved in the membrane binding of AtCPK2. All CDPKs containing an N-myristoylation site also contain a cysteine residue near the myristoylation site, which is a potential palmitoylation site (Milligan et al., 1995; Hrabak, 2000). To investigate whether palmitoylation contributes to AtCPK2 membrane binding, site-directed mutagenesis was used to mutate a cysteine residue to an alanine (C5A) in the CPK2-GUS construct. The C5A mutation reduced the percentage of membrane-bound AtCPK2, but did not abolish it, suggesting that the cysteine residue at position 5 could be important for AtCPK2 membrane association. Since I have not demonstrated that cysteine-5 is the site of palmitic acid attachment, it is too early to conclude that palmitoylation is involved in AtCPK2 membrane association. I also can not rule out the possibility that the glycine-2 and cysteine-5 themselves are involved in membrane binding through other mechanisms such as interacting with membrane-bound proteins.

In conclusion, my results demonstrated that *Arabidopsis* CDPK isoform AtCPK2 is associated with the ER membrane in plants. The region of AtCPK2 necessary for ER targeting is located within the first 10 amino acids of the protein. AtCPK2 is myristoylated *in vitro* and a G2A mutation not only prevented myristoylation, but also decreased membrane binding in plants indicating that myristoylation is at least part of the mechanism by which AtCPK2 associates with ER, but is not the only factor involved. The effect of a C5A mutation in plants suggests that the cysteine residue at position 5

could also be involved in membrane association of AtCPK2. These studies not only advance our knowledge of CDPKs localization and function, but also contribute to our understanding of myristoylation and protein targeting in plant cells.

CHAPTER IV

MEMBRANE ASSOCIATION OF TWO CALCIUM-DEPENDENT PROTEIN KINASES IN *ARABIDOPSIS THALIANA*

INTRODUCTION

Many potential CDPK substrates are membrane proteins and CDPK enzymatic activities have often been identified in membrane fractions. It is important to determine which membranes different CDPKs are targeted to. This information will be crucial for determining the *in vivo* substrates of CDPKs because even though CDPKs may phosphorylate a wide variety of substrates *in vitro*, the real substrates and the CDPK must co-localize *in planta*. In Chapters II and III, I have experimentally demonstrated that AtCPK5 is associated with the plasma membrane and AtCPK2 is associated with the endoplasmic reticulum membrane and that the amino termini of these CDPKs are sufficient for correct membrane targeting. To determine whether other CDPKs with related amino termini are targeted to the same membranes, two more *Arabidopsis* CDPKs were examined. There are 34 predicted genes encoding CDPKs in *Arabidopsis* and they form several subgroups on a sequence tree (Fig. 21). AtCPK1 is most closely related to AtCPK2, while AtCPK6 is most similar to AtCPK5. Although the sequence tree was constructed using kinase domains only, related CDPKs also have similarity in other domains. Sequence similarity at the immediate amino-termini of AtCPK1, 2, 5 and 6 is shown in Figure 22. All four of the CDPKs contain N-myristoylation consensus

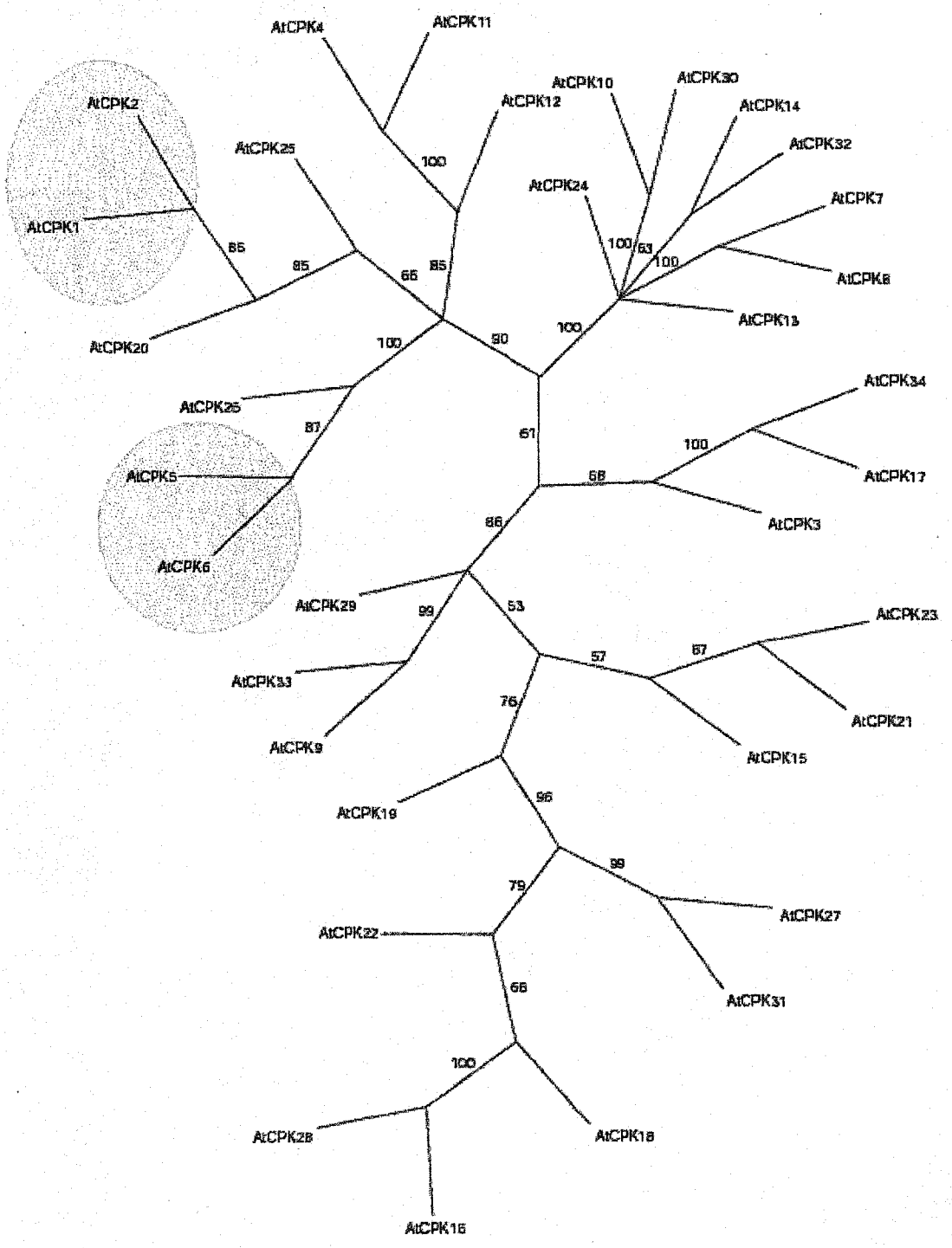

Figure 21. Unrooted sequence tree showing the relationship between the *Arabidopsis* CDPKs. The tree was constructed from sequences of kinase catalytic domains. Alignments were constructed by Clustal W and used to generate a maximum parsimony tree, which was subjected to 100 rounds of bootstrap analysis. Bootstrap values are indicated for each node. The AtCPK1/AtCPK2 group and the AtCPK5/AtCPK6 group are shaded.

A

	1	2	3	4	5	6	7	8	9	10
CPK1	M	G	N	**T**	C	V	G	P	**S**	**R**
CPK2	M	G	N	**A**	C	V	G	P	**N**	**I**
	*	*	*		*		*			

B

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
CPK5	M	G	N	S	C	R	G	S	F	K	D	K	**L**	**D**
CPK6	M	G	N	S	C	R	G	S	F	K	D	K	**I**	**Y**
	*	*	*		*		*							

Figure 22. Comparison of the amino termini of related CDPKs. A, Comparison of the amino termini of AtCPK1 and AtCPK2. B, Comparison of the amino termini of AtCPK5 and AtCPK6. Bold characters indicate amino acids that are different between sequence pairs. Asterisks indicate amino acids that are identical in all four CDPKs.

sequences in their amino terminal regions. Here I investigated the myristoylation and membrane association of AtCPK1 and AtCPK6 as well as the role of the amino termini in membrane targeting.

MATERIALS AND METHODS

The materials and methods described in Chapter II and III are not repeated here.

Plasmid constructs

All constructs were made previously by E. Hrabak and are only briefly described here. In construct CPK6-GUS, a large segment of genomic DNA including 1.1 kb upstream of the AtCPK6 translational start codon and 0.5 kb downstream of the translational stop codon was modified by addition of the GUS sequence (Fig. 23A). The resulting fusion protein contains the entire AtCPK6 protein with the GUS protein inserted in-frame 18 amino acids before the carboxy terminus. The CPK6(14aa)-GUS construct contained the same CPK6 5' region as CPK6-GUS, but only the first 42 nucleotides of the coding sequence. The in-frame GUS coding sequence followed by the *nos* terminator were inserted downstream of the CPK6 sequence (Fig. 23C). The resulting fusion protein contains the first 14 amino acids of the AtCPK6 protein at the amino terminus of the GUS protein. The CPK6(G2A)-GUS and CPK6(14aa/G2A)-GUS constructs are identical to CPK6-GUS and CPK6(14aa)-GUS constructs respectively, except that site-directed mutagenesis of the glycine at position 2 to alanine was done (Fig. 23B and D). In construct CPK1(31aa)-GUS, a fragment containing 1.5 kb of CPK1 genomic sequence upstream from the translational start site and including the first 93 nucleotides of the coding sequence was cloned into pBIN19 upstream of and in-frame with the GUS reporter gene and followed by the *nos* terminator (Fig. 23E). The resulting fusion protein contains the first 31 amino acids of the AtCPK1 protein at the amino terminus of the GUS protein.

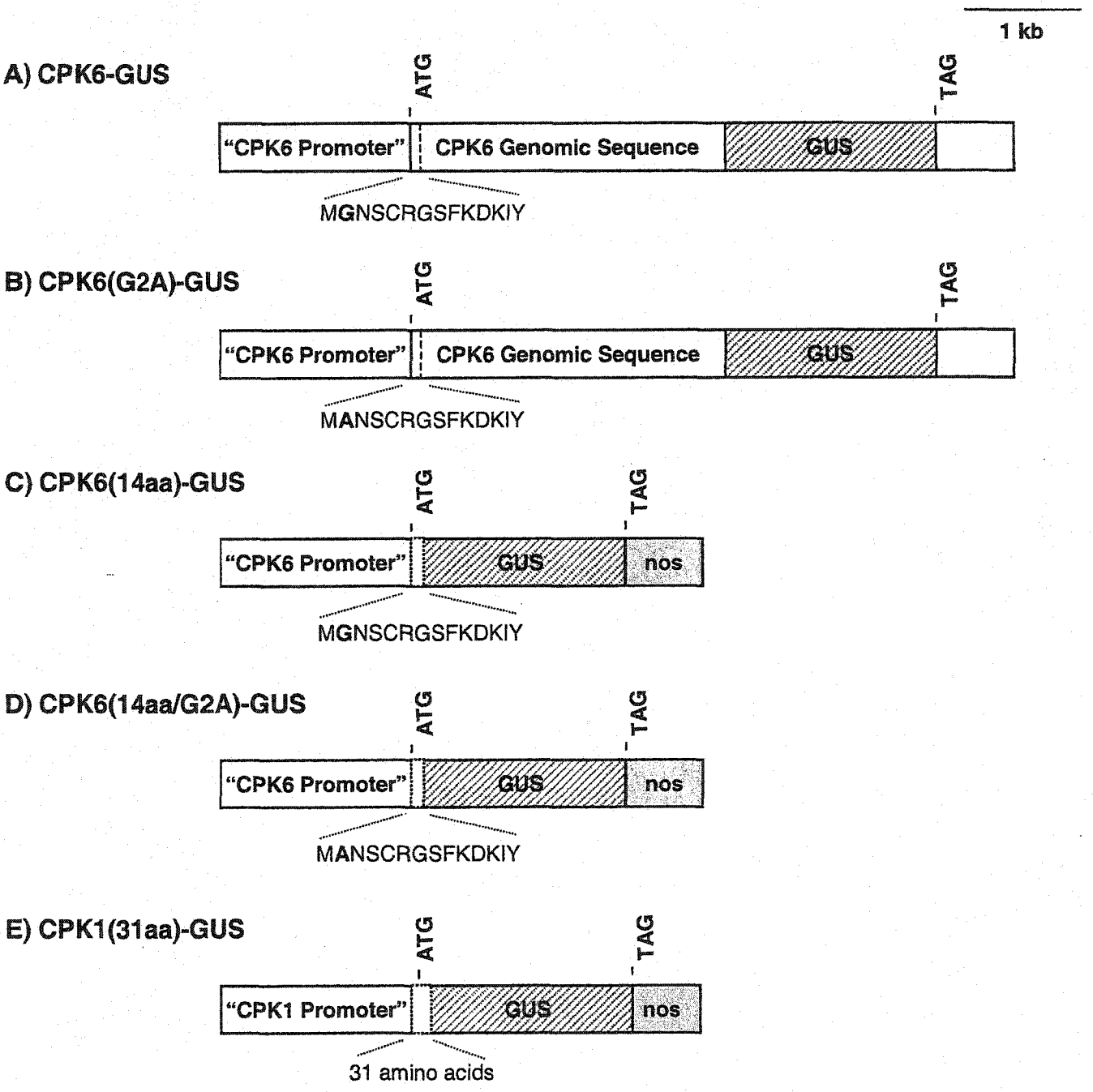

Figure 23. AtCPK6 and AtCPK1 constructs used for plant transformation. A-D, The amino acid sequence encoded by the first 42 nucleotides of the AtCPK6 coding sequence is indicated. E, The dashed box indicates the first 93 nucleotides of the AtCPK1 coding sequence. "CPK6 promoter" and "CPK1 promoter" indicate the sequence upstream of the translational start site and includes both 5' untranslated leader and promoter sequences. Striped boxes are the coding region of GUS. *nos* is a plant termination signal cassette.

