The prenylation status of a novel plant calmodulin directs plasma membrane or nuclear localization of the protein

Manuel Rodriguez-Concepcion,
Shaul Yalovsky, Moriyah Zik¹, Hillel Fromm¹
and Wilhelm Ggrissem²

Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA and ¹Department of Plant Sciences, Weizmann Institute of Science, Rehovot, 76100, Israel

²Corresponding author
e-mail: ggrissem@nature.Berkeley.edu

Post-translational attachment of isoprenyl groups to conserved cysteine residues at the C-terminus of a number of regulatory proteins is important for their function and subcellular localization. We have identified a novel calmodulin, CaM53, with an extended C-terminal basic domain and a CTIL CaaX-box motif which are required for efficient prenylation of the protein *in vitro* and *in vivo*. Ectopic expression of wild-type CaM53 or a non-prenylated mutant protein in plants causes distinct morphological changes. Prenylated CaM53 associates with the plasma membrane, but the non-prenylated mutant protein localizes to the nucleus, indicating a dual role for the C-terminal domain. The subcellular localization of CaM53 can be altered by a block in isoprenoid biosynthesis or sugar depletion, suggesting that CaM53 activates different targets in response to metabolic changes. Thus, prenylation of CaM53 appears to be a novel mechanism by which plant cells can coordinate Ca²⁺ signaling with changes in metabolic activities.

Keywords: calmodulin/isoprenoid/membrane/nucleus/
prenylation

Introduction

Isoprenoids are synthesized in all eukaryotic cells via a key metabolic pathway whose inactivation results in specific G₁ arrest (Goldstein and Brown, 1990), suggesting that mechanisms exist by which cells integrate information about their metabolic status with the regulation of cell division and growth. A small number of regulatory proteins involved in signal transduction, nuclear architecture and vesicular transport are modified by the covalent attachment of the isoprenoid pathway intermediates farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) to conserved C-terminal cysteine residues via a thioether linkage (reviewed in Clarke, 1992; Schafer and Rine, 1992; Zhang and Casey, 1996). These proteins include most members of the Ras superfamily of small GTP-binding proteins, γ subunits of heterotrimeric GTP-binding proteins (G proteins), fungal pheromones, type I inositol-1,4,5-triphosphate 5'-phosphatase, cGMP phosphodiesterases, nuclear lamins and a small group of specific protein kinases and phosphatases (Clarke, 1992; Inglese *et al.*,

1992a,b; Cates *et al.*, 1996; De Smedt *et al.*, 1996). For proteins that have been investigated in detail, it was shown that the hydrophobic isoprenyl groups increase the affinity of the proteins for cell membranes (Hancock *et al.*, 1991; Glomset and Farnsworth, 1994) or facilitate their interaction with other regulatory proteins (Marshall, 1993; Zhang and Casey, 1996).

Two types of geranylgeranyl transferases (GGTase-I and RabGGTase) and a single farnesyl transferase (FTase) catalyze the prenylation reactions in animal and yeast cells (Schafer and Rine, 1992; Zhang and Casey, 1996). FTase and GGTase-I are heterodimeric enzymes that share a common α subunit but have distinct β subunits. Both enzymes recognize a conserved amino acid C-terminal sequence motif known as the CaaX box, in which 'C' represents a conserved cysteine, 'a' represents mostly aliphatic amino acids and 'X' is usually serine, methionine, cysteine, alanine, glutamine or leucine. If 'X' is leucine, the protein is prenylated typically by GGTase-I, while other residues are recognized preferentially by FTase (Moores *et al.*, 1991; Schafer and Rine, 1992). All three types of protein prenyl transferases are present in plants as well (reviewed in Rodriguez-Concepcion *et al.*, 1999). Plant FTase is the best characterized and has been implicated in the regulation of abscisic acid signal transduction (Cutler *et al.*, 1996; Pei *et al.*, 1998) and cell cycle progression (Qian *et al.*, 1996). In addition, plant FTase can restore Ras signaling and mating effectively in the yeast *ram1* Δ mutant in which the chromosomal FTase β subunit gene was deleted, indicating that enzymatic activity and substrate recognition are evolutionarily conserved (Yalovsky *et al.*, 1997). However, in contrast to animal and yeast cells, only very few prenylation substrates have been identified in plants, including ANJ1, a homolog of the bacterial chaperone DnaJ (Zhu *et al.*, 1993), and members of the Rab and Rac protein families (Loraine *et al.*, 1996; Trainin *et al.*, 1996; Yalovsky *et al.*, 1996).

We identified several new plant proteins with CaaX-box motifs that were potential substrates for FTase or GGTase-I (Rodriguez-Concepcion *et al.*, 1999). Among these proteins was petunia CaM53, a novel type of calmodulin (CaM) with an extended basic C-terminal domain and a conserved GGTase-I recognition motif. CaM is a member of a family of structurally related EF-hand Ca²⁺-binding proteins that function as primary receptors for second messenger Ca²⁺. CaM mediates the regulatory effects of Ca²⁺ through two hydrophobic domains that are exposed after Ca²⁺ association (Finn and Forsen, 1995) and that bind to specific domains present in several CaM-binding proteins and enzymes, resulting in the activation of their function. In plant cells, a wide spectrum of signals such as light, hormones, temperature changes, drought, mechanical stimulation or pathogen infection trigger rapid increases in intracellular Ca²⁺ concentration.

This activates signal transduction pathways which coordinate changes in biochemical activities and gene expression, often resulting in cell division and growth (Bush, 1995; Trevaas and Malho, 1997; Snedden and Fromm, 1998). Unlike animals and yeast, however, plants have several CaM and CaM-related proteins, suggesting a broader role for CaM–Ca²⁺ and CaM–target interactions (Snedden and Fromm, 1998), although specific functions have not been reported for any of the CaM proteins. Target proteins in plants known to be regulated by Ca²⁺ and CaM include NAD kinase, glutamate decarboxylase (GAD), membrane Ca²⁺-ATPase and Ca²⁺ channels, protein kinases and nuclear NTPase (reviewed in Snedden and Fromm, 1998; Zielinski, 1998).

Although certain EF-hand proteins such as recoverin can be modified by myristilation (Ames *et al.*, 1997), prenylation of a Ca²⁺-binding protein, and of CaM in particular, has not been reported. The amino acid sequences of all known CaMs predict that they are soluble proteins, although it is interesting to note that in developing plant tissues a substantial amount of CaM and CaM-binding proteins has been found associated with membranes (Collinge and Trevaas, 1989; Fraichard *et al.*, 1996). We demonstrate here that CaM53 is prenylated and localizes to the plasma membrane, which can explain the previously reported results. Most interestingly, CaM53 is found in the nucleus when synthesis of mevalonic acid (MVA) is blocked or after transfer of plants to the dark, suggesting that the subcellular localization of the protein can be modulated in response to metabolic changes.

Results

CaM53 is a novel calmodulin with an extended C-terminal basic domain and a GGTase-I CaaX motif

The petunia *CaM53* cDNA sequence predicts a 184 amino acid protein with an N-terminal 150 amino acid domain that is homologous to other CaM proteins (Figure 1A). The CaM domain of CaM53 is identical to CaM81, a second petunia CaM, although both proteins are encoded by different genes. The CaM domain of CaM53 is also very similar to other CaMs in *Arabidopsis* (AtCaM2, two conservative substitutions in EF-hand IV), rice (OsCaM61, all conservative amino acid substitutions except at position 11 proximal to EF-hand I), human (HsCaM, with only one non-conservative substitution in EF-hand IV) and yeast (ScCMD1, eight non-conservative substitutions).

CaM53 is a novel type of CaM, however, that has a C-terminal extension of 34 amino acids which is rich in lysine and arginine (Figure 1B). The last four amino acids in this domain are CTIL, which comprise a functional CaaX-box substrate for yeast GGTase-I (Caplin *et al.*, 1994). We confirmed that *CaM53* was not a hybrid cDNA produced during cloning because the identical sequence could be obtained by RT-PCR with petunia flower total RNA (data not shown). In addition, CaM61 from rice also contains a similar lysine/arginine-rich C-terminal domain ending in the CaaX-box sequence CVIL (Figure 1A). Finally, polyclonal antibodies raised against the C-terminal domain of CaM53 cross-react with 22 kDa proteins from petunia and potato, and a 24 kDa protein from tomato

Fig. 1. CaM53 is a novel type of CaM. (A) Comparison of *Petunia hybrida* CaM53 with other CaM proteins. Some restriction enzyme sites in the cDNA molecule are marked. The four conserved Ca²⁺-binding EF-hands (present in all CaMs) are indicated I–IV on the CaM53 CaM domain. The positions of arginine and lysine residues in the basic domain are marked by white and black circles, respectively. The CaaX box sequences are highlighted at the C-terminus. Numbers indicate the percentage of similarity (and identity) of the corresponding regions of rice (*Oryza sativa*) CaM61 (DDBJ/EMBL/GenBank accession No. U37936), *Phybrida* CaM81 (M80836), *Arabidopsis thaliana* CaM2 (M38380), and human (*Homo sapiens*) and yeast (*Saccharomyces cerevisiae*) CaMs (M19311 and M14760, respectively) with those in CaM53 (M80831). (B) Amino acid sequence of the C-terminal polybasic domain of CaM53. Basic residues are indicated in bold, and the CaaX box motif is underlined. (C) RNA blot analysis of petunia total RNA from roots (R), stems (S), mature leaves (ML), young leaves (YL), flower buds (FB) and flowers (F). The blot was hybridized with probes corresponding to the CaM53 basic domain and CaM domain, and with an 18S rRNA probe to compare the RNA amounts loaded in each lane.

