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Characterization of GmCaMK1, a member of a soybean calmodulin-binding receptor-like kinase family

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ABSTRACT

Calmodulin(CaM)-regulated protein phosphorylation forms an important component of Ca^{2+} signaling in animals but is less understood in plants. We have identified a CaM-binding receptor-like kinase from soybean nodules, GmCaMK1, a homolog of *Arabidopsis* CRLK1. We delineated the CaM-binding domain (CaMBD) of GmCaMK1 to a 24-residue region near the C-terminus, which overlaps with the kinase domain. We have demonstrated that GmCaMK1 binds CaM with high affinity in a Ca^{2+}-dependent manner. We showed that *GmCaMK1* is expressed broadly across tissues and is enriched in roots and developing nodules. Finally, we examined the CaMBDs of the five-member GmCaMK family in soybean, and orthologs present across taxa.

Structured summary:
MINT-8051564: *AtCRLK2* (uniprotkb:Q9LFV3) *binds* (MI:0407) to *CaM* (uniprotkb:P62199) by *filter binding* (MI:0049)
MINT-8051416: *GmCaMK3* (uniprotkb:C6ZRS6) *binds* (MI:0407) to *CaM* (uniprotkb:P62199) by *filter binding* (MI:0049)
MINT-8051258: *CaM* (uniprotkb:P62199) and *GmCaMK1* (genbank_protein_gi:223452504) *bind* (MI:0407) by *isothermal titration calorimetry* (MI:0065)
MINT-8051400: *GmCaMK2* (uniprotkb:C6ZRY5) *binds* (MI:0407) to *CaM* (uniprotkb:P62199) by *filter binding* (MI:0049)
MINT-8051242, MINT-8051295, MINT-8051313, MINT-8051327, MINT-8051341, MINT-8051355: *GmCaMK1* (genbank_protein_gi:223452504) *binds* (MI:0407) to *CaM* (uniprotkb:P62199) by *filter binding* (MI:0049)
MINT-8051467: *GmCaMK4* (uniprotkb:C6TIQ0) *binds* (MI:0407) to *CaM* (uniprotkb:P62199) by *filter binding* (MI:0049)
MINT-8051276: *CaM* (uniprotkb:P62199) and *GmCaMK1* (genbank_protein_gi:223452504) *bind* (MI:0407) by *comigration in non denaturing gel electrophoresis* (MI:0404)
MINT-8051374: *CaM* (uniprotkb:P62199) and *GmCaMK1* (genbank_protein_gi:223452504) *bind* (MI:0407) by *mass spectrometry studies of complexes* (MI:0069)

1. Introduction

Ca^{2+} is a central second messenger in all eurkaryotes, and intracellular [Ca^{2+}] fluxes have been observed in response to numerous stimuli [1]. Ca^{2+} signals are perceived and interpreted by Ca^{2+}-binding proteins (Ca^{2+} sensors), many of which bind Ca^{2+} through the conserved EF-hand motif [2]. In plants several large families of Ca^{2+} sensors have been studied, including: calmodulin (CaM) and calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) [3,4].

Abbreviations: CaM, calmodulin; CaMBD, CaM-binding domain; CaMBP, CaM-binding protein; CaMK, calmodulin-binding kinase; CaMRLK, CaM-binding RLK; CBL, calcineurin B-like protein; CCaMK, Ca^{2+} and Ca^{2+}/CaM-depedent protein kinase; CDPK, calcium-dependent protein kinase; CIPK, CBL-interacting protein kinase; CML, calmodulin-like protein; CRK, CDPK-related kinase; GmCaMK1, Glycine max CaM-binding RLK; GST, glutathione S-transferase; HRP, horseradish peroxidase; ITC, isothermal titration calorimetry; MALDI, matrix-assisted laser desorption/ionization; Q-tof, quantitative time-of-flight; RLK, receptor-like kinase; qPCR, quantitative real-time PCR

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CDPKs and CBLs (via their corresponding CBL-interacting protein kinase family [CIPKs]) possess catalytic activity, while CaM acts via regulation of many CaM-binding target proteins of diverse function [5,6]. CaM is an archetypal and ubiquitous eukaryotic Ca^{2+}-sensor, and in plants participates in coordinating developmental processes, abiotic and biotic stress response, and the formation of microbial symbioses [4,7]. Although considerable progress has been made in the identification of plant CaM targets, the role of CaM-binding protein kinases (CaMKs) are poorly understood. In contrast, there has been extensive analysis of CaMKs in animals [8]. To date, plant CaMKs identified include kinases that share some sequence homology to CDPK-related kinases (CRKs), the chimeric Ca^{2+} and Ca^{2+}/CaM-depedent protein kinase (CCaMK), and CaM-binding receptor-like kinases (RLKs) [9,10].

Amongst plant CaM-binding kinases, CCaMK is the most thoroughly studied [11–15] and a large body of evidence has demonstrated that CCaMK is critical for both rhizobial nodule formation and mycorrhizal fungal associations [13–18]. Given the importance of Ca^{2+} signals and CaM-binding CCaMK in nodule formation, we screened expressed soybean nodule cDNA using ^{35}S-CaM as a probe to identify nodule-expressed CaM-binding proteins (CaM-BPs). We isolated a CaM-binding RLK (CaMRLK), *Glycine max* CaM-binding RLK (GmCaMK1), which is a homolog of the recently identified CRLK1 of *Arabidopsis thaliana* [19]. Here we present the biochemical characterization of GmCaMK1 and demonstrate that this family of CaMRLKs is conserved in soybean, *Arabidopsis*, and *Medicago truncatula*.