RESULTS

Both AtCPK1 and AtCPK6 are myristoylated

To determine whether AtCPK1 and AtCPK6 are myristoylated, a wheat germ *in vitro* transcription/translation system was used to express both AtCPK1 and AtCPK6 cDNA in the presence of either [^{35}S]methionine or [^{3}H]myristic acid. In the [^{35}S]methionine reactions, a protein of the predicted size for AtCPK1 (~ 68 kDa) and for AtCPK6 (~61 kDa) were synthesized (Fig. 24, lane 1 and 3). When AtCPK1 and AtCPK6 were expressed in the wheat germ system in the presence of [^{3}H]myristic acid, radiolabel was incorporated into the AtCPK1 and AtCPK6 proteins (Fig, 24, lane 2 and 4). Currently, we do not have isoform specific antibodies for AtCPK1 and AtCPK6, so the identity of the synthesized protein could not be confirmed with certainty. These results suggest that both AtCPK1 and AtCPK6 can be myristoylated *in vitro*.

Both AtCPK1 and AtCPK6 are associated with membranes

Since isoform-specific antibodies are not available for AtCPK1 and AtCPK6, a GUS tag was used for specific and quantitative detection of each isoform. I have demonstrated previously that the GUS tag does not interfere with the localization of AtCPK2 and AtCPK5 and it can be used reliably to specifically monitor the location of CDPKs. For AtCPK1, transgenic plants containing the CPK1(31aa)-GUS (Fig. 23E) were used to determine whether AtCPK1 was membrane associated. I have demonstrated that the short amino terminal region of AtCPK2 and AtCPK5 retained the membrane binding and targeting ability of the intact proteins, so it is reasonable to assume that AtCPK1 will behave in a similar manner. For AtCPK6, both the long version CPK6-GUS (Fig. 23A) and the short version CPK6(14aa)-GUS (Fig. 23C) were used to determine whether

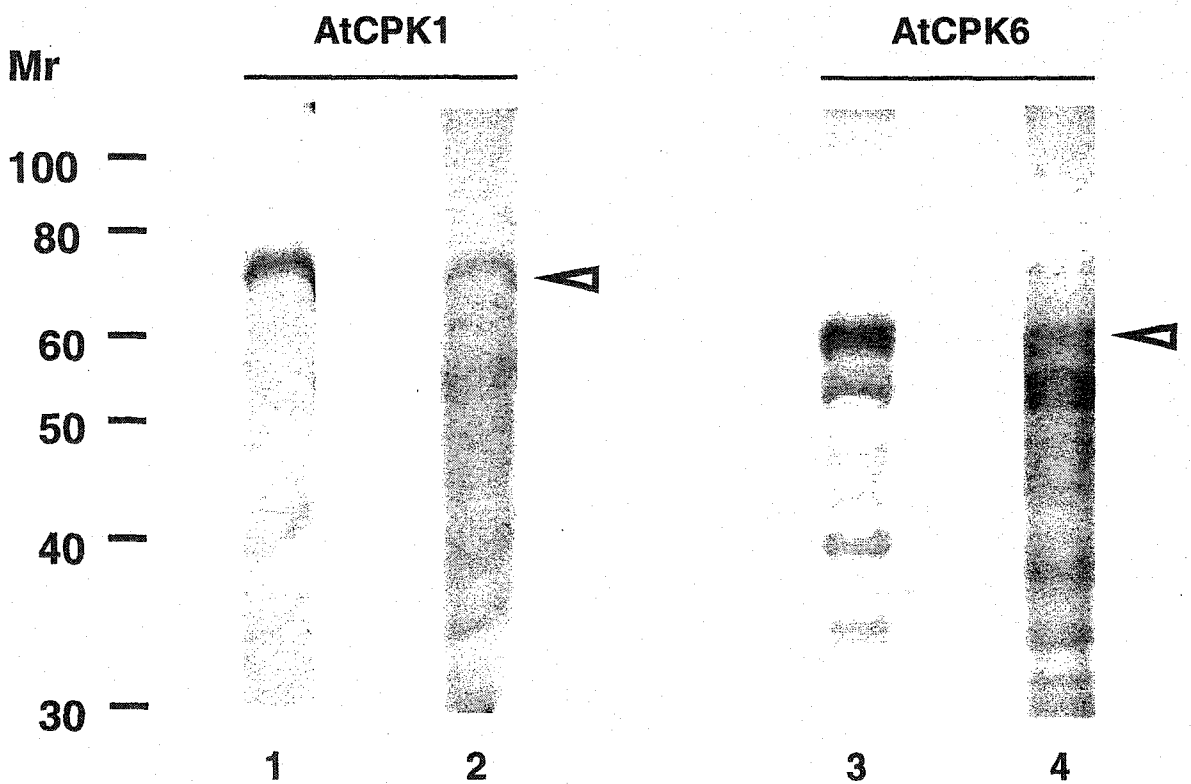

Figure 24. Wheat germ transcription/translation showing that both AtCPK1 and AtCPK6 can be myristoylated *in vitro*. Lane 1 and 3, Protein synthesized in the presence of [^{35}S]methionine. Lane 2 and 4, Protein synthesized in the presence of [^{3}H]myristic acid. Lane 1 and 2, AtCPK1 cDNA was expressed. Lane 3 and 4, AtCPK6 cDNA was expressed. Arrow indicates either AtCPK1 or AtCPK6 protein.

AtCPK6 was associated with membranes. After separating microsomal membranes from soluble fractions, GUS enzyme activity for both fractions was analyzed by the standard fluorometric assay. About 56% of the GUS activity was associated with the membrane fraction in CPK1(31aa)-GUS plants (Table 2). In transgenic plants expressing CPK6-GUS or CPK6(14aa)-GUS, the amount of membrane-bound GUS activity was 51% and 61% respectively and the difference between them is not significant using one-way ANOVA (Table 2). These data suggest that both AtCPK1 and AtCPK6 exist in a membrane associated form.

The amino terminus of AtCPK1 contains ER-targeting information

Within the first 10 amino acids, both AtCPK1 and AtCPK2 contain N-myristoylation consensus sequences and they differ by three amino acids. The amino acid changes at positions 4 and 9 are conservative substitutions and the only major change is at position 10 where AtCPK1 has a basic residue and AtCPK2 contains an aliphatic residue (Fig. 22A). The amino terminal region of AtCPK2 has been shown to contain sufficient information for ER-targeting. Since the amino termini of AtCPK1 and AtCPK2 are so similar, I hypothesized that AtCPK1 might also be targeted to the ER. To examine whether the amino terminus of AtCPK1 conferred ER-targeting ability, total microsomes from CPK1(31aa)-GUS plants were fractionated on sucrose gradients. The distribution of membrane-bound GUS activity closely resembled the distribution of the ER marker ACA2 (Fig. 25), indicating that the amino terminal region of AtCPK1 is sufficient to direct a soluble GUS protein to the ER membrane.

Table 2. Percentage of GUS enzymatic activity detected in the microsomal fraction of transgenic *Arabidopsis* plants containing GUS-tagged CDPK constructs.

Protein Expressed in Transgenic Plants	Membrane-Associated GUS Activity	GUS Activity in Membrane Fraction (nmol min^{-1} mg^{-1})	No. of Independent Transgenic Lines	No. of Independent Experiments
CPK1(31aa)-GUS	56 ± 2	$1.1x10^3$-$1.3x10^3$	2	2
CPK2-GUS	40 ± 2	$6.7x10^1$-$1.5x10^2$	3	7
CPK2(10aa)-GUS	46 ± 2	5.2-8.3	2	4
CPK5(16aa)-GUS	73 ± 7	$1.1x10^4$-$1.8x10^4$	3	4
CPK6-GUS	51 ± 3	$1.5x10^3$-$2.9x10^3$	3	5
CPK6(14aa)-GUS	61 ± 3	$3.3x10^2$-$4.5x10^2$	3	10

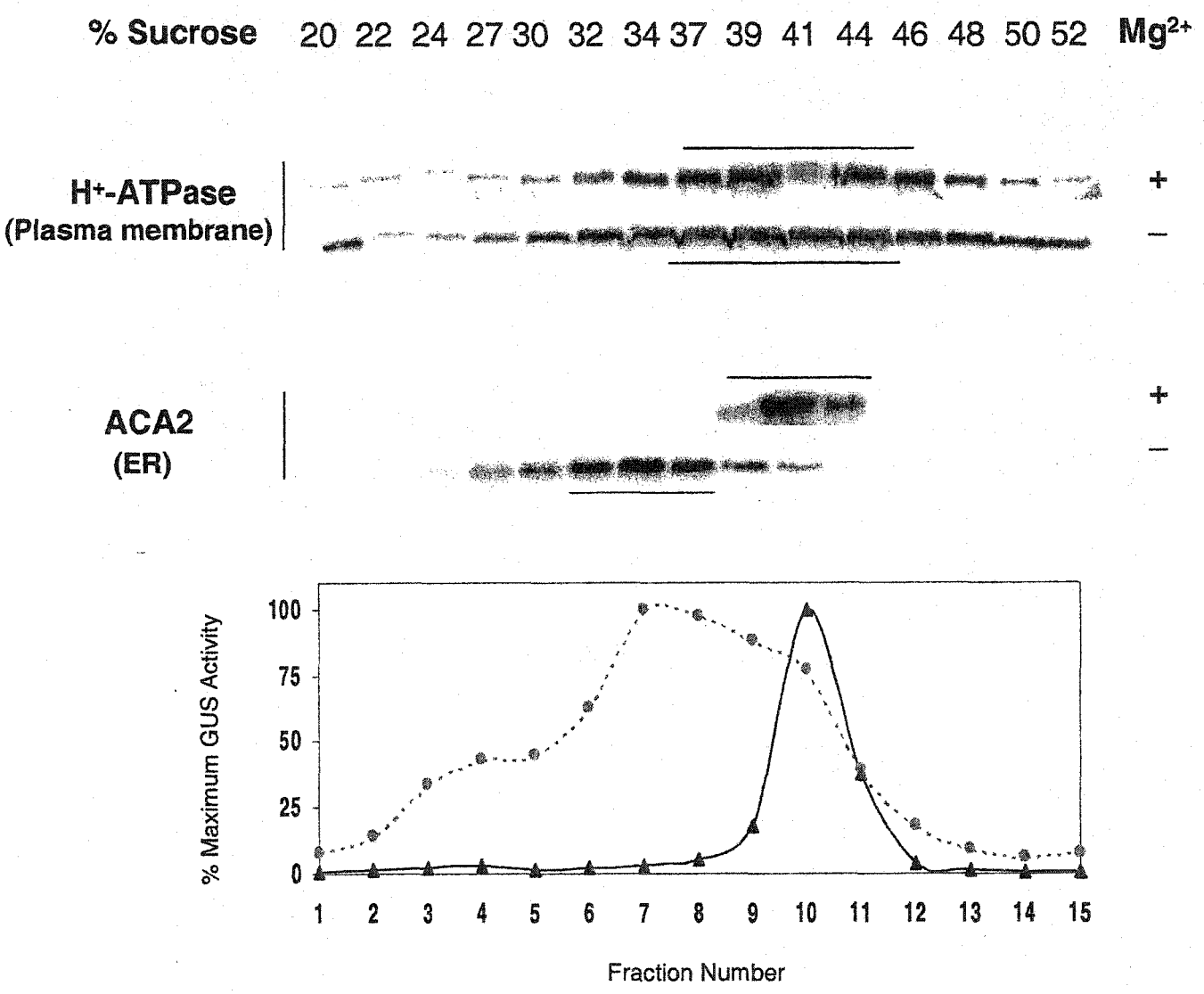

Figure 25. Sucrose gradient fractionation of microsomal membranes from plants expressing CPK1(31aa)-GUS. CPK1(31aa)-GUS co-fractionated with the ER marker. Microsomal membranes were fractionated and analyzed as described in Figure 17. Western blot analysis of membrane fractions analyzed with anti-H^+-ATPase and anti-ACA2 antibodies are shown. Horizontal bars indicate the peak fractions. AtCPK1(10aa)-GUS fusion protein was assayed fluorometrically for GUS enzyme activity. ▲, + Mg^{2+} gradients; •, – Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100%.

The amino terminus of AtCPK6 contains PM-targeting information

A comparison of the amino termini of AtCPK6 and AtCPK5 showed that they differed only at positions 13 and 14, although Leu vs. Ile at position 13 is a conservative change (Fig. 22B). In Chapter II, I demonstrated that the amino terminal region of AtCPK5 is sufficient for PM targeting. To determine whether the similar amino terminus of AtCPK6 retained PM targeting ability, total microsomes from CPK6(14aa)-GUS plants were separated in an aqueous two-phase system. The GUS enzyme activity representing the CPK6(14aa)-GUS fusion protein was slightly enriched in the upper phase indicating that CPK6(14aa)-GUS fusion protein was at least partially associated with the PM (Fig. 26). These data are consistent with the amino terminus of AtCPK6 containing PM-targeting information.