(Figure 2A), suggesting that this novel type of CaM is present widely in plants.

CaM53 transcripts were detected in all petunia tissues with a *CaM53*-specific cDNA probe corresponding to the C-terminal basic domain, but they were most abundant in stems, mature flowers and roots. Lower levels of *CaM53* mRNAs were detected in young and expanded leaves and flower buds (Figure 1C). A cDNA probe from the CaM domain of *CaM53* shows a different pattern that most likely includes highly homologous transcripts from *CaM81* and other putative petunia *CaM* genes as well. Together,

Fig. 2. CaM53 can function as a CaM. (A) Immunoblots with an antibody against the CaM53 basic domain. The migration of protein size standards (kDa) is indicated. Left panel: protein extracts from the shoot apical region of *Arabidopsis thaliana* and the Solanaceae plants tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*) and petunia. Right panel: protein extracts from petunia 15-day-old whole seedlings, incubated in either 1 mM CaCl₂ or 2 mM EGTA before SDS-PAGE. (B) Complementation of *cmd1Δ* yeast with *CaM53*. Yeast strain TDY9-37D (with the *CaM CMD1* gene in a plasmid with the *URA3* marker) was transformed with plasmids expressing CaM53 with either a CTIL (pJR-Cwt) or an STIL (pJR-CmS) C-terminal sequence, or with the plasmid without an insert (pJR1138). Transformants were plated on selective medium containing 5-fluoro-orotic acid (5-FOA) and Ura to select against the presence of *CMD1*. Replica plates were grown at 30 and 37°C. (C) Activation of petunia glutamate decarboxylase (GAD) by petunia CaMs. GAD was purified and its activity determined as described in Materials and methods. The presence (+) or absence (-) of Ca²⁺, CaM81 or CaM53 are indicated. Values represent mean and standard error of 3–5 replicates.

these results confirm that *CaM53* encodes a novel CaM-like protein that is expressed widely in plants.

CaM53 is a functional calmodulin

The mobility of CaM53 in SDS-PAGE is altered by addition of Ca²⁺ (Figure 2A), a shift that is characteristic of CaM and other EF-hand proteins (Burgess *et al.*, 1980). To confirm that CaM53 can function as CaM, we expressed the protein in the *Saccharomyces cerevisiae cmd1Δ* mutant

in which the single gene for CaM is disrupted (Davis *et al.*, 1986). Disruption of *CMD1* is lethal, but it is rescued in the yeast strain TDY9-37D by expression of *CMD1* from plasmid pTD17 containing the *URA3* marker. TDY9-37D cells were transformed with an expression vector to express either wild-type CaM53 or CaM53mS, in which cysteine in CTIL was mutated to serine and thus cannot serve as a substrate for GGTase-I. Expression of both full-length CaM53 and CaM53mS was confirmed by immunoblot analysis with the anti-CaM53 antibody (data not shown). Transformants were plated on 5-fluoro-orotic acid (5-FOA) to select for cells in which pTD17 was lost. Figure 2B shows that only transformants expressing either CaM53 or CaM53mS grew at 30 and 37°C, indicating that *CaM53* can complement the *cmd1Δ* mutant independent of the presence of a functional CaaX motif in the protein.

CaM activity of CaM53 was also confirmed by assaying the ability of the protein to activate petunia GAD. Addition of CaM53 activated GAD >20-fold in a Ca²⁺-dependent manner (Figure 2C), consistent with its activation by other CaMs (Snedden *et al.*, 1996). Activation of GAD by CaM53 was less pronounced compared with CaM81, suggesting that the basic domain might affect the interaction between CaM53 and GAD. We cannot exclude, however, that the reduced activity could be the result of different amounts of active protein in the purified fractions used in the enzyme assays. Together, the results demonstrate that CaM53 functions as a CaM, thus establishing it as a member of a new family of CaMs with a basic C-terminal domain and a GGTase-I prenylation motif.

CaM53 is a substrate for prenylation by plant GGTase-I

The CaaX-box sequence CTIL is present in proteins that are prenylated efficiently by yeast GGTase-I (Caplin *et al.*, 1994). We used CaM53 expressed in *Escherichia coli* for *in vitro* prenylation assays to determine whether CTIL can function as a substrate for plant protein prenyltransferases. Figure 3A shows that full-length CaM53, but not CaM53mS, was an efficient substrate for prenylation by purified recombinant plant GGTase-I. To confirm this result, the C-terminal 84 amino acids or the CTIL sequence of CaM53 were fused to GST, producing GST-PCaM53 and GST-CTIL. As controls, we constructed the corresponding mutant proteins, GST-PCaM53mS and GST-STIL. Both GST-PCaM53 and GST-CTIL were substrates for GGTase-I, but were prenylated less efficiently by GGTase-I when FPP was used as the prenyl group donor (Figure 3A). These GST fusion proteins were only poor substrates for purified recombinant plant FTase, confirming that the CTIL motif is specific for GGTase-I. No prenylation was detected with the STIL mutant proteins, establishing that prenylation of CaM53 depends on the functional CTIL motif. In addition, the prenylation of GST-CTIL indicates that the CaaX box sequence is sufficient for prenylation by GGTase-I, although upstream amino acid sequences are likely to be required for a more efficient prenylation of the protein, as suggested by the relative intensity of the fluorography bands in Figure 3A.

To confirm that CaM53 is a substrate for GGTase-I *in vivo*, CaM53 and the mutant protein CaM53mS were expressed in *Nicotiana benthamiana* plants using a potato virus X (PVX) vector system (Chapman *et al.*, 1992).

Fig. 3. CaM53 is prenylated *in vitro* and *in vivo*. (**A**) Different wild-type (containing an intact CTIL CaaX box) and mS (mutated to STIL) versions of chimeric CaM53 were expressed in *E. coli* and used as substrates for plant protein prenyltransferases. Prenylation reactions were carried out with (1) 20 µg of total protein from bacteria expressing CaM53, (2) purified wt and mS versions of GST-PCaM53 (from the *Pst*I site of CaM53 cDNA fused to GST) or (3) purified GST-CTIL and GST-STIL. The reaction mix also contained [³H]GGPP or [³H]FPP and recombinant plant GGTase-I or FTase (symbols + and - indicate the presence or absence of these components). After electrophoresis and fluorography, exposure was carried out for 20 h. The migration of protein size standards (kDa) is indicated. (**B**) Leaf explants from 20 d.p.i. *N. benthamiana* plants inoculated with RNA from PVX, PVX-CaM53 or PVX-CaM53mS were fed with [³H]MVA and used for immunoblot analysis with a specific anti-CaM53 antiserum (left panel). Labeled proteins in the same extracts were detected by fluorography, exposing the film for 16 days (right panel). The migration of protein size standards (kDa) is indicated.

Infected leaves were detached from plants 20 days post-inoculation (d.p.i.) and incubated as explants in the presence of mevinolin (Alberts *et al.*, 1980) to inhibit HMG CoA reductase (HMGR) and to deplete cells of MVA, the precursor for the synthesis of FPP and GGPP (Goldstein and Brown, 1990). Subsequent addition of [³H]MVA resulted in strong labeling of CaM53 (Figure 3B). Plants transfected with PVX-CaM53mS accumulated a comparable level of CaM53mS, but no labeling of the protein was detected. Based on this result, we conclude that CaM53 is a substrate for GGTase-I in plants and that prenylation *in vivo* requires the intact CTIL sequence motif.

Ectopic expression of CaM53 and CaM53mS produces distinct phenotypes

All transfected plants ectopically expressing CaM53 were significantly altered in their growth morphology as com-

pared with plants infected with PVX alone or plants expressing CaM53mS (Figure 4A). At 40 d.p.i., CaM53 plants were severely stunted as a result of reduced internode length and stem thickening. Beginning at 35 d.p.i., leaves showed strong curling, chlorosis and extensive necrotic sectors (Figure 4B). Although growth effects were also visible in plants expressing CaM53mS (which appeared to be more bushy, probably because of reduced apical dominance), stunting and necrosis were not observed (Figure 4A). Since accumulation of CaM53 and CaM53mS appears to be similar in the transfected plants but only CaM53 is geranylgeranylated *in vivo*, it is likely that the difference in phenotype results from the prenylation status of the proteins.