2. Materials and methods

2.1. Plant growth conditions

G. max L. cv. Djakal or cv. Boyer seeds were sown in Waikerie river sand, inoculated with *Bradyrhizobium japonicum* USDA 110 one-day after planting, and fertilized twice weekly with nutrient solution lacking nitrogen [20]. Plants were grown in a glasshouse (24–28 °C) under long day conditions (16/8 h day/night). *A. thaliana* (ecotype Col-0) seeds were grown in soil in a growth chamber under long day (16/8 h) conditions at 22 °C and supplemented weekly with 1 g/l 20–20–20 fertilizer.

2.2. Preparation and screening of soybean nodule cDNA library

We prepared a cDNA expression library from soybean nodules (*G. max* L. cv. Boyer) at various developmental stages using the UniZapII kit (Stratagene) according to manufacturer's instructions. This nodule cDNA library was then screened for CaMBPs using ^{35}S-methionine labeled CaM (in all cases recombinant petunia CaM81 – a conserved plant CaM with 100% amino acid identity to *Arabidopsis* CaM2) in the presence of 1 mM Ca^{2+} as previously described [21].

2.3. Cloning and expression of GmCaMK1

Serial truncations of *GmCaMK1* were subcloned from the *GmCaMK1* clone isolated from the cDNA library screen and expressed as glutathione S-transferase (GST)-fusion proteins in the pGEX4T3 vector (Amersham) using *Escherichia coli* BL21 pLysS cells grown in LB media at 37 °C. Supplementary Table 1 lists the relevant PCR primers used for subcloning procedures. Recombinant proteins used in kinase assays were purified to near-homogeneity (Supplementary Fig. 3) by glutathione-agarose affinity chromatography and/or Ca^{2+}-dependent CaM-agarose affinity chromatography or phenyl-sepharose chromatography (in the case of CaM).

A full length *GmCaMK1* cDNA was obtained from the SoyBase database (clone Gm_c1065-5498), from which a fragment encoding residues 29-431 was amplified by PCR, subcloned into a pET21 vector, expressed in *E. coli* BL21 pLysS cells, and purified by Ca^{2+}-dependent CaM-agarose affinity chromatography as described [22].

2.4. CaM-binding assays

CaM-binding overlay assays were performed as described previously ([22]. Briefly, proteins were either separated by SDS–PAGE and transferred to nitrocellulose or purified proteins were blotted directly onto nitrocellulose (spot blot assays) according to figure legends. Membranes were blocked in 25 mM Tris–Cl, pH 7.5 with 5% (w/v) skim milk powder at 4 °C overnight, probed with either ^{35}S-methionine CaM81 (150 nM) or horseradish peroxidase (HRP)-conjugated CaM (HRP::CaM, 25–100 nM as indicated in figure legends) in 25 mM Tris–Cl, pH 7.5, 1% milk and either 1 mM $CaCl_2$ or 5 mM EGTA for 60 min. HRP::CaM conjugation and bioluminescent overlay assays were performed as described [23]. Membranes were washed in buffer without milk and imaged using chemiluminescence reagents with either X-ray film (Kodak MR-1) or a Typhoon phosphorimager (GE).

2.5. Isothermal titration calorimetry (ITC)

ITC was performed using a VP-ITC calorimeter (Microcal, LLC, Northampton, MA) at 30 °C in CaM-binding buffer (25 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$). A 26mer peptide (364-KRPSMRDIVQVLTRILKSRHQRNHHH-389) containing the CaM-binding domain (CaMBD) of GmCaMK1 was synthesized (GenScript) and used for binding analysis with CaM. CaM (114 μM) was titrated into the cell containing 14.5 μM CaMBD peptide in 49 injections of 6 μl each with a 300-s equilibration interval between injections. Using Origin 7.0 software (OriginLab Corp., Northampton, MA), the best fit for the ITC binding isotherm was obtained with the two sets of sites model, following removal of the initial injection point and subtraction of the heat of dilution as determined from the final 20 injections of the isotherm post-saturation of both binding sites. Amino acid analysis (Advanced Protein Technology Centre, Toronto) was used to quantify peptide and CaM concentrations for ITC samples.

2.6. Non-dissociating PAGE

CaM-binding peptide shift gels were carried out as described previously [22]. Briefly, CaM was incubated with GmCaMK1 CaM-BD peptide at varying molar ratios in 25 mM Tris–Cl, pH 7.5, 0.1 mM $CaCl_2$ (or 2 mM EGTA) for 30 min at 25 °C, separated by non-dissociating 12.5% acrylamide PAGE (ND-PAGE) in the presence of 0.1 mM $CaCl_2$ (or 2 mM EGTA) at 25 mA and 25 °C, then stained with Coomassie brilliant blue R-250.

2.7. Mass spectrometry analysis of CaM–peptide complex

For all mass spectrometry analyses, CaM and GmCaMK1 peptide were combined in 25 mM Tris–Cl, 1 mM $CaCl_2$, pH 7.5 to a final concentration of 75 μM (sample ~50 μl). For denaturing matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, recrystallized sinapinic acid (Sigma) was freshly prepared at a concentration of 10 mg/ml in 70% acetonitrile, 0.1% trifluoroacetic acid, 10 mM diammonium citrate. One microliter of this solution was applied to each sample spot on the MALDI target and allowed to air dry. Samples were mixed 1:1 with matrix solution prepared previously and 1 μl was applied to a target position. The spots were allowed to air dry. Each sample was washed on the target with 1.5 μl of ice cold 0.1% trifluoroacetic acid for 5 s. The wash was removed and discarded. Spectra were acquired and analyzed on a Voyager DePro (Applied Biosystems Corporation) using Data