A G2A mutation affects the membrane association of AtCPK6

In the N-myristoylation consensus sequence, the amino terminal glycine residue is absolutely required and mutation of that glycine prevents myristoylation and affects membrane binding ability of many myristoylated proteins. To investigate the effect of a G2A mutation on AtCPK6 membrane binding, transgenic plants expressing either CPK6(G2A)-GUS (Fig. 23B) or CPK6(14aa/G2A)-GUS (Fig. 23D) were tested. The amount of GUS activity in the membrane fraction was reduced to 15% in CPK6(G2A)-GUS plants compared to 61% in CPK6-GUS plants and reduced to 10% in CPK6(14aa/G2A)-GUS plants compared to 51% in CPK6(14aa)-GUS plants (Fig. 27). The G2A mutation dramatically decreased the proportion of AtCPK6 in the membrane fraction indicating that myristoylation contributes to the membrane binding of AtCPK6, but we can not rule out the possibility that other factors may be involved because there

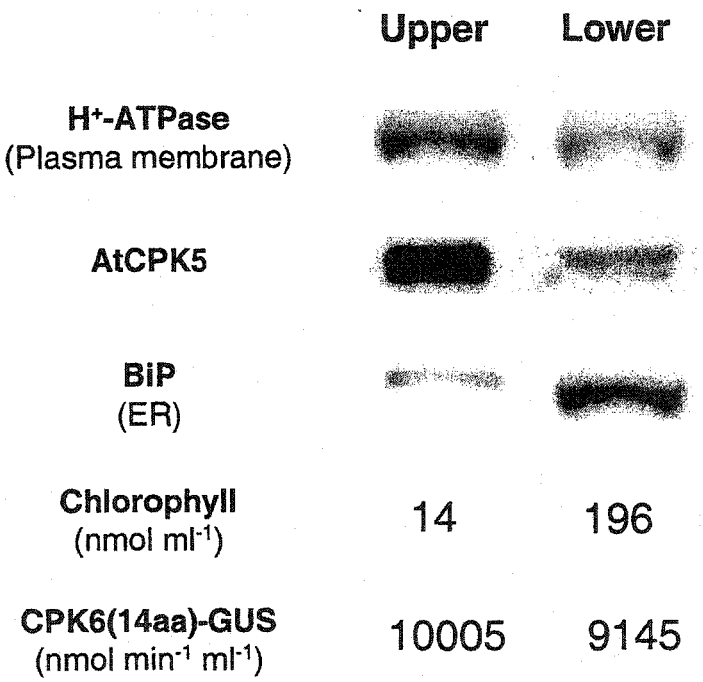

Figure 26. Aqueous two-phase partitioning of microsomal membranes from CPK6(14aa)-GUS transgenic plants. CPK6(14aa)-GUS fusion protein was slightly enriched in the upper phase. Equal proportions of the upper and lower phases were separated by SDS-PAGE and assayed by immunoblotting with specific antibodies. Chlorophyll absorbance (thylakoid marker) was measured spectrophotometrically and CPK6(14aa)-GUS fusion protein was assayed fluorometrically for GUS enzyme activity.

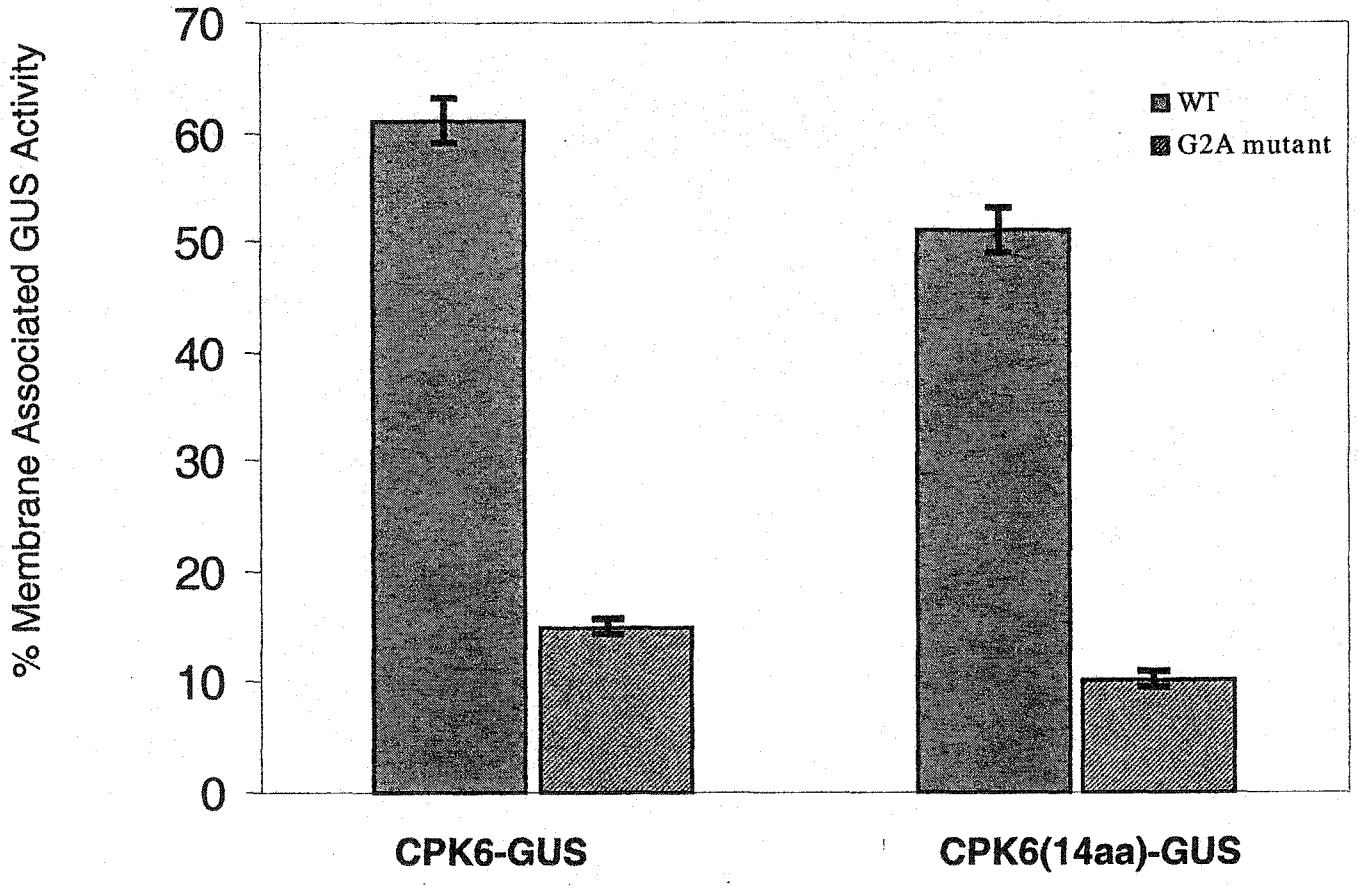

Figure 27. Effect of G2A mutation on membrane binding of AtCPK6. Transgenic plants expressing either CPK6-GUS or CPK6(14aa)-GUS were examined. Soluble proteins and total microsomes were separated by ultracentrifugation and GUS activity of both soluble and microsomal fractions was assayed. Solid bars are wild type proteins. Hatched bars are G2A mutants. In 2-3 independent transgenic lines, total membrane-bound GUS activity (nmol min^{-1} mg^{-1}) was $1.5x10^3$-$2.9x10^3$ in CPK6-GUS plants, $7.1x10^2$-$1.2x10^3$ in CPK6(G2A)-GUS plants, $3.3x10^2$-$4.5x10^2$ in CPK6(14aa)-GUS plants and $1.1x10^2$-$2.3x10^2$ in CPK6(14aa)(G2A)-GUS plants.

was a significant amount of GUS activity still associated with the membrane fraction in the G2A mutants.

DISCUSSION

Many CDPKs contain an N-myristoylation consensus sequence at their amino termini and myristoylation is known to promote protein-membrane interaction. In fact, membrane-associated CDPK enzymatic activities have been reported many times (Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998; Martin and Busconi, 2001). Membrane binding could allow better access to CDPK substrates and/or enable the enzyme to be rapidly activated by the incoming calcium. Since no transmembrane domains have been reported in CDPKs, the addition of a myristate group to the N-terminus of the protein could facilitate the previously reported CDPK membrane association.

I have demonstrated that two *Arabidopsis* CDPKs, AtCPK1 and AtCPK6, can be myristoylated in a cell-free wheat germ coupled transcription/translation system and that these two kinases are membrane associated in transgenic plants expressing a GUS-tagged CDPK. Mutation of the N-terminal glycine to alanine (G2A), which is known to prevent myristoylation, significantly decreased the membrane association of AtCPK6 in plants. These results suggest that myristoylation is an important mechanism for membrane association of AtCPK6.

On a sequence tree of all 34 *Arabidopsis* CDPKs, AtCPK1 and AtCPK2 are most closely related, as are AtCPK5 and AtCPK6 (Fig. 21). Moreover, they share high similarity in amino acid sequence in the immediate amino terminus which contains the N-myristoylation consensus sequence (Fig. 22). I have demonstrated that the N-terminus of AtCPK2 or AtCPK5 can direct the specific targeting of GUS to the ER or plasma membrane respectively. To investigate whether other CDPKs with amino termini of

related to AtCPK2 and AtCPK5 provide the same specificity of membrane binding, transgenic plants expressing the fusion protein containing the amino terminal region of AtCPK1 or AtCPK6 fused with GUS were examined. The N-terminus of AtCPK1 was able to target the soluble GUS protein to the ER membrane and the N-terminus of AtCPK6 could direct the PM targeting of GUS indicating that CDPKs with similar amino termini are targeted to the same membranes and that the immediate N-terminus of CDPKs is sufficient for membrane localization.

Combining all the data in Chapters II, III and IV, I have conclusively demonstrated that CDPKs with N-myristoylation consensus sequences can be myristoylated *in vitro* and that myristoylation is important for CDPK membrane binding, although it may not be the only mechanism. Different *Arabidopsis* CDPKs are associated with different membranes and the specific membrane targeting information resides in the amino terminal region of CDPKs. I have reported the first endoplasmic reticulum-localized CDPKs (AtCPK2 and AtCPK1) and two plasma membrane localized CDPKs (AtCPK5 and AtCPK6). It is not known how myristoylated proteins are directed to a specific membrane. One potential explanation is that these four CDPKs are not only myristoylated, but also palmitoylated, since they all have a cysteine at position 5 which represents a potential palmitoylation site (Fig. 22). A rice CDPK was demonstrated to be palmitoylated at cysteine 4 and 5, and mutation of either or both decreased membrane binding (Martin and Busconi, 2000). Although I have not demonstrated palmitoylation, a C5A mutation of AtCPK2 decreased the membrane binding comparable to that observed in the rice CDPK. Most dually acylated proteins are associated with the PM and one explanation for this phenomenon is the "kinetic bilayer trapping" hypothesis. This

hypothesis states that a myristoyl group allows a protein to transiently interact with multiple intracellular membranes but when it reaches the PM, it will be palmitoylated by a PM-bound palmitoyltransferase and remain stably attached to the PM. In my case, AtCPK5 and AtCPK6 could be targeted to the PM using this default pathway while the ER targeting of AtCPK1 and AtCPK2 could be mediated by interaction with a specific receptor on the ER that recognizes the amino terminus of CDPKs. To test this hypothesis, it will be of interest to determine which specific residues at the amino terminus of CDPKs are required for specifying membrane targeting. Comparing the amino termini of four CDPKs, the major differences between ER-targeted CDPKs and PM-targeted CDPKs are at position 6 and 8 (Fig. 22). If mutations at these positions change the localization of CDPKs, it will be good evidence that those amino acids play a role in specifying membrane targeting.

My studies contribute to our overall understanding of CDPK function and complement current efforts in the field to determine the *in vivo* substrates of this important group of unique plant proteins. My work on the basic mechanisms of CDPK membrane binding and specific targeting advance our knowledge of myristoylation and the targeting of peripheral membrane proteins in plants. Finally, the finding that the amino terminus of CDPKs contains membrane targeting information could be useful for targeting other proteins to different membranes by protein engineering.

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APPENDICES

APPENDIX I

OVEREXPRESSION OF AtCPK2 IN *ARABIDOPSIS THALIANA*

Constitutive expression of specific genes is commonly used as a tool to study the function of the gene when no mutants are available. To investigate the biological function of AtCPK2 in *Arabidopsis* plants, transgenic plants were produced that contained the AtCPK2 cDNA under control of the strong, constitutive cauliflower mosaic virus 35S promoter (Fig. A). The transgene was introduced into a wild-type background of four *Arabidopsis* ecotypes: Columbia (Col), Landsberg *erecta* (Ler), Nossen (No-O) and RLD. Transgenic plants were selected by kanamycin resistance and the presence of the transgene was further confirmed by PCR with specific primers as described in the previous chapters. Overexpression of AtCPK2 protein in transgenic plants was confirmed by immunoblotting using isoform specific antibodies (data not shown).

Arabidopsis is a facultative long-day (LD) plant, which means that plants flower earlier under LDs than under short days (SDs). Both wild type and overexpression plants were grown either under LDs (22°C, 18 hour light cycle) or under SDs (22°C, 8 hour light cycle). As the plants grew, the percentage of plants showing a visible primary inflorescence (bolt) was recorded. Bolting (internode elongation) takes place after the fate change of lateral meristems and is an indication of transition from vegetative phase to reproductive phase (Koornneef et al., 1998). For each ecotype, three independent transgenic lines and more than 20 individual plants were examined.

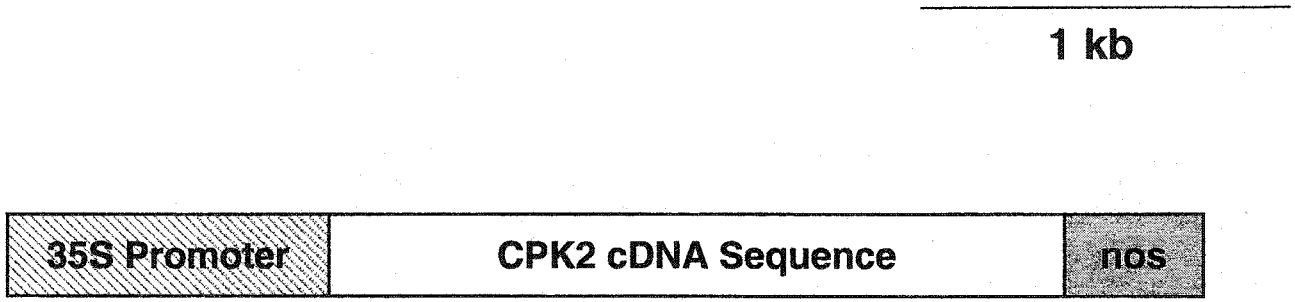

Figure A. Schematic diagram of the 35S-CPK2 construct.