It was also possible that the phenotype observed in CaM53 plants was caused by expressing high levels of a prenylated protein that interfered with normal cell and/or membrane functions. To exclude this possibility, *N. benthamiana* plants were transfected with PVX constructs designed to express sGFP, a soluble, codon-optimized synthetic green fluorescent protein (Chiu *et al.*, 1996), fused to either the entire basic domain (sGFP-BDCaM53) or only the CTIL sequence (sGFP-CTIL) of CaM53. Figure 4C shows that no apparent phenotype was observed in transfected plants expressing these proteins, confirming that the growth defects caused by ectopic expression of CaM53 resulted from the CaM activity of the prenylated protein.

CaM53 localizes to the plasma membrane, but CaM53mS is transported to the nucleus

Prenylated proteins generally are localized to cell membranes but remain soluble when the cysteine residue in the CaaX motif is mutated to serine (Casey *et al.*, 1989; Hancock *et al.*, 1991). To investigate the role of CaM53 prenylation in the subcellular localization of the protein, CaM53 and CaM53mS were fused to the C-terminus of sGFP and transiently expressed in transfected protoplasts (Figure 5). Most of the sGFP-CaM53 protein in protoplasts from tobacco XD cells or petunia leaves was found associated with the plasma membrane (Figure 5E and I). This localization was specific to sGFP-CaM53 because sGFP alone was distributed uniformly in the cytoplasm (Figure 5C). In contrast, protoplasts that expressed sGFP-CaM53mS showed intense nuclear GFP fluorescence, suggesting that failure to prenylate the protein results in localization of sGFP-CaM53mS to the nucleus (Figure 5G and J). A small number (<15%) of protoplasts expressing sGFP-CaM53 had low levels of fluorescence in the nucleus as well, most likely as the result of strong expression of sGFP-CaM53 that most probably exceeded the prenylation capacity of the cell, thus resulting in nuclear localization of unmodified protein.

Previous results showed that binding of myristoylated EF-hand proteins to membranes is dependent on their Ca²⁺-induced conformation (Ames *et al.*, 1997). To distinguish between a role for the CaM53 CaM and basic C-terminal domains in the subcellular localization of the protein, we constructed sGFP-SCaM53 and sGFP-SCaM53mS mutant proteins from which EF-hands I and II had been deleted. Figure 5 shows that this did not affect the fluorescence pattern generated by the proteins, indicating that the intact CaM domain is not required

Fig. 4. Growth alterations in plants ectopically expressing CaM53. (A) *Nicotiana benthamiana* plants 40 days after infection with transcripts from PVX, PVX-CaM53 or PVX-CaM53mS. (B) Magnification of the apical region of a 40 d.p.i. plant expressing CaM53. The arrow indicates necrotic tissue. (C) Plants at 40 d.p.i. with PVX constructs expressing sGFP-CTIL (with only the CTIL sequence fused to sGFP) or sGFP-BDCaM53 (with the entire basic domain of CaM53 fused to sGFP).

for subcellular localization. Similarly, incubation of the transfected protoplasts in CaCl_2 or EGTA did not change the fluorescence pattern (data not shown), suggesting that Ca^{2+} -induced changes in the conformation of the CaM domain are probably not required for membrane and nuclear localization of CaM53 and CaM53mS, respectively. It appears, therefore, that prenylation of CaM53 functions to retain the plasma membrane association of the protein.

The CaM53 basic C-terminal domain is required for both plasma membrane and nuclear localization

The CTIL sequence is sufficient to direct prenylation by GGTase-I (Figure 3A), although addition of this sequence alone to sGFP produced a fluorescence pattern in transfected protoplasts typical of unmodified sGFP (Figure 5). To establish the role of the CaM53 C-terminal domain in membrane targeting of the prenylated protein *in vivo*, we determined the localization of sGFP-BDCaM53 and sGFP-CTIL expressed in PVX-infected *N.benthamiana* leaves. Figure 6 shows that the fluorescence of sGFP-BDCaM53 was restricted to the periphery of most epidermis cells, consistent with a plasma membrane association of the protein. Interestingly, in trichomes and a small number of epidermal and stomata cells, fluorescence was also detected in the nucleus (Figure 6C and D). In contrast, fluorescence of sGFP-CTIL was not localized specifically to the plasma membrane or the nucleus of leaf stomata, epidermal cells or trichomes (Figure 6A and B). This is consistent with the results from protoplasts, in which most of the sGFP-CTIL fusion protein accumulates in the cytoplasm (Figure 5D). Together, the results suggest that the GGTase-I CaaX-box motif itself is not sufficient to direct sGFP to the plasma membrane, either as a result of inefficient prenylation *in vivo* or because additional proximal sequences in the basic C-terminal domain of CaM53 are required for plasma membrane targeting. Thus, the plasma membrane localization of CaM53 is dependent on both the C-terminal lysine/arginine-rich domain and a functional CTIL sequence.

To confirm the subcellular localization of CaM53 and CaM53mS observed by sGFP fluorescence, extracts from PVX-transfected *N.benthamiana* leaves expressing the full-length proteins were separated into nuclear and extra-nuclear fractions. Figure 7A shows that while nearly all CaM53 was found in the extra-nuclear fraction, a sig-

nificant amount of CaM53mS partitioned into the fraction enriched in nuclei. A parallel fractionation to separate soluble and membrane fractions confirmed that CaM53 is associated with the membrane fraction but, as expected, CaM53mS is a soluble protein. These cell fractionation results are consistent with the fluorescence observed from the sGFP-CaM53 and sGFP-CaM53mS proteins and confirm that nuclear localization of CaM53 is facilitated by the basic domain in the absence of the prenyl modification. Taking these results together, we conclude that the prenylation status of CaM53 in conjunction with the basic C-terminal domain determines the subcellular localization of the protein.

Nuclear localization of CaM53 can be induced by mevinolin and dark-shift

The different subcellular localization of CaM53 could be regulated by the cellular concentration of GGPP, in which case CaM53 could function as a sensor of cytoplasmic isoprenoid biosynthesis. To investigate this possibility, we determined the localization of the endogenous CaM53 in petunia seedlings as a function of isoprenoid synthesis, using the specific inhibitor mevinolin to block HMGR *in vivo*. This approach required rapid separation of the total seedling extract into nuclear and extra-nuclear fractions because CaM53 is unstable in protein extracts even in the presence of proteinase inhibitors (data not shown). In control seedlings, most of the CaM53 was localized in the extra-nuclear fraction (Figure 7B). In contrast, a significantly higher level of CaM53 was detectable in the nuclear fraction of mevinolin-treated seedlings, demonstrating that inhibition of MVA synthesis is sufficient to direct the endogenous protein to the nucleus. Based on these results, we can also exclude that the nuclear localization of CaM53mS in transfected protoplasts (Figure 5) and *N.benthamiana* plants (Figure 7A) is simply a consequence of ectopic expression of high levels of this protein.

To test whether prenylation and, therefore, localization of CaM53 could be a function of the metabolic status of plant cells, we subjected leaves from *N.benthamiana* plants expressing the reporter sGFP-BDCaM53 protein to a light-dark regime. As expected from previous results, Figure 8 shows that sGFP-BDCaM53 localizes to the plasma membrane of epidermis cells from leaf explants exposed to light for 3 days, except in some patches of a

Fig. 5. Subcellular localization of sGFP-CaM53 in protoplasts. Different sGFP-CaM53 fusion proteins were detected by green fluorescence using CLSM in protoplasts 24 h after transfection. Bars correspond to 5 μ m in (G) and 10 μ m in the rest. (A) Bright field image of a typical tobacco XD protoplast. (B) A tobacco XD protoplast stained with DAPI to show the localization of the nucleus. (C–H) Tobacco XD protoplasts expressing sGFP (C), sGFP-CTIL (D), sGFP-CaM53 (E), sGFP-SCaM53 (with only CaM53 EF-hands III and IV, from the *SacI* site, fused to sGFP) (F), sGFP-CaM53mS (G, region around the nucleus) or sGFP-SCaM53mS (H). (I) and (J) Petunia leaf protoplasts expressing sGFP-CaM53 (I) or sGFP-CaM53mS (J). Red fluorescence corresponds to chloroplasts.

few cells in which green fluorescence was detected in the nucleus as well (Figure 8A and B). In contrast, incubation of leaves in the dark for 3 days resulted in nuclear localization of sGFP-BDCaM53 (Figure 8C), suggesting that the protein fails to become prenylated under these conditions. However, dark exposure of the leaf explants on a medium containing 2% sucrose prevented nuclear localization of sGFP-BDCaM53 (Figure 8D), suggesting that in the presence of the carbon source isoprenoid synthesis and prenylation of the protein are maintained. Together, the results suggest that the CaM53 C-terminal basic domain together with the CTIL CaaX-box can direct

Fig. 6. The CaM53 basic domain is important for subcellular localization in plants. Leaves from the *N.benthamiana* plants shown in Figure 4C were observed directly using CSLM. (A and B) Plant expressing sGFP-CTIL. (A) Leaf epidermis; (B) trichome. (C and D) Plant expressing sGFP-BDCaM53. (C) Leaf epidermis; the arrows indicate the position of nuclei. (D) Trichome. Bars indicate 10 μ m.

the subcellular localization of CaM53 as a function of the metabolic status of plant cells.