Under both LDs and SDs, ecotypes Col and Ler bolted earlier than ecotypes RLD and No-O (Figs. B and C). Since *Arabidopsis* has a wide distribution and differences found among ecotypes grown under the same environmental conditions are considered to reflect adaptations to different natural environments (Alonso-Blanco et al., 1998). Under LDs, AtCPK2 overexpression plants showed a slightly delayed bolting phenotype comparing to wild type and this effect was more obvious when plants were grown under SDs (Figs. B and C). This data indicates that AtCPK2 or perhaps a related CDPK may be involved in the transition from vegetative phase to reproductive phase of plants.

The interpretation of the phenotype of AtCPK2 overexpression plants is complicated. First, the overexpression phenotype shows the ability of AtCPK2 to delay bolting, but it does not prove that AtCPK2 is necessary for the timing of the transition. Second, overexpression of AtCPK2 protein allows ecotopic expression, which may lead to artifactual protein localization due to a lack of required targeting elements in certain cell type. Third, increased abundance of AtCPK2 protein may overload a targeting pathway and cause significant missorting of membranes. Now with the recent availability of AtCPK2 knock-out lines, AtCPK2 mutant plants could be analyzed in parallel with the overexpression plants to see whether they have the opposite phenotype. The analysis of mutant plants will give us more direct evidence of the function of AtCPK2 *in planta*.

Alonso-Blanco, C., El-Assal, S.E., Coupland, G., and Koornneef, M. (1998). Analysis of natural alleic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. Genetics **149**, 749-764.

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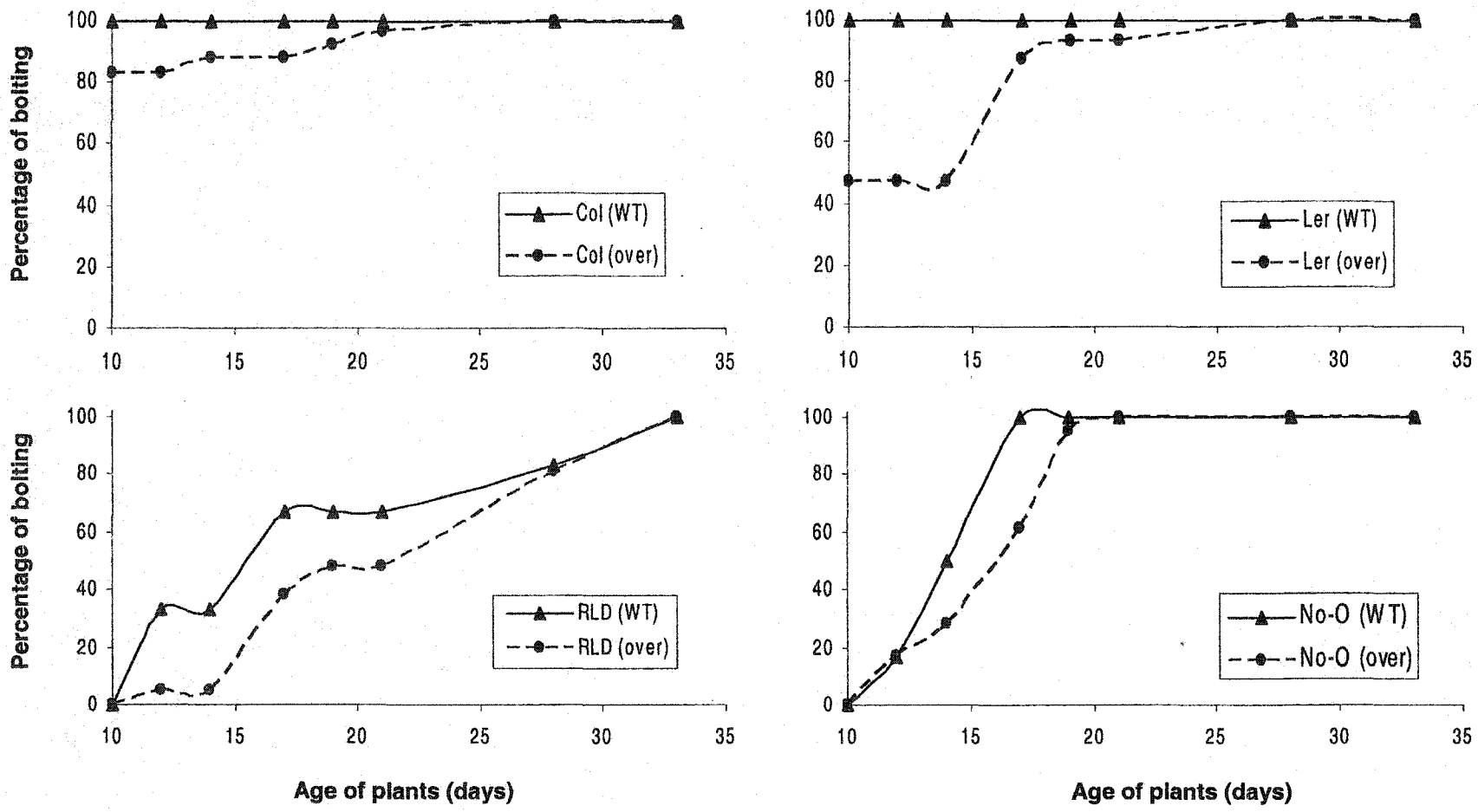

Figure B. Comparison of bolting time for wild type *Arabidopsis* plants and *Arabidopsis* plants containing the 35S-CPK2 over-expression construct under long days (LDs). Percentage of plants showing a visible bolt was recorded.

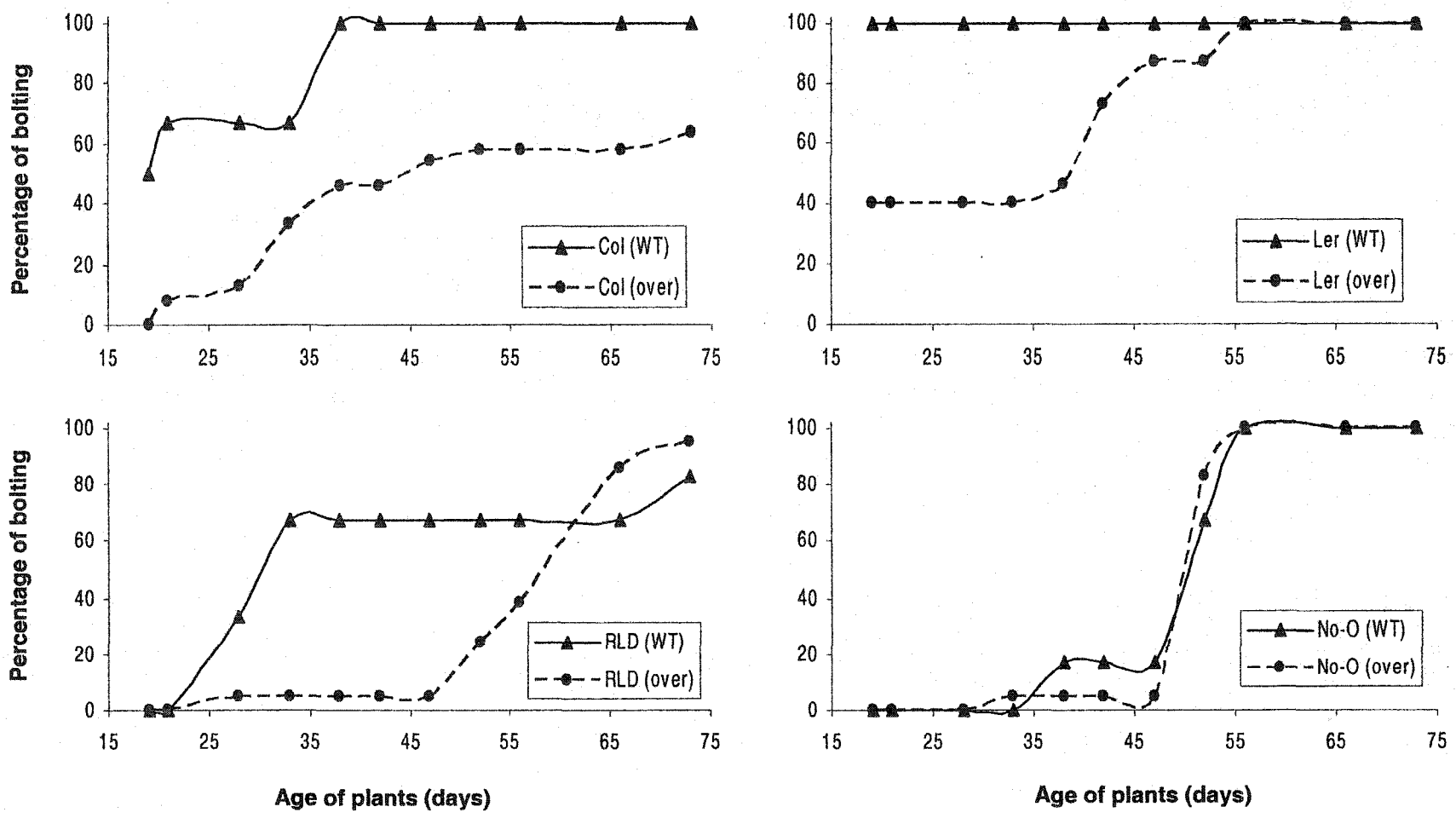

Figure C. Comparison of bolting time for wild type *Arabidopsis* plants and *Arabidopsis* plants containing the 35S-CPK2 over-expression construct under short days (SDs). Percentage of plants showing a visible bolt was recorded.

APPENDIX II

An Arabidopsis Calcium-Dependent Protein Kinase Is Associated with the Endoplasmic Reticulum[1]

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Arabidopsis contains 34 genes that are predicted to encode calcium-dependent protein kinases (CDPKs). CDPK enzymatic activity previously has been detected in many locations in plant cells, including the cytosol, the cytoskeleton, and the membrane fraction. However, little is known about the subcellular locations of individual CDPKs or the mechanisms involved in targeting them to those locations. We investigated the subcellular location of one Arabidopsis CDPK, AtCPK2, in detail. Membrane-associated AtCPK2 did not partition with the plasma membrane in a two-phase system. Sucrose gradient fractionation of microsomes demonstrated that AtCPK2 was associated with the endoplasmic reticulum (ER). AtCPK2 does not contain transmembrane domains or known ER-targeting signals, but does have predicted amino-terminal acylation sites. AtCPK2 was myristoylated in a cell-free extract and myristoylation was prevented by converting the glycine at the proposed site of myristate attachment to alanine (G2A). In plants, the G2A mutation decreased AtCPK2 membrane association by approximately 50%. A recombinant protein, consisting of the first 10 amino acids of AtCPK2 fused to the amino-terminus of β-glucuronidase, was also targeted to the ER, indicating that the amino terminus of AtCPK2 can specify ER localization of a soluble protein. These results indicate that AtCPK2 is localized to the ER, that myristoylation is likely to be involved in the membrane association of AtCPK2, and that the amino terminal region of AtCPK2 is sufficient for correct membrane targeting.

The predominant calcium-stimulated protein kinase activity in plant extracts is attributed to calcium-dependent protein kinases (CDPK), a group of enzymes identified only in plants and some protists. Calcium-stimulated kinase activity has been detected in both the soluble and microsomal fractions of plant cells. In many cases, CDPK-like activity was associated with the plasma membrane; for example, in oat (*Avena sativa*; Schaller et al., 1992), red beet (*Beta vulgaris*; Baizabal-Aguirre and de la Vara, 1997), zucchini (*Cucurbita pepo*; Verhey et al., 1993), and tobacco (*Nicotiana tabacum*; Iwata et al., 1998). In other studies, calcium-stimulated kinase activity was reported to be associated with plant microsomes, although the specific membrane was not determined (Battey, 1990; Klimczak and Hind, 1990; Abo-El-Saad and Wu, 1995; MacIntosh et al., 1996; Martin and Busconi, 2000). Thus, it is possible that CDPKs are associated with other cellular membranes in addition to the plasma membrane.

Whereas the roles of individual CDPKs have not yet been elucidated, it has been hypothesized that each CDPK isoform is functionally specialized. Several lines of evidence support this hypothesis. First, three soybean (*Glycine max*) CDPKs have different susceptibilities to protein kinase inhibitors (Lee et al., 1998). Second, these soybean CDPKs differ in their calcium-binding properties (Lee et al., 1998). Third, Arabidopsis CDPKs have overlapping but distinct expression patterns (E. Hrabak, unpublished data). In addition, it is possible that CDPKs could be targeted to different subcellular locations, thereby enabling them to interact with different substrates.

All CDPK proteins contain three domains with well-characterized functions: the Ser/Thr kinase catalytic, autoregulatory, and calcium-binding domains (Harmon et al., 2000). The fourth amino-terminal variable domain is the most divergent region of these proteins, ranging in length from 20 to 200 amino acids and usually exhibiting little sequence similarity between different CDPK isoforms. The function of the variable domain is largely unknown but the majority of CDPK proteins contain a potential myristoylation site at the beginning of the variable domain (Harmon et al., 2000; Hrabak, 2000).

Myristate, a C14:0 fatty acid, can be covalently attached to the amino-terminal Gly residue of a protein when the Gly is found in the context of a short myristoylation consensus sequence (Towler et al., 1988). Many myristoylated proteins are membrane associated but they also can be soluble or alternate between membrane and cytosol (Johnson et al., 1994; Bhatnagar and Gordon, 1997). In addition to a role in mediating protein-lipid interactions, myristoylation can be important for protein-protein interactions or

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protein stability (Yonemoto et al., 1993; Kennedy et al., 1996; Herberg et al., 1997; Taniguchi, 1999).

Myristoylation, catalyzed by *N*-myristoyltransferase (NMT), has been intensively studied in fungal and animal cell systems (for review, see Johnson et al., 1994; Bhatnagar and Gordon, 1997). In contrast, until recently there were few examples of protein myristoylation in plants (Thompson and Okuyama, 2000). An Arabidopsis NMT gene has been cloned and shown to myristoylate amino-terminal peptides derived from a CDPK and from the Fen kinase (Qi et al., 2000). Ellard-Ivey et al. (1999) demonstrated in vitro myristoylation of a CDPK from zucchini and confirmed the requirement for an amino-terminal Gly residue. Rice (*Oryza sativa*) CDPK OsCPK2 was shown to be myristoylated in a heterologous maize (*Zea mays*) protoplast system and this acyl modification was critical for membrane binding (Martin and Busconi, 2000). The importance of myristoylation for correct protein function in plants was suggested by mutation of the putative myristoylation site in the tomato (*Lycopersicon esculentum*) *Fen* gene, which abolished its ability to confer sensitivity to the insecticide fenthion (Rommens et al., 1995). In addition, the myristoylation site of the Arabidopsis SOS3 protein was required for its role in salt tolerance (Ishitani et al., 2000).

Many proteins involved in signal transduction in eukaryotes are myristoylated, including the alpha subunits of heterotrimeric G proteins, members of the Src family of Tyr protein kinases, and the protein phosphatase calcineurin (Casey, 1995; Resh, 1996; Taniguchi, 1999; Thompson and Okuyama, 2000). Acyl groups, and the properties conferred by these hydrophobic modifications, are critical for the proper functioning of these proteins in signaling pathways. In plants, many signaling pathways are known to involve CDPKs, including the response to drought stress, the regulation of carbon and nitrogen metabolism, and the control of seed germination (for review, see Harmon et al., 2000; Hrabak, 2000). Because many CDPKs have predicted acylation sites, the subcellular localization of these enzymes and the role of myristoylation in membrane binding and protein function is of interest. We have focused on Arabidopsis CDPK isoform 2 (AtCPK2) which does not contain any significant transmembrane domains, signal sequences, or targeting signals when analyzed by the PSORT program for predicting protein localization (Nakai and Kanehisa, 1992). However, AtCPK2 does have a predicted amino-terminal myristoylation consensus sequence (Towler et al., 1988).

In this paper, we demonstrate that the AtCPK2 protein in plant cells is associated with the ER membrane. To our knowledge, this is the first example of a CDPK localized to the endoplasmic reticulum (ER). AtCPK2 can be myristoylated in vitro and mutation of the myristoylation site prevents addition of the fatty acid. Mutation of the myristoylation site also decreases membrane association of AtCPK2 in plants. The first 10 amino acids of AtCPK2 are shown to be sufficient to direct a soluble protein to the ER membrane, indicating that this region can be used for protein targeting.

RESULTS

AtCPK2 Genomic Clones

A clone containing the *AtCPK2* genomic region was isolated from a genomic library by low-stringency hybridization and a 5.8-kb region of this clone was sequenced (see GenBank accession no. AF286222 for the complete sequence). The predicted open reading frame contained all of the characteristic features of a CDPK (Hrabak, 2000), including a calmodulin-like domain with four predicted calcium-binding EF hands. AtCPK2 also contains the largest amino-terminal variable domain (187 amino acids) of any CDPK characterized to date (Hrabak, 2000). The genomic sequence, rather than the cDNA, was chosen for these experiments because we have evidence that regions downstream of the promoter are important for full expression of *AtCPK2* (E. Hrabak, unpublished data).

For some experiments where it was important to accurately quantitate levels of AtCPK2 protein or distinguish between the endogenous AtCPK2 and a modified transgenic protein, constructs were made in which the *AtCPK2* gene was tagged with the β-glucuronidase (GUS) reporter gene. pCPK2-GUS contains an *AtCPK2* genomic DNA fragment of 4.9 kb into which the GUS coding sequence was inserted in-frame at an introduced *Bam*HI site (Fig. 1A). This plasmid contains 1.6 kb of DNA upstream of the translation start codon and 0.6 kb downstream of the translation stop codon. Although the precise transcription start site for the *AtCPK2* gene is not known, this construct is predicted to contain the entire *AtCPK2* promoter and 5′-untranslated region because there is less than 1.1 kb of intergenic DNA between the *AtCPK2* translational start codon and the stop codon of the preceding gene, based on the completed Arabidopsis genome sequence. pCPK2-GUS should also contain sufficient DNA downstream of

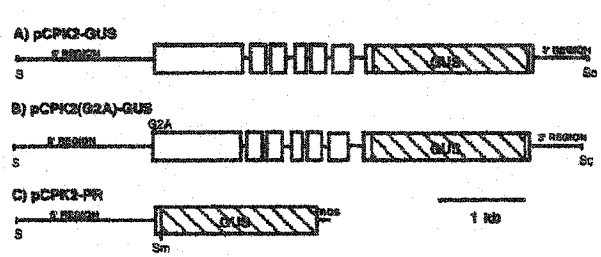

Figure 1. AtCPK2 constructs used for plant transformation. Lines represent introns or non-coding regions. White boxes are *AtCPK2* coding regions. Striped boxes are the coding region for GUS. Most vector sequences are not shown. S, *Sal*I; Sm, *Sma*I; Sc, *Sac*I.

the stop codon to encompass the typical plant polyadenylation signals (Li and Hunt, 1997) because the 3' region of this clone is 385 bp larger than the 3' region of the largest cDNA clone identified. In the resulting 138-kD CPK2-GUS fusion protein, the GUS protein is fused to the AtCPK2 protein at a position ten amino acids from the carboxy terminus of AtCPK2.

Membrane Association of AtCPK2 in Plants

To eliminate the possibility of detecting multiple CDPK isoforms simultaneously and to accurately quantitate levels of a single isoform, transgenic plants expressing the CPK2-GUS fusion protein were used in initial experiments to assess membrane localization. Two-week-old transgenic plants were homogenized, debris was removed by low-speed centrifugation, and the membranes were pelleted by ultracentrifugation. GUS enzyme activity was assayed in both the soluble and membrane fractions using a sensitive fluorimetric assay. Controls for these experiments included wild-type plants and transgenic plants expressing the GUS protein alone. No GUS activity was detected in extracts from wild-type plants. In plants expressing the GUS protein alone, 2% of the GUS activity was found in the membrane fraction (data not shown), which probably represents protein trapped in vesicles during homogenization or nonspecifically bound to membranes. In extracts from CPK2-GUS transgenic plants, 40% of the GUS activity was detected in the membrane fraction (Table I), providing evidence that some of the AtCPK2 protein in these plants is membrane associated.

Treatments to Dissociate AtCPK2 from Membranes

To investigate the interaction of AtCPK2 with membranes, isolated membranes from plants containing GUS-tagged AtCPK2 constructs were incubated in the presence of buffer alone or buffer containing a chelating agent (EDTA), high ionic strength (NaCl), a nonionic detergent (Triton X-100), or an ionic detergent (SDS). After 30 min, the samples were recentrifuged and the amount of GUS activity in the pellet and supernatant was determined with the GUS fluorimetric assay (Fig. 2). In transgenic plants expressing CPK2-GUS protein, approximately 75% of the membrane-associated GUS activity remained in the pellet after treatment of the membranes with buffer containing EDTA or NaCl, whereas the remainder of the CPK2-GUS protein was now found in the supernatant. These results were not significantly different from membranes treated with buffer alone (Fig. 2), indicating that AtCPK2 protein may exist in an equilibrium between soluble and membrane-bound states. Almost all of the GUS activity was released from the membranes of these transgenic plants by detergent treatment. Thus, treatments that disrupt ionic or electrostatic interactions were not effective at dissociating AtCPK2 from membranes, whereas treatments that disrupted most types of hydrophobic interactions efficiently solubilized AtCPK2. Because Triton X-100 was able to release AtCPK2 from membranes, AtCPK2 is unlikely to be associated with detergent-resistant membranes or lipid rafts (Moffett et al., 2000).

AtCPK2 Is Associated with the ER Membrane

Because calcium-stimulated protein kinase activity previously has been detected in association with the plasma membrane fraction in plants (Schaller et al., 1992; Verhey et al., 1993; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998), we used aqueous two-phase partitioning to analyze membranes from wild-type plants (Fig. 3). This technique enriches for plasma membranes in the upper phase and other cellular membranes in the lower phase. Both phases were analyzed by immunoblotting to locate AtCPK2 protein, as well as markers for the ER, mitochondrial, vacuolar, and plasma membranes. Golgi membranes were assayed enzymatically for latent UDPase, whereas chloroplast membranes were detected by measuring chlorophyll concentration. The plasma membrane marker, as expected, was enriched in the upper phase, whereas ER and mitochondrial markers, as well as AtCPK2 protein, were located in the lower phase (Fig. 3). All other membrane markers

Table I. *Membrane association of GUS-tagged AtCPK2 proteins in transgenic plants*

After low-speed centrifugation, plant extracts were ultracentrifuged to pellet microsomes. The supernatant after ultracentrifugation contains primarily soluble proteins, whereas the pellet contains primarily membrane-bound proteins. Both the supernatant and the pellet were assayed fluorimetrically for GUS enzymatic activity.

Protein Expressed in Transgenic Plants	No. of Independent Experiments	No. of Independent Transgenic Lines	GUS-Specific Activity in Membrane Fraction[a]	Membrane-Associated GUS Activity[b]
				%
CPK2-GUS	7	3	67.1–154.2	40 ± 2.3
CPK2(G2A)-GUS	7	3	37.5–76.6	18 ± 1.7
CPK2-PR	4	2	5.2–8.3	46 ± 2

[a] Specific activity units are nmol methylumbelliferone produced min^{-1} mg $protein^{-1}$. [b] Values were calculated from the total enzyme activity data and are presented as mean ± SD.

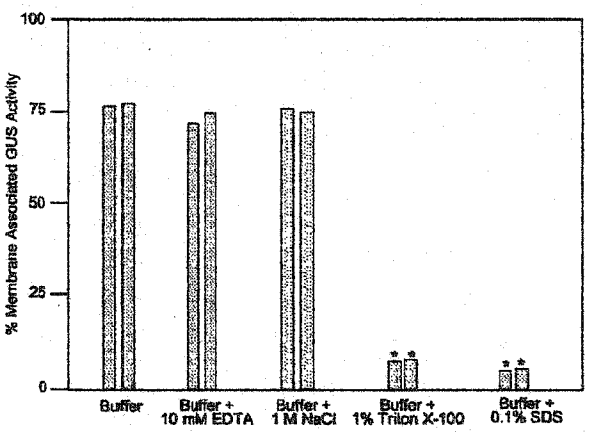

Figure 2. AtCPK2 associated with the membrane fraction after various treatments. Microsomal membranes were isolated from transgenic plants expressing CPK2-GUS. Membrane pellets were homogenized in resuspension buffer alone or resuspension buffer containing EDTA, NaCl, Triton X-100, or SDS and incubated at 4°C for 30 min before repelleting. The resulting supernatant and pellet were assayed for GUS activity to assess the effect of the treatment on AtCPK2 membrane binding. Data shown are the percentage of GUS activity remaining in the pellet. Results from two independent experiments are shown. Asterisks indicate values that were significantly different from the buffer control ($P \leq 0.05$)

were also found predominantly in the lower phase (data not shown). These results indicate that AtCPK2 is not associated with the plasma membrane.

To localize AtCPK2 to a specific cellular membrane, Suc gradients were used to separate microsomes based on their buoyant density. Microsomes and Suc gradients were prepared in buffers containing EDTA alone ($-Mg^{2+}$) or EDTA plus excess $MgCl_2$ ($+Mg^{2+}$). In the presence of Mg^{2+}, ribosomes remain associated with the ER membranes that migrate at 40% to 46% (w/w) Suc. Chelation of Mg^{2+} by EDTA dissociates the ribosomes, shifting the ER membranes to a lower Suc concentration (Lord, 1987). Gradient fractions were analyzed as described for the phase partitioning experiments.

In the presence of Mg^{2+}, AtCPK2 was detected in fractions containing 41% to 46% (w/w) Suc, similar to the ER, chloroplast, and mitochondrial markers. This sedimentation pattern could be easily distinguished from vacuolar, Golgi, and plasma membranes (Fig. 4), but did not permit the unambiguous localization of AtCPK2. In the absence of Mg^{2+}, the ER markers BiP (a major ER-resident binding protein) and ACA2, as well as AtCPK2, shifted to 33% to 37% (w/w) Suc, consistent with a change in buoyant density of the ER after dissociation of ribosomes (Fig. 4). Although we consistently observed that the absence of Mg^{2+} broadened the sedimentation profiles for some of the membrane marker proteins, there was not a shift of the peak fractions to the extent observed for AtCPK2 or the ER membrane markers. Data from the Suc gradients are consistent with localization of membrane-associated AtCPK2 to the ER membrane.

All of the membrane marker proteins detected in this study are integral membrane proteins with the exception of BiP, which is associated with the lumenal face of the ER but does not contain any membrane-spanning domains. In our experiments, some BiP protein is usually detected near the top of the gradients (22%–24% [w/w] Suc). This most likely represents BiP that dissociated during resuspension of the pelleted membranes before loading onto the gradients. It is probably not a result of proteolysis because BiP shows no apparent change in M_r in different gradient fractions. Some AtCPK2 is usually detected in these low-density gradient fractions also, consistent with our previous results that some AtCPK2 protein is solubilized during resuspension in buffer (Fig. 2). The other membrane protein markers, which represent integral membrane proteins, are not found in these low-density gradient fractions.