Discussion

In this report, we demonstrate that CaM53 is a novel type of plant CaM with an extended polybasic C-terminal domain and a CaaX-box motif which is modified by GGTase-I prenylation to target the protein to the plasma membrane. Our studies show that CaM53 binds Ca^{2+} , activates GAD (a known CaM target in plants) and complements the lethal yeast *cmd1Δ* mutant, confirming that the protein functions as a typical CaM. These results explain previous observations of membrane-associated CaM in plants (Collinge and Trewavas, 1989; Fraichard *et al.*, 1996). The presence of the extended polybasic C-terminal domain in CaM53 is intriguing because it establishes a novel mechanism by which this CaM can be relocated to the nucleus in the absence of the prenyl group. Although the specific target proteins for CaM53 remain to be identified, our results clearly show that this novel type of CaM probably functions in specific Ca^{2+} -activated pathways either at the plasma membrane or in the nucleus, depending on the metabolic status of the cell and the prenylation of the protein.

Fig. 7. Subcellular localization of CaM53 in plants. **(A)** Leaf tissue from 20 d.p.i. *N.benthamiana* plants expressing either the wt or the mS version of CaM53 were used for fractionation of the cell components (Materials and methods). Equivalent volumes of each fraction were loaded on a 12.5% SDS–polyacrylamide gel. Immunoblot analysis was carried out with specific antibodies against CaM53 and the tomato nuclear protein MSI1. Upper panels: nuclear fractionation. T, total; E, extra-nuclear; N, nuclear. Lower panels: membrane fractionation. T, total; S, S100 (soluble); P, P100 (insoluble). **(B)** Two-week-old petunia seedlings were incubated for 24 h either with or without mevinolin (an inhibitor of the synthesis of MVA, the precursor for the production of FPP and GGPP). Nuclear fractionation was carried out afterwards as described in Materials and methods. Immunoblot analysis of the isolated fractions was carried out with specific antibodies against CaM53 and the tomato nuclear protein MSI1. T, total; E, extra-nuclear; N, nuclear.

The CaM53 C-terminal domain is required for efficient prenylation and subcellular targeting

To demonstrate that CaM53 is prenylated *in vivo*, we used a PVX vector system for ectopic expression of the protein. This approach established conditions under which wild-type CaM53, but not the CaaX box mutant protein, was labeled efficiently *in vivo*. Based on the CTIL sequence and *in vitro* labeling experiments shown in Figure 3A, we expect that CaM53 is modified by geranylgeranylation *in vivo* as well, although we did not analyze the functional group directly. Our results indicate that the CTIL sequence can also be prenylated by FTase with low efficiency, consistent with previously reported cross-specificity of FTase and GGTase-I for both prenyl and protein substrates (Moores *et al.*, 1991; Caplin *et al.*, 1994; Yalovsky *et al.*, 1997). Although the CTIL sequence was sufficient for prenylation by FTase and GGTase-I, *in vitro* geranylgeranylation was more efficient when either a C-terminal

Fig. 8. Distribution of sGFP-BDCaM53 in leaf explants. Leaf explants from the *N.benthamiana* plants shown in Figure 4C were observed using CSLM after incubation for 72 h in the following conditions: **(A)** light, 1× MS medium with 2% mannitol; **(B)** light, 1× MS medium with 2% sucrose; **(C)** dark, 1× MS medium with 2% mannitol; **(D)** dark, 1× MS medium with 2% sucrose. Green fluorescence corresponds to sGFP-BDCaM53, and red fluorescence corresponds to chloroplasts. Bars correspond to 100 μm.

fragment or the full-length CaM53 protein were used as substrates, based on the relative intensity of the fluorography bands (Figure 3A). Kinetic studies showed that the affinity of purified plant GGTase-I for GST-PCaM53 is ~10-fold higher than for GST-CTIL *in vitro* (D.Caldelari and W.Gruissem, unpublished data). Thus, the CTIL-proximal polybasic sequence increases the affinity of CaM53 or the GFP fusion proteins for GGTase-I, as was demonstrated for a similar basic domain in K-rasB (James *et al.*, 1995). We cannot exclude, however, that the CaM domain itself affects the interaction between the CTIL substrate and the active site in GGTase-I. Our results also show that the CTIL sequence alone is not sufficient for efficient *in vivo* membrane targeting (Figures 5 and 6). Most of the ectopically expressed sGFP-CTIL protein remains in the cytoplasm, although the fusion protein can be prenylated *in vitro*. Addition of the C-terminal polybasic domain in sGFP-BDCaM53 targets most of the protein to the plasma membrane. This is similar to results previously reported for K-rasB (Hancock *et al.*, 1990), suggesting that the cluster of lysine and arginine residues in the

C-terminal domain facilitates interactions between the CaaX-box domain and GGTase-I as well as the plasma membrane through electrostatic interactions with acidic phospholipids.

In the absence of prenylation, however, CaM53 and the GFP fusion proteins containing the CaM53 C-terminal polybasic domain localize to the nucleus, although the proteins do not contain consensus nuclear localization sequences. Nuclear transport most probably is facilitated by the lysine/arginine-rich sequence in the C-terminal basic domain, as has been demonstrated for other proteins (Gorlich and Matta, 1996). Therefore, the C-terminal domain appears to have a dual role in efficient prenylation and subcellular localization. Unlike methylation of CaM which can affect its interaction with target proteins (Roberts *et al.*, 1992), our preliminary results indicate that prenylation of CaM53 does not significantly affect its ability to activate GAD *in vitro* (data not shown). This does not exclude, however, a function for the prenylated polybasic C-terminal domain in modulating the activity of CaM53 or its interaction with other proteins *in vivo*, as was found for Ras in animal cells and yeast where farnesylation of the proteins facilitates interactions with guanine nucleotide exchange factor (Marshall, 1993; Porfiri *et al.*, 1994) or adenylyl cyclase (Kuroda *et al.*, 1993).

Differential localization of CaM53 causes specific morphogenetic effects

The growth defects in plants ectopically expressing prenylated CaM53 resulted from alterations in the level and/or activity of the membrane-associated CaM rather than effects on membrane function caused by high concentrations of a prenylated protein. This was confirmed using the sGFP-BDCaM53 chimeric protein which was prenylated efficiently *in vitro* and localized to the plasma membrane *in vivo* (Figure 6), but did not result in visible phenotypic changes when ectopically expressed from the PVX vector (Figure 4). Responses to several stimuli in plants, including mechanical perturbations, light and pathogen attack, are tightly associated with rapid influxes of Ca^{2+} through plasma membrane-localized Ca^{2+} channels, some of which are stimulated by CaM (Kurosaki *et al.*, 1994; Bush, 1995; Askerlund *et al.*, 1997; Zimmermann *et al.*, 1997; Xu and Heath, 1998). CaM plasma membrane targets also include other ion channels, receptors, proton pumps and other components of signal transduction located in or near the plasma membrane. Co-localization of regulatory components on a two-dimensional surface may enhance their interactions by at least a factor of 10^6 as a result of the high local concentrations of proteins tethered to membranes (Gelb, 1997). We expect that overexpression of CaM53 changes the local concentrations of membrane-bound CaM, thus disrupting the normal balance of Ca^{2+} sensors and target proteins that could explain the observed alteration of normal morphogenetic responses. Previous reports have shown that disruption of CaM and target protein interactions produces developmental phenotypes similar to those resulting from ectopic expression of CaM53. For example, overexpression of VU-3 (a CaM mutant in Lys115 which cannot be methylated) in transgenic tobacco is associated with hyperactivation of NAD kinase and an enhanced production of active oxygen

species, and causes stunted growth (Roberts *et al.*, 1992). Similarly, ectopic expression of GADAC (a petunia mutant GAD from which the C-terminal CaM-binding domain was deleted) in tobacco also causes stunted growth (Baum *et al.*, 1996). Alternatively, the phenotype observed in plants expressing plasma membrane-bound prenylated CaM53 may be caused by the loss of regulation of CaM53-specific signaling pathways, as we will discuss below.

The strikingly different effect on plant growth caused by ectopic expression of CaM53mS (Figure 4) clearly results from the constitutive nuclear localization of the non-prenylated protein (Figure 7A) where it most probably interacts with different target proteins. It is well established for plants that signals which alter cytoplasmic Ca^{2+} concentrations also elicit changes in gene expression, although the precise mechanisms of this control are still unknown. It has been shown, however, that CaM3, a specific *Arabidopsis* CaM isoform, can bind the transcription factor TGA3 and enhances its interaction with a specific regulatory element in the *Cam-3* promoter *in vitro* (Szymanski *et al.*, 1996). Although most nuclear proteins whose activities are regulated by Ca^{2+} /CaM are unknown, we would expect that constitutive ectopic expression of CaM53mS will affect interactions with target proteins in the nucleus, thus causing altered morphogenetic responses. Together, our experiments provide the first evidence that plants may control the temporal and spatial activity of specific Ca^{2+} signaling pathways by prenylation of novel CaMs.

CaM53 subcellular localization is cell specific and can be regulated by changes in metabolic activities

The plasma membrane or nuclear localization of sGFP-BDCaM53 in transfected plants is cell- rather than tissue-dependent (Figures 6 and 8), suggesting that cell-specific controls regulate prenylation and subcellular localization of CaM53. Although protein prenylation has now been established as a conserved modification in many eukaryotic cells (reviewed in Rodriguez-Concepcion *et al.*, 1999), the cellular mechanisms that control the prenylation status of proteins and the activities of prenyltransferases are not well understood. Our results show that a block in MVA synthesis in petunia seedlings does not significantly affect the synthesis or stability of CaM53, but results in nuclear localization of the protein (Figure 7). Thus, the plasma membrane or nuclear localization of CaM53 may be part of a signal transduction pathway that reports the activities of HMG CoA reductase enzymes which are tightly controlled in plants (Gillaspy *et al.*, 1993; Rodriguez-Concepcion and Gruissem, 1999; Jelesko *et al.*, 1999, and references therein). Similarly, farnesylation and membrane association of Ras recently was reported to be controlled by the level of isoprenoid synthesis in cultured cardiac myocytes (Gadbut *et al.*, 1997), thus providing additional support for cross-talk between this biochemical pathway and prenylated regulatory proteins.

The shift to nuclear localization of CaM53 is reminiscent of the recently reported translocation of human CaM to the nucleus in hippocampal neurons (Deisseroth *et al.*, 1998). This translocation follows a localized rise in Ca^{2+} near the cell plasma membrane and results in the phosphorylation of CREB by CaMKIV, a nuclear CaM-

dependent kinase. The mechanism of translocation into the nucleus is not known, but may include facilitated translocation of CaM by association with another protein (Deisseroth *et al.*, 1998). Although a similar mechanism cannot be excluded for non-prenylated CaM53 in plant cells, we have found that translocation into the nucleus is not likely to be dependent on Ca^{2+} (Figure 5) and that the polybasic C-terminal domain may be sufficient for this translocation. Although reversible membrane association of geranylgeranylated Rab proteins and farnesylated rhodopsin kinase is part of their mode of regulation (Inglese *et al.*, 1992b; Pfeffer *et al.*, 1995), our results currently do not distinguish between relocation of prenylated plasma membrane-bound CaM53 or transport of newly synthesized but non-prenylated CaM53 to the nucleus. They suggest, however, that irrespective of the underlying mechanism, CaM53 targeting is a novel control by which plant cells may differentially activate Ca^{2+} -dependent signaling pathways.

A role for CaM53 in sugar-sensing and sugar-mediated signal transduction?

The localization of sGFP-BDCaM53 in cells of leaf explants can be manipulated by light and sucrose (Figure 8), suggesting that localization of CaM53 could be controlled by the sugar status of the cell. Sugar-sensing and sugar-mediated signal transduction in plants are cell-autonomous processes (Smeekens and Rook, 1997), and this may also explain the cell type-specific localization of ectopically expressed sGFP-BDCaM53 (Figures 6 and 8). The high levels of *CaM53* transcripts in assimilate sinks such as stems, roots and flowers also suggest a role for CaM53 in sugar-sensing signal transduction pathways. Moreover, the morphological changes in *N.benthamiana* plants expressing CaM53 are strikingly similar to phenotypes of transgenic plants in which sugar regulation has been altered. For example, accumulation of high levels of carbohydrates in leaf cells induced by ectopic expression of either apoplastic invertase (von Schaewen *et al.*, 1990; Dickinson *et al.*, 1991; Heineke *et al.*, 1992) or cytosolic pyrophosphatase (Sonnewald, 1992; Lerchl *et al.*, 1995), or by reduced expression of sucrose transporter (Riesmeier *et al.*, 1994) caused stunted growth, decreased stem internode length, and caused leaf curling, chlorosis and necrosis. Since ectopic expression of CaM53mS does not induce the same morphogenetic alterations, we suggest that high levels of prenylated CaM53 in the plasma membrane mimic a situation of increased sugar accumulation in leaf cells.

The mechanisms of sugar sensing in plants are not well understood, although it has been established that Ca^{2+} and CaM are involved in the transduction of the signal initiated by activated sugar sensors (reviewed in Smeekens and Rook, 1997). In tobacco leaf cells, cytoplasmic Ca^{2+} increases during incubation with sugars, and it has been suggested that a plasma membrane-associated sugar-inducible Ca^{2+} -dependent Ser/Thr protein kinase could regulate the activity of sugar transporters in the plasma membrane (Ohto and Nakamura, 1995). In addition, the expression of a CaM-like protein was induced in assimilate-accumulating transgenic tobacco (Herbers *et al.*, 1995), reinforcing the role of Ca^{2+} signaling in sugar sensing. Plant homologs of the yeast SNF1 Ser/Thr kinase, which

has a central role in the regulation of glucose repression of transcription, can also phosphorylate plant HMGR, resulting in inactivation of the enzyme (reviewed in Halford and Hardie, 1998). Control of HMGR activity by sugar-regulated SNF1-like kinases would alter flux through the isoprenoid pathway and, therefore, affect the subcellular localization of CaM53. The different subcellular localization of sGFP-BDCaM53 in dark-incubated leaf explants in the absence and presence of sucrose (Figure 8) is consistent with such a model, although further experimental work is necessary to establish a role for CaM53 in sugar sensing and signaling. Taking all these aspects together, we suggest that CaM53 is an example of a novel prenylated CaM which can coordinate the metabolic activity of plant cells with Ca^{2+} -activated pathways in the plasma membrane and the nucleus.

Materials and methods

RNA analysis

Isolation of total RNA from petunia tissues and hybridizations were carried out as described (Rodríguez-Concepción and Gruissem, 1999). *CaM53* probes were prepared from cDNA fragments corresponding to either the CaM domain (obtained by cleavage with *Bgl*II and *Msc*I) or the basic domain (*Msc*I–*Bam*HI fragment, containing the entire cDNA region encoding the basic domain and the 3'-untranslated region). Purified restriction fragments were labeled randomly with [^{32}P]dCTP using the Stratagene Prime-It kit.

Glutamate decarboxylase activity and calmodulin mobility shift assays

Recombinant petunia proteins GAD, CaM81 and CaM53 were expressed in *E.coli* and purified essentially as described (Fromm and Chua, 1992; Snedden *et al.*, 1996). Activity was measured in a 1,3-bis-Tris-propane-HCl buffer, pH 7.3, as described (Snedden *et al.*, 1996), except that all reactions contained 0.25 mM EGTA. Ca^{2+} (as CaCl_2) and CaM were added, where indicated, to concentrations of 1 mM and 50 nM, respectively.

Two-week-old petunia seedlings obtained as described below were homogenized in 100 mM Tris-HCl pH 7.8, 10 mM β -mercaptoethanol and 1× PIC [proteinase inhibitors cocktail, from a 100× ethanol stock containing 17 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.16 mg/ml benzamidine and 0.1 mg/ml 1,10-phenanthroline, and a 100× water stock with 0.05 mg/ml antipain, aprotinin, pepstatin and leupeptin] on ice. Samples were incubated for 2 min either in 1 mM CaCl_2 or in 2 mM EGTA, and then denatured by boiling in 1× SDS loading buffer (Laemmli, 1970), and loaded on a 12.5% SDS-PAGE for electrophoresis. CaM53 proteins were detected by immunoblot analysis as described below.

Antibody production and immunoblot analysis

A synthetic peptide of 36 amino acids corresponding to the CaM53 C-terminal basic domain (from Asn148 to Leu183) was conjugated to a carrier protein (keyhole limpet hemocyanin; KLH) and injected (three injections of ~100 μg peptide per injection) into rabbits. The rabbits were bled ~10 days after each injection. After removal of the clot, the serum was divided into aliquots and stored at -70°C .

Immunoblot analyses were carried out as described (Yalovsky *et al.*, 1997), with the following modifications. Unless otherwise stated, plant tissue was homogenized directly in 1× SDS loading buffer (Laemmli, 1970) at 1 mg/ μl , and boiled immediately for 5 min. Electrophoresed proteins were transferred to nitrocellulose membranes (Protran BA83, Schleicher & Schuell), which were rinsed immediately in phosphate-buffered saline (PBS; 50 mM sodium phosphate pH 7.2, 150 mM NaCl) for 10 min and incubated in PBS containing 0.4% (v/v) glutaraldehyde for 45 min with shaking at room temperature. After two rinses for 10 min in PBS, immunodetections were carried out using the polyclonal antibodies raised against CaM53 and against the tomato nuclear protein MSII (Ach *et al.*, 1997) at a dilution of 1:1000.