AtCPK2 Protein Tagged with GUS Localizes to the ER

To determine whether a GUS tag would affect the localization of AtCPK2, Suc gradients were performed on membranes from transgenic plants expressing CPK2-GUS protein (Fig. 5). As expected, the ER marker protein ACA2 was detected at 41% to 46% (w/w) Suc in the presence of Mg^{2+} and shifted to lower buoyant density when the Mg^{2+} was chelated by EDTA. The location of the plasma membrane marker protein was similar in the presence and

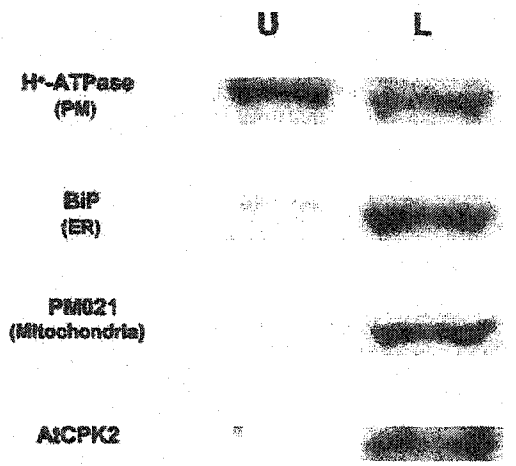

Figure 3. Two-phase separation of membranes from wild-type Columbia plants showing AtCPK2 accumulation in the lower phase. Equal proportions of the upper and lower phases were separated by SDS-PAGE and assayed by immunoblotting with antibodies specific for AtCPK2, H^+-ATPase (plasma membrane marker), BiP (ER marker), and PM021 (mitochondrial membrane marker). U, Upper phase; L, lower phase.

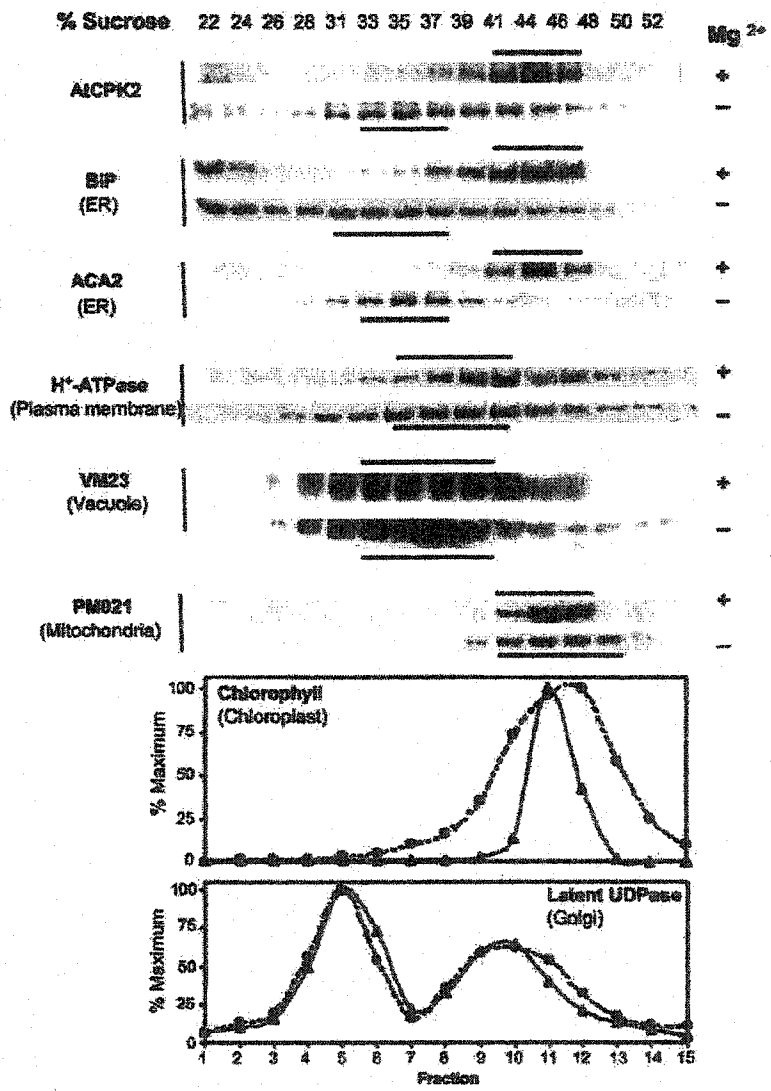

Figure 4. Suc gradient fractionation of membranes from wild-type Columbia plants showing colocalization of AtCPK2 with ER markers. Fractions from parallel gradients, with and without Mg^{2+}, were separated by SDS-PAGE and assayed by immunoblotting with antibodies specific for AtCPK2, BiP and ACA2 (ER markers), H^+-ATPase (plasma membrane marker), Vm23 (vacuolar membrane marker), or PM021 (mitochondrial membrane marker). Horizontal bars indicate the peak fractions. Graphs show chlorophyll absorbance (chloroplast marker) and enzyme assay data for latent UDPase (Golgi marker). , Mg^{2+} gradients; , Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100%.

absence of Mg^{2+}, as were all other membrane markers tested (data not shown). Sedimentation of the membranes containing the wild-type AtCPK2 protein, detected by immunoblotting, was most similar to that of the ER membrane markers. Distribution of GUS enzyme activity, representing the CPK2-GUS fusion protein, closely resembled the distribution of ACA2 and wild-type AtCPK2. These results indicate that the 600-amino acid GUS tag did not interfere with localization of the AtCPK2 protein to the ER membrane (Fig. 5). Because the CPK2-GUS fusion protein is localized in a manner comparable with the wild-type AtCPK2 protein, we conclude that transgenic plants expressing CPK2-GUS can be used interchangeably with wild-type plants to monitor the location of AtCPK2.

AtCPK2 Is Myristoylated in Vitro

The AtCPK2 protein has no predicted transmembrane domains that would account for its observed membrane localization, but it does contain a predicted amino-terminal myristoylation sequence (MGNACVGPN). To determine if the AtCPK2 protein could be a substrate for plant NMT, plasmid pCPK2-ORF, which contains the *AtCPK2* coding sequence downstream of the viral T7 promoter, was used in an in vitro myristoylation experiment. A coupled transcription-translation system from wheat germ was used to transcribe and translate the *AtCPK2* cDNA sequence after addition of T7 RNA polymerase. Wheat germ extract has been shown to contain NMT activity (Heuckeroth et al., 1988; Ellard-Ivey et al., 1999). The reactions were performed in the pres-

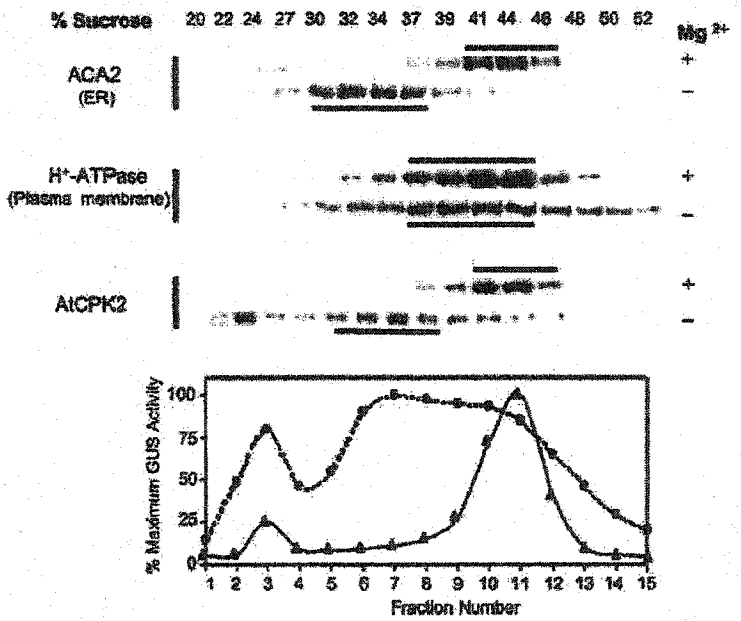

Figure 5. CPK2-GUS, a full-length AtCPK2 protein tagged with GUS, is localized to the ER in transgenic Arabidopsis plants. Fractions from parallel Suc gradients, with and without Mg^{2} , were separated by SDS-PAGE and assayed by immunoblotting with antibodies specific for various membrane markers. AtCPK2-GUS fusion protein was assayed fluorimetrically. Horizontal bars indicate the peak fractions. , Mg^{2+} gradients; , Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100% which corresponds to 188 nmol min^{-1} mL^{-1} for the $+Mg^{2+}$ gradients and 76 nmol min^{-1} mL^{-1} for the - Mg^{2+} gradients. Total GUS activity loaded onto the gradient was 654 nmol min^{-1} for the $+Mg^{2+}$ gradients and 734 nmol min^{-1} for the - Mg^{2+} gradients.

ence of either [^{35}S]Met for detection of total protein synthesis or [^{3}H]myristate to detect myristoylated proteins. A prominent protein of approximately 80 kD was synthesized in the [^{35}S]Met-containing reaction (Fig. 6A, lane 2). The identity of this protein was confirmed by immunoblot analysis with AtCPK2-specific antibody (Fig. 6C, lane 2). A protein of similar size was labeled in the [^{3}H]myristate-containing reaction (Fig. 6B, lane 2). These results indicate that the AtCPK2 protein can be myristoylated in vitro.

No proteins recognized by AtCPK2 antibodies were detected by immunoblotting of mock reactions that contained no plasmid template (Fig. 6C, lane 1). Likewise, no radiolabeled proteins were synthesized when the plasmid template was omitted from the reaction mix (Fig. 6, A and B, lane 1). The smaller proteins routinely observed in [^{35}S]Met-labeled reactions (Fig. 6A, lane 2) might be AtCPK2 degradation products or translation products from alternative start sites within the CPK2 mRNA. Because they are

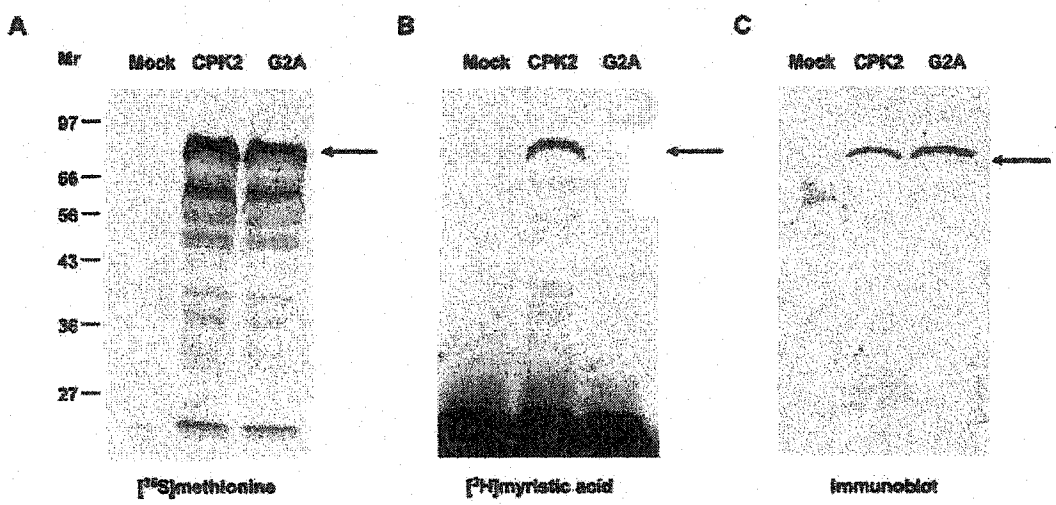

Figure 6. In vitro transcription and translation reactions demonstrate that AtCPK2 can be myristoylated, whereas a G2A mutation prevents myristoylation. Mock reactions contained no plasmid template, CPK2 reactions contained pCPK2-ORF, and G2A reactions contained pCPK2(G2A). A, Proteins synthesized in the presence of [^{35}S]Met. B, Proteins synthesized in the presence of [^{3}H]myristate. C, Proteins detected by immunoblotting with antiserum against the first 90 amino acids of AtCPK2 fused to GST.

not myristoylated and are not detected by immunoblotting, these proteins probably represent amino-terminally truncated products produced from pCPK2-ORF.

A G2A Mutation Prevents Myristoylation of AtCPK2 in Vitro

To confirm that the amino-terminal Gly of AtCPK2 was the site of myristoylation, site-directed mutagenesis was used to convert the second position Gly of pCPK2-ORF to an Ala (G2A) to create the plasmid pCPK2-G2A. A coupled transcription-translation reaction was performed in the presence of either [^{35}S]Met or [^{3}H]myristate using pCPK2-G2A as the template. The G2A mutation did not affect the synthesis of the AtCPK2 protein (Fig. 6A, lane 3 and Fig. 6C, lane 3), but prevented the addition of [^{3}H]myristate (Fig. 6B, lane 3), consistent with the Gly at position 2 of the native AtCPK2 protein being the site of myristoylation.

Effect of CPK2(G2A) Mutation in Plants

To investigate the effect of a G2A mutation in Arabidopsis, transgenic plants were created using the pCPK2(G2A)-GUS construct (Fig. 1B). pCPK2(G2A)-GUS is identical to pCPK2-GUS except for a single nucleotide change that converted the second codon from Gly to Ala. The amount of GUS activity in the membrane fraction from plants expressing CPK2(G2A)-GUS was reduced to 18%, compared with 40% in plants expressing CPK2-GUS (Table I). Suc gradients were used to determine the location of CPK2(G2A)-GUS protein in these plants. The distribution of membrane-bound GUS activity paralleled the distribution of the ER membrane markers (data not shown). Thus, although mutation of the myristoylation site decreased the proportion of AtCPK2 in the membrane fraction, the G2A mutation did not affect ER localization.