Bacterial vectors and protein expression

The 0.75 kb *CaM53* cDNA (DDBJ/EMBL/GenBank accession No. M80831) was cloned into the *Nde*I and *Bam*HI sites of the pET3b

expression vector, under the control of the T7 promoter, to create pET-Cwt. A fragment of *CaM53* was amplified by PCR using pET-Cwt as a template and the primer PCM (5'-TAGGAATCTCTGCAGCTG-AGCTTCGCC-3', which anneals overlapping the *Pst*I site of *CaM53* cDNA, Figure 1A), together with either RCMwt (5'-CCTCGAGTGG-ATCACAGTATTGTGCATCGATCTCTC-3', for the wild-type version of the CaaX box, CTIL), or RCMmS (5'-CCTCGAGTGGATCACAGTATTGTGCTTCGATCTCTC-3', for the mS version, STIL). The wild-type and mS PCR fragments were subcloned into pGEX-4T-1 (Pharmacia) at *Eco*RI-*Xho*I sites. The resulting constructs (pGEX-PCwt and pGEX-PCmS) contained an in-frame fusion of GST with the last 84 amino acid residues of the corresponding *CaM53* version: GST-PCaM53 and GST-PCaM53mS. The mS PCR fragment was also cloned in the pCR2.1 vector (Invitrogen) to create pCR-PCmS. This plasmid was digested with *Hinc*II and *Spe*I, and ligated to the *CaM53* *Hinc*II-*Xba*I fragment from pET-Cwt. The digestion of the new pCR-CmS with *Nde*I and *Xho*I released the full-length *CaM53mS* fragment, which was subcloned in the same sites of pET-Cwt to create pET-CmS, encoding *CaM53mS*.

The constructs to express GST with the additional four amino acid sequence CTIL or STIL were made using the following oligonucleotides: CMwt'E' (5'-AATTGTGCACAATACTGTGA-3') and CMwt'X' (5'-TCGATCACAGTATTGTGCAC-3') for CTIL, and CMm'E' (5'-AATTGTGCACAATACTGTGA-3') and CMm'X' (5'-TCGATCACAGTATTGTGCTTC-3') for STIL. The oligonucleotides 'E' and 'X' of each version (wt or mS) were mixed together, heated to 94°C for 5 min, and then left at room temperature for 3 h to anneal. The annealed oligonucleotides were ligated to the plasmid pGEX-4T-1, previously digested with *Eco*RI and *Xho*I, and gel-purified to create pGEX-CTIL and pGEX-STIL, encoding GST-CTIL and GST-STIL, respectively.

The full-length *CaM53* and *CaM53mS* proteins were expressed in *E. coli* strain BL21-DE3 cotransformed with pUBS520-argU (courtesy of R. Mattes, Stuttgart, Germany). The GST fusion proteins were expressed in *E. coli* XL1Blue and purified with glutathione-Sepharose 4B following the protocol supplied by Pharmacia.

Yeast complementation assay

The plasmids pET-Cwt and pET-CmS were digested with *Xba*I and *Xho*I, and the inserts released were directionally cloned into the *Spe*I and *Xho*I sites of pJR1138 for expression of the corresponding full-length *CaM53* versions in yeast. The plasmid pJR1138 (C.E. Trueblood, unpublished data) is identical to pJR1136 (Yalovsky *et al.*, 1997) but it contains the *LEU2* instead of the *TRP1* marker. The resulting plasmids pJR-Cwt and pJR-CmS were used to transform the *S. cerevisiae* strain TDY9-37D [*MATα ade5 his3-Δ200 leu2-3,112 trp1-901 ura3-52 lys2Δ::HIS3 cmd1Δ::TRP1* + pTD17 (high copy number, *URA3*, *CMD1*)], in which the only genomic *CaM* gene, *CMD1*, is disrupted by a functional *TRP1* gene. Following transformation, cells were plated on minimal medium supplemented with an amino acid mixture without Leu and Trp. To select for transformants in which pTD17 was substituted for the plasmid expressing *CaM53*, colonies were grown at 30 and 37°C on selective media with Ura and 5-FOA, a compound which is toxic for cells expressing the *URA3* gene (Boeke *et al.*, 1987).

In vitro prenylation

Prenylation reactions were carried out as described (Yalovsky *et al.*, 1997), with the following changes. A 20 µg aliquot of total cell protein (in the case of bacteria transformed with the constructs encoding full-length *CaM53*) or different amounts of purified GST fusion proteins were mixed with the prenylation buffer containing [³H]GGPP or [³H]FPP. Recombinant α and β subunits from *Arabidopsis thaliana* GGTase-I and tomato (*Lycopersicon esculentum*) FTase were co-expressed in the baculovirus system and purified to be used as the source for protein prenyltransferase activity (S. Yalovsky, D. Caldelari and W. Gruissem, unpublished data). The reaction mixtures were incubated for 30 min at 30°C and then denatured with SDS buffer and loaded in 10% SDS-PAGE (Laemmli, 1970). After electrophoresis, gels were either stained

with Brilliant Blue G (Sigma) or treated with a fluorographic reagent (Amplify, Amersham), dried and exposed to XAR film (Kodak).

GFP constructs and protoplast assays

A new version of the pRTL2-sGFP vector, which has the synthetic sGFP gene under the control of the 35S promoter (Chiu *et al.*, 1996), was constructed using a PCR-based approach. The new vector, pGFP-MRC, incorporated the multiple cloning site shown in Figure 9 to create C-terminal fusions with the sGFP protein.

The nearly full-length *CaM53* cDNA fragments released from pET-Cwt and pET-CmS after digestion with *Bgl*II (Figure 1A) and *Bam*HI were cloned in the same sites of pGFP-MRC, creating pGFP-Cwt and pGFP-CmS, to express the fusion proteins sGFP-*CaM53* and sGFP-*CaM53mS*, respectively. Similarly, *Sac*I-*Bam*HI fragments (Figure 1A) from pET-Cwt and pET-CmS were also cloned in pGFP-MRC to construct pGFP-SCwt and pGFP-SCmS, encoding sGFP-*SCaM53* and sGFP-*SCaM53mS*. A PCR fragment corresponding to the basic domain of *CaM53* was obtained using pET-Cwt as a template and the primers BCM (5'-CCAGGATCCCCATGATGGCCAATAGGAGG-3') and RCMwt (see above). This fragment was cloned in-frame with the sGFP gene to construct pGFP-BCwt, encoding the fusion protein sGFP-BDCaM53. A CTIL C-terminal sequence was fused to sGFP in a similar way to that described for GST-CTIL by ligating the annealed oligonucleotides CM'GFP' (5'-CTAGGTGCACAATACTGTGATCG-ATGCA-3') and CMwt'X' to the *Spe*I and *Nsi*I sites of pGFP-MRC.

Protoplasts were obtained as described from *Nicotiana tabacum* XD culture cells (McLean *et al.*, 1995) and from *Petunia hybrida* Mitchell young leaves (Jones *et al.*, 1990). After counting, protoplasts were diluted in electroporation buffer (McLean *et al.*, 1995) to a concentration of 2.5 × 10⁶ cells/ml. Transformation with the plasmids pGFP-MRC, pGFP-Cwt, pGFP-CmS, pGFP-SCwt, pGFP-SCmS and pGFP-CTIL was carried out by electroporation as described (McLean *et al.*, 1995). After incubation for 24 h at 25°C, protoplast green fluorescence corresponding to the fusion proteins was examined using a Nikon Optiphot/Molecular Dynamics Confocal Laser Scanning Microscope (CSLM). Excitation light at 488 nm and 514 nm was attenuated to 10% transmittance. From sample, light was passed through a 530 nm long-pass barrier filter and a 595 nm beam splitter. Detectors were set at 610 nm for chlorophyll and 530 nm for GFP fluorescence. Serial confocal sections (60× or 100× magnification, 1.5 µm thick, 256 × 256 pixels) were collected. Protoplast nuclei were visualized by staining the DNA with 20 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma). Images were exported as TIFF files and processed for printing using Adobe Photoshop.

Ectopic expression in plants and in vivo prenylation

For ectopic expression using the PVX vector, the plasmids pET-Cwt and pET-CmS were digested with *Xba*I and *Bam*HI, and the resulting full-length *CaM53* inserts were blunt-ended with Klenow, gel purified, and cloned in the *Eco*RV site of the PVX genome. Similarly, the inserts encoding sGFP-CTIL and sGFP-BDCaM53 were cloned in the *Eco*RV site after release from the corresponding pGFP plasmids by digestion with *Dra*I and *Fsp*I. The resulting plasmids were linearized with *Spe*I and *in vitro* transcribed. RNA was diluted 1:10 and inoculated in young greenhouse-grown *N. benthamiana* plants (with three or four leaves) as described (Chapman *et al.*, 1992). At least three plants were inoculated with each construct in three independent experiments. Protein expression was monitored by immunoblot or, in the case of the sGFP fusion proteins, by observing leaves directly by CSLM.

Leaves from *N. benthamiana* plants expressing the petunia *CaM53* protein were cut from the plant at 20 d.p.i. and immediately incubated as explants in MSM solution, containing 1 × MS salts (Gibco-BRL) and 25 µM mevinolin (a gift from Merck), at room temperature, with continuous light. After 7 h, each explant was transferred to a tube containing 50 µCi of [³H]mevalonolactone (ARL) in 50 µl of MSM and incubated at room temperature until no solution was left in the tube (1.5–2 h). At this point, 2 ml of fresh MSM solution was added to the

Fig. 9. The multiple cloning site, incorporated into the vector pGFP-MRC, to create COOH-terminal fusions with the sGFP protein.

tubes with the explants, and they were left at room temperature, with continuous illumination, for an additional 12 h. After *in vivo* labeling, 250 mg of each of the explants were homogenized in 250 µl of 1× SDS loading buffer (Laemmli, 1970) and boiled for 5 min. A 12.5% SDS–polyacrylamide gel was loaded with 12 µl of denatured proteins (corresponding to 12 µg of leaf tissue) for immunoblot analysis, and 50 µl for fluorography, as described above.

At 20 d.p.i., leaves expressing sGFP–BDCaM53 were taken from plants and incubated as explants in a solution containing 1× MS salts with 2% (w/v) mannitol or 2% (w/v) sucrose. Explants were incubated at 25°C in a growth chamber either in the dark or in continuous light for 72 h. Localization of fluorescence corresponding to the sGFP–BDCaM53 protein was observed directly by CSLM as described above.

Subcellular protein fractioning

Petunia seeds were germinated on filter paper soaked in water and incubated in Petri dishes for 15 days in a growth chamber. Eight grown seedlings were then transferred to plates with filter paper soaked either in 50 µM mevinolin or in water and were left in the light for another 24 h. All subsequent steps were done at 4°C, and all the fractions were mixed with protein loading buffer (Laemmli, 1970) and boiled immediately. The seedlings were homogenized in 50 µl of nuclei isolation buffer [NIB; 10 mM MES–KOH pH 6, 10 mM NaCl, 5 mM EDTA, 0.6% Triton X-100, 0.25 M sucrose and 1× proteinase inhibitor cocktail (PIC)] and filtered through two layers of Miracloth. A part of the crude homogenate was used as total cell extract. Nuclei were purified in a discontinuous NIB–Percoll gradient as described (Sakamoto and Nagatani, 1996). The upper phase of the gradient (extra-nuclear extract) was also collected. Absence of contamination in the nuclear fraction was analyzed as described (Sakamoto and Nagatani, 1996) by staining the preparation with DAPI to visualize the nuclei under a Zeiss Axiophot epifluorescence microscope.

Leaf tissue from 20 d.p.i. *N.benthamiana* plants expressing either CaM53 or CaM53mS was frozen and ground in liquid nitrogen to a fine powder with a pestle and mortar. All subsequent steps were done at 4°C. Part of this powder was used for nuclei isolation as described above. The rest of the powder was homogenized with TEB buffer (100 mM Tris–HCl pH 7.8, 5 mM EDTA, 10 mM β-mercaptoethanol and 1× PIC), vortexed with acid-washed silanized sand, and fractionated into soluble and insoluble cell fractions as described (Loraine *et al.*, 1996). A fraction of the extract was removed before centrifugation at 100 000 g and used as total cell extract. The pellet corresponding to the insoluble fraction was dissolved in TEB by sonicating twice for 2 s.

In both fractioning experiments, the volumes of each fraction (total, extra-nuclear and nuclear, or total, soluble and insoluble) were kept equal during the experiment. Following fractionation, equal volumes of the fractions were separated electrophoretically by 12.5% SDS–PAGE and analyzed by immunoblot.

Acknowledgements

We thank Drs D.Caldelari for the purified recombinant plant GGTase-I; T.Rubio for providing materials and advice on PVX infections; G.Baum for analyzing the CaM53 antibodies; J.Thorner and T.Davis for the TDY9-37D yeast strain; S.Ruzin and D.Schichnes for technical support on CSLM; P.Taranto for the anti-LeMS11 antibody; and Russell Jones and Sheng Luan for comments on the manuscript. We are also grateful to the greenhouse staff for the care of the plants, and the members of the Gruijssem lab for their help and advice. M.R.C. was supported by a postdoctoral fellowship from the Spanish M.E.C., and S.Y. by the U.C. Cancer Research Laboratory NIH training grant. This work was supported by DOE grant 85ER13375 to W.G.

References

Ach, R.A., Taranto, P. and Gruijssem, W. (1997) A conserved family of WD-40 proteins binds to the retinoblastoma protein in both plants and animals. *Plant Cell*, **9**, 1595–1606.

Alberts, A.W. *et al.* (1980) Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc. Natl Acad. Sci. USA*, **77**, 3957–3961.

Ames, J.B., Ishima, R., Tanaka, T., Gordon, J.I., Stryer, L. and Ikura, M. (1997) Molecular mechanics of calcium–myristoyl switches. *Nature*, **389**, 198–202.

Askerlund, P. (1997) Calmodulin-stimulated Ca^{2+} -ATPases in the

vacuolar and plasma membranes in cauliflower. *Plant Physiol.*, **114**, 999–1007.

Baum, G., Lev-Yadun, S., Fridmann, Y., Arazi, T., Katsnelson, H., Zik, M. and Fromm, H. (1996) Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. *EMBO J.*, **15**, 2988–2996.

Boeke, J.D., Trueheart, J., Natsoulis, G. and Fink, G.R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.*, **154**, 164–175.

Burgess, W.H., Jemiolo, D.K. and Kretsinger, R.H. (1980) Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta*, **623**, 257–270.

Bush, D.S. (1995) Calcium regulation in plant cells and its role in signaling. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **46**, 95–122.

Caplin, B.E., Hettich, L.A. and Marshall, M.S. (1994) Substrate characterization of the *Saccharomyces cerevisiae* protein farnesyltransferase and type-I protein geranylgeranyltransferase. *Biochim. Biophys. Acta*, **1205**, 39–48.

Casey, P.J., Solski, P.A., Der, C.J. and Buss, J.E. (1989) p21ras is modified by a farnesyl isoprenoid. *Proc. Natl Acad. Sci. USA*, **86**, 8323–8327.

Cates, C.A., Michael, R.L., Stayrook, K.R., Harvey, K.A., Burke, Y.D., Randall, S.K., Crowell, P.L. and Crowell, D.N. (1996) Prenylation of oncogenic human PTPCAAX protein tyrosine phosphatases. *Cancer Lett.*, **110**, 49–55.

Chapman, J., Kavanagh, T. and Baulcombe, D. (1992) Potato virus X as a vector for gene expression in plants. *Plant J.*, **2**, 549–557.

Chiu, W.-L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. *Curr. Biol.*, **6**, 325–330.

Clarke, S. (1992) Protein isoprenylation and methylation at carboxy-terminal cysteine residues. *Annu. Rev. Biochem.*, **61**, 355–386.

Collinge, M. and Trewavas, A.J. (1989) The location of calmodulin in the pea plasma membrane. *J. Biol. Chem.*, **264**, 8865–8872.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in *Arabidopsis*. *Science*, **273**, 1239–1241.

Davis, T.N., Urdea, M.S., Masiarz, F.R. and Thorner, J. (1986) Isolation of the yeast calmodulin gene: calmodulin is an essential protein. *Cell*, **47**, 423–431.

Deisseroth, K., Heist, E.K. and Tsien, R.W. (1998) Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature*, **392**, 198–202.

De Smedt, F., Boom, A., Pesesse, X., Schiffmann, S.N. and Erneux, C. (1996) Post-translational modification of human brain type I inositol-1,4,5-trisphosphate 5-phosphatase by farnesylation. *J. Biol. Chem.*, **271**, 10419–10424.

Dickinson, C.D., Altabella, T. and Chrispeels, M.J. (1991) Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiol.*, **95**, 420–425.

Finn, B.E. and Forsen, S. (1995) The evolving model of calmodulin structure, function and activation. *Structure*, **3**, 7–11.

Fraichard, A., Perotti, E., Gavin, O. and Chanson, A. (1996) Subcellular localization, distribution and expression of calmodulin in *Zea mays* roots. *Plant Sci.*, **118**, 157–165.

Fromm, H. and Chua, N.-H. (1992) Cloning of plant cDNAs encoding calmodulin-binding proteins using ^{35}S -labeled recombinant calmodulin as a probe. *Plant Mol. Biol. Rep.*, **10**, 199–206.

Gadbut, A.P., Wu, L., Tang, D., Papageorge, A., Watson, J.A. and Galper, J.B. (1997) Induction of the cholesterol metabolic pathway regulates the farnesylation of RAS in embryonic chick heart cells: a new role for Ras in regulating the expression of muscarinic receptors and G proteins. *EMBO J.*, **16**, 7250–7260.

Gelb, M. (1997) Protein prenylation, et cetera: signal transduction in two dimensions. *Science*, **275**, 1750–1751.

Gillaspy, G., Ben-David, H. and Gruijssem, W. (1993) Fruits: a developmental perspective. *Plant Cell*, **5**, 1439–1451.

Glomset, J.A. and Farnsworth, C.C. (1994) Role of protein modification reactions in programming interactions between ras-related GTPases and cell membranes. *Annu. Rev. Cell Biol.*, **10**, 181–205.