The First 10 Amino Acids of AtCPK2 Are Sufficient for ER Localization

To determine whether the amino terminus of AtCPK2 contains the ER-targeting information, we tested whether this region was able to direct a soluble protein to the ER membrane. Arabidopsis plants were stably transformed with DNA from plasmid pCPK2-PR containing 1.6 kb of *AtCPK2* genomic DNA upstream of the translational start site followed by the first 30 bp of the *AtCPK2* coding sequence in a translational fusion with the GUS gene and *nos* terminator (Fig. 1C). These plants expressed the GUS protein preceded by the first 10 amino acids of AtCPK2. This 10-amino acid region was chosen because it contains the myristoylation consensus sequence as defined by Towler et al. (1988). The proportion of GUS activity found in the membrane fraction from plants expressing the CPK2-PR protein was 46%, which is similar to the results from plants expressing CPK2-GUS, the full-length CPK2 protein tagged with GUS (Table I). The specific activity of the GUS enzyme detected in extracts from plants expressing CPK2-PR was consistently lower than in extracts from plants containing the full-length CPK2-GUS constructs (Table I). The lower activity may be because of missing regulatory sequences downstream of the promoter that are required for higher levels of expression or to differences between Arabidopsis ecotypes because CPK2-PR transgenic plants are in the RLD genetic background, whereas the CPK2-GUS plants are in the Columbia ecotype. Regardless of the reason for the lower expression levels in CPK2-PR plants, the results demonstrate that the first 10 amino acids of AtCPK2 were sufficient to allow direct membrane targeting of the normally soluble GUS protein.

Suc gradients were used to determine the location of CPK2-PR protein in microsomes from transgenic CPK2-PR-expressing plants. As observed previously for the wild-type AtCPK2 protein (Fig. 4) and for plants expressing CPK2-GUS (Fig. 5), the distribution of membrane-bound GUS activity was most similar to the location of the ER membrane marker (Fig. 7). Thus, a 10-amino acid region from the amino terminus of AtCPK2 was sufficient to direct localization of the GUS protein to the ER in a manner indistinguishable from the intact AtCPK2 protein.

DISCUSSION

CDPKs are known to be involved in many cellular processes such as pollen tube growth (Moutinho et al., 1998), mobilization of starch during seed germination (Ritchie and Gilroy, 1998), regulation of actin tension (Grabski et al., 1998), plant defense (Romeis et al., 2000), and responses to water stress (Shinozaki and Yamaguchi-Shinozaki, 1997). However, the details of how specific CDPKs function in plant cells are not well understood. It has been proposed that individual CDPKs may function in specific cell types, respond to different calcium concentrations, or be targeted to specific subcellular locations (Harmon et al., 2000; Hrabak, 2000).

Targeting of a protein kinase to a membrane can serve to increase the local concentration of the enzyme manyfold and to enhance the phosphorylation of substrate proteins found at that location, while limiting interaction with proteins in other parts of the cell. CDPK substrates include both soluble proteins, such as nitrate reductase (Douglas et al., 1998) and Suc phosphate synthase (Huber et al., 1996), and integral membrane proteins, such as the ER-localized calcium pump ACA2 (Hwang et al., 2000), a vacuolar chloride channel (Pei et al., 1996), and a proton pump and a potassium channel in the plasma membrane

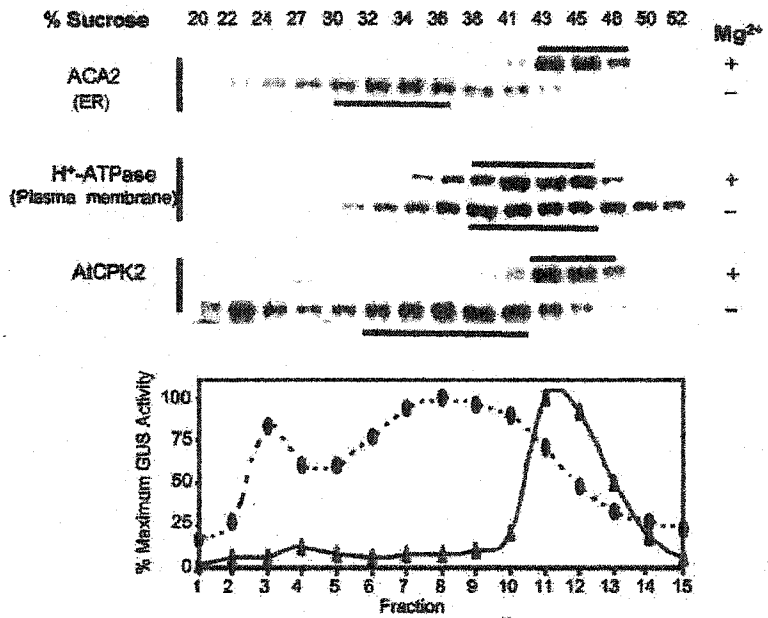

Figure 7. CPK2-PR, consisting of the GUS protein preceded by the first 10 amino acids of AtCPK2, is localized to the ER in transgenic Arabidopsis plants. Fractions from parallel Suc gradients, with and without Mg^{2} , were separated by SDS-PAGE and assayed by immunoblotting with antibodies specific for various membrane markers. AtCPK2-GUS fusion protein was assayed fluorimetrically. Horizontal bars indicate the peak fractions. , $+Mg^{2+}$ gradients; , - Mg^{2+} gradients. The fraction with the highest activity was assigned a value of 100% that corresponds to 41.2 nmol min^{-1} mL^{-1} for the $+Mg^{2+}$ gradients and 21.8 nmol min^{-1} mL^{-1} for the - Mg^{2+} gradients. Total GUS activity loaded onto the gradient was 146 nmol min^{-1} for the + Mg^{2+} gradients and 199 nmol min^{-1} for the - Mg^{2+} gradients.

(Schaller and Sussman, 1988; Li et al., 1998; Lino et al., 1998). In addition, calcium-stimulated protein kinase activity has been detected in many subcellular locations, including the cytosol (Battey, 1990; Klimczak and Hind, 1990; Putnam-Evans et al., 1990; Das-Gupta, 1994; MacIntosh et al., 1996; Frylinck and Dubery, 1998), the nucleus (Li et al., 1991), the cytoskeleton (Putnam-Evans et al., 1989), and the plasma membrane (Schaller et al., 1992; Verhey et al., 1993; Baizabal-Aguirre and de la Vara, 1997; Iwata et al., 1998).

We investigated the membrane localization of AtCPK2 from Arabidopsis. Initial evidence that AtCPK2 was membrane associated was obtained using transgenic plants expressing AtCPK2 tagged with the GUS reporter protein. The sensitive fluorometric GUS assay enabled specific and quantitative detection of AtCPK2 in the presence of other members of the CDPK family. When plant extracts were separated into membrane and soluble fractions, about 40% of the GUS enzyme activity was detected in the insoluble fraction. A similar distribution has been reported for myristoylated Src kinase (Resh, 1989). Some AtCPK2 was removed from the membrane fraction by treatment with buffer but removal was not increased in the presence of EDTA or NaCl, indicating that ionic or electrostatic interactions are not essential for binding. Comparable results have been reported for the Src protein (Resh, 1989) and a CDPK from tobacco (Romeis et al., 2000), which suggests that these proteins may exist in an equilibrium between membrane-bound and soluble forms. The GUS enzyme can also be assayed histochemically. Microscopic examination of these plants revealed that the colored product of the histochemical assay accumulated slowly, an indication that AtCPK2 expression levels are low. Expression was limited to a few cell types during vegetative growth such as developing trichomes and cells in the elongation zone of the root (E. Hrabak, unpublished data). The restricted expression of AtCPK2 explains why the native AtCPK2 protein could only be detected on western blots using the most sensitive chemiluminescent reagents currently available. The fluorimetric GUS assay was similar in sensitivity to chemiluminescence and provided a separate means to confirm localization studies.

To determine the specific membrane(s) to which AtCPK2 was bound, the membrane fraction of wild-type and transgenic plants was further analyzed by aqueous two-phase partitioning and Suc density gradients. The phase partitioning experiments indicated that AtCPK2 was not associated with the plasma membrane, but was enriched in the intracellular membranes. Suc gradient fractionation in buffer containing Mg^{2+} was able to narrow the location of AtCPK2 to several membrane types with similar buoyant densities. Incorporation of EDTA into the buffers used to prepare plant microsomes and Suc gradients allowed us to distinguish ER membranes from other membranes based on a characteristic buoyant density shift and demonstrated that the distribution of AtCPK2 most closely paralleled the distribution of the ER membrane markers.

Whereas many acylated proteins are associated with the plasma membrane in yeast or animal cells, few myristoylated proteins have been localized to the ER. Because the ER lumen is a site of calcium sequestration (Malho et al., 1998; Trewavas, 1999), localized calcium release could activate CDPKs located on or

near the ER. Whereas the specific substrate(s) of AtCPK2 have not yet been identified, the ER-localized calcium pump ACA2 has been shown to be an in vitro substrate for a closely related CDPK, AtCPK1 (Hong et al., 1999; Hwang et al., 2000). It is tempting to speculate that AtCPK2 might be one of the kinases that phosphorylates ACA2 in plants, although it is not yet known if these two proteins are expressed in the same cell types.

Although most CDPKs contain one slightly hydrophobic region in the kinase domain, there is no evidence that CDPKs are integral membrane proteins. One mechanism that can allow membrane binding of peripheral membrane proteins is acquisition of a hydrophobic domain, like prenyl, myristate, or palmitate groups or glycosylphosphatidylinositol anchors (Casey, 1995). Whereas CDPKs do not contain prenylation or glycosylphosphatidylinositol anchor motifs, the majority of CDPKs, including AtCPK2, contain potential amino-terminal myristoylation and palmitoylation sites (Hrabak, 2000).

An NMT gene has recently been cloned and characterized from Arabidopsis (Qi et al., 2000) and wheat germ extract contains NMT activity (Heuckeroth et al., 1988), providing evidence that plants contain the enzyme needed to perform the myristoylation reaction. We demonstrated that AtCPK2 could be myristoylated in vitro using a cell-free wheat germ extract to transcribe and translate the AtCPK2 protein in the presence of radiolabeled myristic acid. Whereas these results are not definitive proof that AtCPK2 is myristoylated in vivo, they indicate that AtCPK2 is a substrate for plant NMT. This approach has been used previously to demonstrate that CDPKs from zucchini (Ellard-Ivey et al., 1999) and from rice (Martin and Busconi, 2000) were able to be myristoylated. An AtCPK2 protein containing a G2A mutation could not be myristoylated, indicating that the amino-terminal Gly residue was the site of myristate attachment.

Based on the in vitro myristoylation assays, we predict that the CPK2(G2A)-GUS protein would not be myristoylated in plants. This prediction is supported by experiments with transgenic plants expressing CPK2(G2A)-GUS in which membrane-bound GUS activity was reduced from 40% to 18% by the G2A mutation. This value is above the 2% background level for membrane binding of the GUS protein alone, indicating that a significant portion of AtCPK2 is still membrane associated in the G2A mutant. There are at least two potential explanations for these results. First, all Arabidopsis CDPKs that contain a myristoylation consensus sequence also contain at least one nearby Cys residue that may serve as a palmitoylation site (Hrabak, 2000). Palmitate is more hydrophobic than myristate and binds more tightly to lipid bilayers (Shahinian and Silvius, 1995; Bhatnagar and Gordon, 1997) and therefore most palmitoylated proteins are membrane bound (Resh, 1996). Modification by both myristate and palmitate has been demonstrated for many acylated proteins, including heterotrimeric G protein alpha subunits and Src family proteins (Milligan et al., 1995; Resh, 1996), and recently a rice CDPK, OsCPK2, was shown to be both myristoylated and palmitoylated in a maize protoplast expression system (Martin and Busconi, 2000). Because preventing myristoylation usually decreases or inhibits palmitoylation (Galbiati et al., 1994; Hallak et al., 1994; Wilson and Bourne, 1995; Morales et al., 1998), the decreased membrane binding of the G2A mutant may reflect incomplete palmitoylation. The second potential explanation is that ER targeting of AtCPK2 might be mediated by interaction with specific receptor(s) in or associated with the ER membrane that recognize the AtCPK2 amino terminus. In this case, the hydrophobicity of the myristoyl group might function to facilitate initial membrane binding leading to association with the receptor. This scenario is attractive because it would help to explain how AtCPK2 is targeted specifically to the ER, rather than to many of the other membrane types in the cell as might be expected if the binding was strictly a result of hydrophobic interactions. Thus, the lower membrane binding observed for the G2A mutant could be because of a decreased tendency of the non-myristoylated protein to be close to membranes.

Because the myristoylation site of AtCPK2 is located at the amino terminus of the protein, we investigated whether the first 10 amino acids of AtCPK2 were sufficient to direct the soluble GUS protein to the ER membrane. Several other proteins whose amino termini are sufficient for specific membrane binding have been described including poliovirus VP4 (Martin-Belmonte et al., 2000), CAP23/NAP22 (Takasaki et al., 1999), $p59^{fyn}$ (Gauen et al., 1992), and $p60^{src}$ (Resh and Ling, 1990). In addition, acylated amino termini can target green fluorescent protein to specific subcellular locations (McCabe and Berthiaume, 1999). In our experiments, the percentage of total GUS activity in extracts of transgenic plants that was associated with the membrane fraction was similar in plants expressing either the full-length AtCPK2 protein tagged with GUS (CPK2-GUS) or the GUS protein preceded by the first 10 amino acids of AtCPK2 (CPK2-PR). These results demonstrated that the first 10 amino acids of AtCPK2 retained the membrane targeting ability of the intact protein and indicated that this region may be useful for targeting other proteins to the ER.

In this report, we have demonstrated that Arabidopsis CDPK isoform AtCPK2 is associated with the ER membrane in plants. AtCPK2 is myristoylated in vitro and a G2A mutation prevented myristoylation and decreased membrane binding in plants. These findings support the hypothesis that myristoylation contributes to membrane association of AtCPK2, but is not the only factor involved. The region of AtCPK2

necessary for ER targeting is located within the first 10 amino acids of the protein. An understanding of the role of acylation in the proper functioning of this kinase awaits more information about the function of AtCPK2 in plants. However, the identification of a CDPK associated with the ER membrane presents many directions for future research. It will be of interest to determine whether AtCPK2 binds to or phosphorylates substrates on the ER, to understand the function of the membrane-bound and soluble forms of AtCPK2, and to delineate the specific residue(s) at the amino terminus of AtCPK2 that are required for ER targeting.