Goldstein, J.L. and Brown, M.S. (1990) Regulation of the mevalonate pathway. *Nature*, **343**, 425–430.

Gorlich, D. and Mattaj, W. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.

Halford, N.F. and Hardie, D.G. (1998) SNF1-related protein kinases: global regulators of carbon metabolism in plants? *Plant Mol. Biol.*, **37**, 735–748.

- Hancock, J.F., Paterson, H. and Marshall, C.J. (1990) A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell*, **63**, 133–139.
- Hancock, J.F., Cadwallader, K., Paterson, H. and Marshall, C.A. (1991) CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *EMBO J.*, **10**, 4033–4039.
- Heineke, D., Sonnewald, U., Bussis, D., Gunter, G., Leidreiter, K., Wilke, I., Raschke, K., Willmitzer, L. and Heldt, H.W. (1992) Apoplastic expression of yeast-derived invertase in potato. *Plant Physiol.*, **100**, 301–308.
- Herbers, K., Moenke, G., Badur, R. and Sonnewald, U. (1995) A simplified procedure for the subtractive cDNA cloning of photoassimilate-responding genes: isolation of cDNAs encoding a new class of pathogenesis-related proteins. *Plant Mol. Biol.*, **29**, 1027–1038.
- Inglese, J., Koch, W.J., Caron, M.G. and Lefkowitz, R.J. (1992a) Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature*, **359**, 147–150.
- Inglese, J., Koch, W.J., Caron, M.G. and Lefkowitz, R.J. (1992b) Isoprenylation of a protein kinase: requirement of farnesylation/ α -carboxyl methylation for full enzymatic activity of rhodopsin kinase. *J. Biol. Chem.*, **267**, 1422–1425.
- James, G.L., Goldstein, J.L. and Brown, M.S. (1995) Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic *in vitro*. *J. Biol. Chem.*, **270**, 6221–6226.
- Jesko, J.G., Jenkins, S.M., Rodriguez-Concepcion, M. and Gruissem, W. (1999) Regulation of tomato HMG1 during cell proliferation and growth. *Planta*, in press.
- Jones, R.W., Jackson, A.O. and Morris, T.J. (1990) Defective-interfering RNAs and elevated temperatures inhibit replication of tomato bushy stunt virus in inoculated protoplasts. *Virology*, **176**, 539–545.
- Kuroda, Y., Suzuki, N. and Kataoka, T. (1993) The effect of posttranslational modifications on the interaction of Ras2 with adenyl cyclase. *Science*, **259**, 683–686.
- Kurosaki, K., Kaburaki, H. and Nishi, A. (1994) Involvement of plasma membrane-located calmodulin in the response decay of cyclic nucleotide-gated cation channel of cultured carrot cells. *FEBS Lett.*, **340**, 193–196.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lerchl, J., Geigenberger, P., Stitt, M. and Sonnewald, U. (1995) Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants. *Plant Cell*, **7**, 259–270.
- Loraine, A.E., Yalovsky, S., Fabry, S. and Gruissem, W. (1996) Tomato Rab1A homologs as molecular tools for studying Rab geranylgeranyl transferase in plant cells. *Plant Physiol.*, **110**, 1337–1347.
- Marshall, C.J. (1993) Protein prenylation: a mediator of protein–protein interactions. *Science*, **259**, 1865–1866.
- McLean, B.G., Zupan, J. and Zambryski, P.C. (1995) Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco cells. *Plant Cell*, **7**, 2101–2114.
- Moores, S.L., Schaber, M.D., Mosser, S.D., Rands, E., O'Hara, M.B., Garsky, V.M., Marshall, M.S., Pompliano, D.L. and Gibbs, J.B. (1991) Sequence dependence of protein isoprenylation. *J. Biol. Chem.*, **266**, 14603–14610.
- Ohto, M.A. and Nakamura, K. (1995) Sugar-induced increase of calcium-dependent protein kinases associated with the plasma membrane in leaf tissues of tobacco. *Plant Physiol.*, **109**, 973–981.
- Pei, Z.M., Ghassemian, M., Kwak, C.M., McCourt, P. and Schroeder, J.I. (1998) Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. *Science*, **282**, 287–290.
- Pfeffer, S.R., Dirac-Svestrup, A.B. and Soldati, T. (1995) Rab GDP dissociation inhibitor: putting Rab GTPases in the right place. *J. Biol. Chem.*, **270**, 17057–17059.
- Porfiri, E., Evans, T., Chardin, P. and Hancock, J.F. (1994) Prenylation of ras proteins is required for efficient hSOS1-promoted guanine nucleotide exchange. *J. Biol. Chem.*, **269**, 22672–22677.
- Qian, D., Zhou, D., Ju, R., Cramer, C.L. and Yang, Z. (1996) Protein farnesyltransferase in plants: molecular characterization and involvement in cell cycle control. *Plant Cell*, **8**, 2381–2394.
- Riesmeier, J.W., Willmitzer, L. and Frommer, W.B. (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *EMBO J.*, **13**, 1–7.
- Roberts, D.M., Besl, L., Oh, S.-H., Masterson, R.V., Schell, J. and Stacey, G. (1992) Expression of a calmodulin methylation mutant affects the growth and development of transgenic tobacco plants. *Proc. Natl Acad. Sci. USA*, **89**, 8394–8398.
- Rodriguez-Concepcion, M. and Gruissem, W. (1999) Arachidonic acid alters tomato HMG expression and fruit growth, and induces HMGR-independent lycopene accumulation. *Plant Physiol.*, **119**, 41–48.
- Rodriguez-Concepcion, M., Yalovsky, S. and Gruissem, W. (1999) Protein prenylation in plants: old friends and new targets. *Plant Mol. Biol.*, in press.
- Sakamoto, K. and Nagatani, A. (1996) Nuclear localization activity of phytochrome B. *Plant J.*, **10**, 859–868.
- Schafer, W.R. and Rine, J. (1992) Protein prenylation: genes, enzymes, targets and functions. *Annu. Rev. Genet.*, **30**, 209–237.
- Smeekens, S. and Rook, F. (1997) Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiol.*, **115**, 7–13.
- Snedden, W.A. and Fromm, H. (1998) Calmodulin, calmodulin-related proteins and plant responses to the environment. *Trends Plant Sci.*, **3**, 299–304.
- Snedden, W.A., Koutsia, N., Baum, G. and Fromm, H. (1996) Activation of a recombinant petunia glutamate decarboxylase by calcium–calmodulin or by a monoclonal antibody which recognizes the calmodulin binding domain. *J. Biol. Chem.*, **271**, 4148–4153.
- Sonnewald, U. (1992) Expression of *E.coli* inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning. *Plant J.*, **2**, 571–581.
- Szymanski, D.B., Liao, B. and Zielinski, R. (1996) Calmodulin isoforms differentially enhance the binding of cauliflower nuclear proteins and recombinant TGA3 to a region derived from the *Arabidopsis Cam-3* promoter. *Plant Cell*, **8**, 1069–1077.
- Trainin, T., Shmuel, M. and Delmer, D.P. (1996) *In vitro* prenylation of the small GTPase Rac13 on cotton. *Plant Physiol.*, **112**, 1491–1497.
- Trewavas, A.J. and Malho, R. (1997) Signal perception and transduction: the origin of the phenotype. *Plant Cell*, **9**, 1181–1195.
- von Schaewen, A., Stitt, M., Schmidt, R., Sonnewald, U. and Willmitzer, L. (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J.*, **9**, 3033–3044.
- Xu, H. and Heath, M.C. (1998) Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. *Plant Cell*, **10**, 585–597.
- Yalovsky, S., Loraine, A.E. and Gruissem, W. (1996) Specific prenylation of tomato Rab proteins by geranylgeranyl type-II transferase requires a conserved cysteine–cysteine motif. *Plant Physiol.*, **110**, 1349–1359.
- Yalovsky, S., Trueblood, C.E., Callan, K.L., Narita, J.O., Jenkins, S.M., Rine, J. and Gruissem, W. (1997) Plant farnesyltransferase can restore yeast ras signaling and mating. *Mol. Cell. Biol.*, **17**, 1986–1994.
- Zhang, F.L. and Casey, P.J. (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.*, **65**, 241–269.
- Zhu, J.-K., Bressan, R.A. and Hasegawa, P.M. (1993) Isoprenylation of the plant molecular chaperone Anj1 facilitates membrane association and function at high temperature. *Proc. Natl Acad. Sci. USA*, **90**, 8557–8561.
- Zielinski, R.N. (1998) Calmodulin and calmodulin-binding proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 697–725.
- Zimmermann, S., Nurnberger, T., Frachisse, J.M., Wirtz, W., Guern, J., Hedrich, R. and Scheel, D. (1997) Receptor-mediated activation of a plant calcium-permeable ion channel involved in pathogen defense. *Proc. Natl Acad. Sci. USA*, **94**, 2751–2755.

Received September 4, 1998; revised and accepted February 4, 1999