MATERIALS AND METHODS

DNA cloning was done in *Escherichia coli* strain DH5 (Life Technologies, Rockville, MD). Standard molecular cloning techniques were used throughout, according to Sambrook et al. (1989). GUS activity was determined with a fluorimetric assay as previously described (Gallagher, 1992). Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

AtCPK2 cDNA Clones

To identify an AtCPK2 cDNA clone, a size-fractionated Arabidopsis ecotype Columbia cDNA library (Schindler et al., 1992) was screened by hybridization with an AtCPK2 genomic DNA fragment as the probe. Three 1.6-kb clones (pE1-5, pE17-3, and pE17-4) were isolated but all of them were missing approximately 400 bp at the beginning of the open reading frame, as predicted from the genomic sequence. A 1.4-kb region of cDNA beginning at the translation initiation codon was amplified by PCR using the cDNA library as template, an upstream primer 5 GGATCCATGGGTAATGCT containing an introduced *Bam*HI site, and the downstream primer 5 GGTTAGTCTTCG. The amplified 5 region and the truncated cDNA clone pE1-5 overlapped in a region containing a unique *Bgl*II restriction site, which was used to ligate both segments of the AtCPK2 coding sequence to create the plasmid pCPK2-ORF containing the entire AtCPK2 coding sequence. The GenBank accession number for the AtCPK2 cDNA sequence is U31833.

An *AtCPK2* cDNA clone with a G2A mutation was made by PCR with pCPK2-ORF as the template, a forward primer with a single base change (bold font) to convert the Gly at codon 2 to an Ala (5 AGTGGATCCATGGCTAATGCTTGCGT), and a reverse primer in the pBluescript vector. The PCR product was recloned into pBluescript to give pCPK2-G2A and sequenced to confirm the G2A mutation.

AtCPK2 Genomic Clones

An *AtCPK2* genomic clone was isolated by hybridization of a cosmid library of Arabidopsis ecotype Columbia genomic DNA in the vector pOCA18 (Olszewski et al., 1988) under low stringency conditions with a radiolabeled DNA probe corresponding to a portion of the closely related *AtCPK1* gene (Harper et al., 1993). An 11-kb *Xba*I fragment containing the *AtCPK2* genomic region was subcloned from one of the hybridizing cosmids to pBluescript (Stratagene, La Jolla, CA) and designated pgAK19. A 5.6-kb region of pgAK19 was sequenced on both strands using either Sequenase 2.0 (U.S. Biochemicals, Cleveland) for manual sequencing or Dye-deoxy Terminators (Perkin-Elmer Applied Biosystems, Foster City, CA) for automated sequencing.

To facilitate detection of the AtCPK2 protein in wildtype plant extract, the *AtCPK2* genomic sequence (including 1.6 kb upstream of the translational start site) was tagged near the end of the coding sequence with the 1.8-kb GUS (*uidA*) gene. A 2-kb *Eco*RI fragment from the 3 end of pgAK19 was subcloned to pALTER-1 (Promega Corp., Madison, WI). Site-directed mutagenesis was performed according to the manufacturer's instructions using the oligonucleotide 5 GGGAGGACCTCTGAAGATGGATCCAGAGAACAGCATTAGCATTTCTC in which bold type indicates nucleotide changes from the original sequence. This procedure introduced a *Bam*HI restriction site 30 bp upstream of the *AtCPK2* stop codon. The 2-kb *Eco*RI fragment was sequenced to confirm that no unintended errors had been introduced by the mutagenesis procedure. To eliminate the unsequenced upstream and downstream regions from pgAK19, a multistep process was used to construct a clone that contained most of the 5.6-kb sequenced region. pCPK2-PR (described below) was digested with *Mlu*I and *Xba*I and ligated to a 6-kb *Mlu*I-*Xba*I fragment from pgAK19 to yield pPGS. pPGS contains the 5.6-kb sequenced region as well as 1.3 kb of downstream DNA. The 2-kb *Eco*RI fragment in pPGS was replaced with the *Eco*RI fragment containing the introduced *Bam*HI site to yield pPGS-M and the fragment was confirmed to be in the correct orientation by restriction enzyme digestion. pPGS-M was digested with *Sal*I and *Kpn*I and the 4.9-kb fragment containing 1.6 kb of *AtCPK2* sequence upstream of the start codon, the entire coding sequence including introns, and 0.6 kb of *AtCPK2* sequence downstream from the stop codon was cloned into pUC18 that had been digested with *Sal*I and *Kpn*I. A GUS cassette (DeWitt et al., 1996) with *Bam*HI ends was ligated into the introduced *Bam*HI site, which fused the GUS sequence in frame with the *AtCPK2* reading frame, to yield pGMG-GUS. Orientation and correct fusion of the cassettes were confirmed by sequencing through the fusion junctions. Finally, the tagged *AtCPK2* genomic construct was subcloned into the vector pBIN19 as a *Sal*I-*Sac*I fragment to yield pCPK2-GUS.

To create an *AtCPK2* genomic clone with Ala (GCT) instead of Gly (GGT) as the second codon (G2A mutation), site-directed mutagenesis was performed with the QuikChange kit (Stratagene) according to manufacturer's instructions using pGMG-GUS as the template DNA. The mutated *AtCPK2* gene was subcloned into the vector pBIN19 as described above and named pCPK2(G2A)-GUS.

To fuse the promoter and first 10 codons of AtCPK2 to the GUS reporter gene, PCR was used to amplify a 1.6-kb region upstream of the *AtCPK2* genomic sequence. A *Sall* site was added at the 5 end of the upstream primer 5 ACTGTCGACTTATATGTCTTCATATCTCT and a *Sma*I site was added to the downstream primer at a position immediately after the 10th full codon of the coding sequence 5' CCACCCGGGAAATGTGGTGTCCAACGCA. Products from the PCR reaction were cloned into pBluescript to yield pPRM and sequenced to confirm that no PCR errors had occurred. The *Sall-Sma*I fragment was then cloned upstream of the GUS reporter gene in pBI101.2 (CLONTECH, Palo Alto, CA) to produce pCPK2-PR. The correct reading frame across the translational fusion junction was confirmed by DNA sequencing.

Production of Transgenic Plants and Plant Culture

Arabidopsis ecotype RLD roots were transformed as described previously (Valvekens et al., 1988), followed by plant regeneration. Arabidopsis ecotype Columbia plants were transformed using a vacuum infiltration procedure (Bent and Clough, 1998). Transformed plants were confirmed to contain the correct transgene using a rapid PCR method (Klimyuk et al., 1993).

Plants for membrane isolation were grown from surface-sterilized seeds in liquid Murashige and Skoog basal medium (Sigma, St. Louis), pH 5.7, containing Gamborg's B-5 vitamins and 1% (w/v) Suc at 100 rpm, 22°C, and an 18-h-light/dark cycle.

AtCPK2-Specific Antibodies

AtCPK2 rabbit polyclonal antibody was made against a purified fusion protein consisting of the first 90 amino acids of the 185 amino acid AtCPK2 variable domain fused to glutathione S-transferase in vector pGEX-KT (Hakes and Dixon, 1992). Recombinant protein expressed in *E. coli* was purified on a glutathione-agarose matrix (Pharmacia, Piscataway, NJ) and used for immunization of New Zealand white female rabbits. The antibody did not cross-react with AtCPK1 protein, the CDPK isoform most closely related to AtCPK2, or with AtCPK4 or AtCPK5 protein.

Membrane Isolation

Cellular membranes were prepared as previously described with minor modifications (Schaller and DeWitt, 1995). All procedures were conducted at 4°C. Two-week-old, liquid-grown Arabidopsis plants were homogenized in a mortar and pestle in 1 to 2 mL of homogenization buffer (50 mM Tris-HCl, pH 8.2; 20% [v/v] glycerol; 1 mM phenylmethylsulfonyl fluoride; 1 mM dithiothreitol; 10 μg mL^{-1} leupeptin; 1 μg mL^{-1} pepstatin; and 10 μg mL^{-1} aprotinin) per gram of tissue. Homogenates were filtered through Miracloth and centrifuged at 5,000*g* for 5 min. The supernatant was centrifuged at 125,000*g* for 30 min to pellet microsomes. The remaining supernatant contains primarily soluble proteins.

Membrane-Binding Assays

To investigate the membrane binding affinity of AtCPK2, microsomal membranes were resuspended at 0.5 mg mL^{-1} in resuspension buffer (25 mM Tris-HCl, pH 7.5; 10% [w/v] Suc; and protease inhibitors as described above) alone or in resuspension buffer containing one of the following: 10 mM EDTA, 1 M NaCl, 1% (v/v) Triton X-100, or 0.1% (w/v) SDS. After incubation at 4°C for 30 min, samples were re-centrifuged at 125,000*g* at 4°C for 30 min to pellet membrane vesicles. The supernatants were saved and the pellets were resuspended in resuspension buffer. Results were analyzed via one-way ANOVA in Systat 9.0. Treatment means were compared with the control via Dunnett's test.

Two-Phase Separation

An aqueous two-phase system (Larsson, 1983) was used to separate plasma membrane from intracellular membranes. Total membranes, prepared as described above, were resuspended in 200 μL of SPK buffer (0.33 M Suc, 5 mM KPO_4, and 3 mM KCl, pH 7.8) and added to a 4-g phase system prepared in the same buffer. The final composition of the phase system was 6.3% (w/w) dextran (M_r = 413,000) and 6.3% (w/w) polyethylene glycol (M_r = 3,350). After thorough mixing by inverting the tube 20 to 30 times, the phases were separated by centrifugation at 1,400*g* for 5 min. The upper phase (enriched for plasma membrane) was removed to a clean tube and repartitioned twice with lower phase. Likewise, the lower phase, containing primarily intracellular membranes, was repartitioned twice with upper phase. Fresh upper and lower phase were obtained from a bulk-phase system of identical composition prepared separately. The final upper and lower phases were diluted with buffer containing 10 mM Tris-HCl (pH 7.0), 1 mM EGTA, and 1 mM EDTA and centrifuged at 125,000*g* for 30 min. Pellets were resuspended in equal volumes of SPK buffer. After separation by SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membranes and analyzed by immunoblotting as described below.

Suc Gradient Fractionation

Membrane pellets were resuspended in 1 mL of resuspension buffer per 10 g wet weight of starting material using a ground glass homogenizer. Resuspended membranes were layered onto linear Suc gradients (20% to 50% [w/w]) prepared in centrifugation buffer (10 mM Tris-HCl, pH 7.6, and protease inhibitors). For density gradients performed in the presence of Mg^{2+}, 2 mM EDTA and 5 mM $MgCl_2$ were added to homogenization, resuspension, and centrifugation buffers. For density gradients performed in the absence of Mg^{2+}, 5 mM EDTA was added to homogenization and resuspension buffers, whereas 2 mM EDTA was used in centrifugation buffer. After centrifugation in a swinging bucket rotor at 125,000*g* for 16 h at 4°C, 1-mL fractions were collected.

The Suc concentration of each fraction was measured with a refractometer (Fisher Scientific, Pittsburgh). Protein

concentrations were determined according to the method of Lowry as described previously (Schaller and DeWitt, 1995). Chlorophyll, a marker for chloroplast thylakoid membranes, was measured spectrophotometrically (Schaller and DeWitt, 1995). Latent UDPase, a marker for Golgi membranes, was assayed as described by Schaller and DeWitt (1995) using the Malachite Green method to detect released phosphate. All other membrane markers were detected by immunoblot analysis. After separation of proteins on 10% (w/v) SDS-polyacrylamide gels (Sambrook et al., 1989), proteins were electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and the membranes were blocked overnight at 4°C in TBS (20 mM Tris-HCl, pH 7.6, and 137 mM NaCl) containing 2% (w/v) nonfat dry milk. The PVDF membranes were then treated sequentially with primary antibodies in TBST (TBS containing 0.05% [v/v] Tween 20) containing 2% (w/v) nonfat dry milk for 1 h, TBST for 5 min (3 times), secondary antibodies in TBST containing 2% (w/v) nonfat dry milk for 45 min, TBST for 5 min (three times), and TBS for 5 min. Immunodecorated proteins were detected with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) on x-ray film. Quantitation of immunodetected proteins was done using the NIH Image program. Membranes were stripped between detections following manufacturer's instructions. Antisera were used at the following dilutions: plasma membrane H^+-ATPase (DeWitt and Sussman, 1995), 1:10,000 (v/v); ER lumenal binding protein BiP (gift of Maarten Chrispeels, University of California, San Diego), 1:1,000 (v/v); ER integral membrane protein ACA2 (*Arabidopsis* calcium ATPase; Harper et al., 1998), 1:2,000 (v/v); mitochondrial membrane protein β-ATPase D (Luethy et al., 1993), 1:100 (v/v); vacuolar membrane protein VM23 (Maeshima, 1992), 1:3,000 (v/v); and AtCPK2 variable domain antibody (described above), 1:10,000 (v/v).

In Vitro Myristoylation Assays

A cell-free wheat germ extract system was used to transcribe and translate the *AtCPK2* gene in the presence of either radiolabeled methionine to assess total protein synthesis or radiolabeled myristate to detect myristoylated proteins. One microgram of plasmid, linearized with *Eco*RI, was used as the template for transcription by T7 RNA polymerase in the TNT Coupled Transcription-Translation Wheat Germ Extract System (Promega Corp.). Control reactions contained no plasmid. Reactions were prepared according to the manufacturer's instructions in the presence of either 50 μCi of [9,10-^{3}H]myristic acid (54 Ci mmol^{-1}; Amersham, Piscataway, NJ) or 10 μCi of 1-[^{35}S]Met (1,000 Ci mmol^{-1}; Amersham). Immediately before beginning the 1.5-h reaction, the [^{3}H]myristic acid was dried under nitrogen and resuspended by vortexing in DEPC-treated water at a concentration of 10 μCi μL^{-1}. Reaction products were separated on 10% (w/v) SDS-polyacrylamide gels (Sambrook et al., 1989), stained with Coomassie Blue, and then treated with Entensify autoradiography enhancer (New England Nuclear, Boston) before detection on x-ray film.

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