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An S-locus receptor-like kinase in plasma membrane interacts with calmodulin in *Arabidopsis*

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ABSTRACT

Calmodulin-regulated protein phosphorylation plays a pivotal role in amplifying and diversifying the action of calcium ion. In this study, we identified a calmodulin-binding receptor-like protein kinase (CBRLK1) that was classified into an S-locus RLK family. The plasma membrane localization was determined by the localization of CBRLK1 tagged with a green fluorescence protein. Calmodulin bound specifically to a Ca^{2+}-dependent calmodulin binding domain in the C-terminus of CBRLK1. The bacterially expressed CBRLK1 kinase domain could autophosphorylate and phosphorylates general kinase substrates, such as myelin basic proteins. The autophosphorylation sites of CBRLK1 were identified by mass spectrometric analysis of phosphopeptides.

Structured summary:
MINT-6800947: *CBRLK1* (uniprotkb:Q9ZT06) and *AtCaM2* (uniprotkb:P25069) *bind* (MI:0407) by *electrophoretic mobility shift assay* (MI:0413)
MINT-6800966: *AtCaM2* (uniprotkb:P25069) and *CBRLK1* (uniprotkb:Q9ZT06) *bind* (MI:0407) by *competition binding* (MI:0405)
MINT-6800930: *CBRLK1* (uniprotkb:Q9ZT06) *binds* (MI:0407) to *AtCaM2* (uniprotkb:P25069) by *far Western blotting* (MI:0047)
MINT-6800978: *AtCaM2* (uniprotkb:P25069) *physically interacts* (MI:0218) with *CBRLK1* (uniprotkb:Q9ZT06) by *cytoplasmic complementation assay* (MI:0228)

1. Introduction

Plants are continually bombarded by various external stimuli and, consequently, have evolved a large array of mechanistic responses to environmental changes. In plants, calcium ion (Ca^{2+}) functions as a major second messenger in the signal transduction pathway responsive to many biotic and abiotic stresses [1]. One of the earliest cellular responses to external stimuli is a rapid increase in concentration of cytoplasmic-free Ca^{2+} [2]. The elevated cytosolic Ca^{2+} concentration initiates cellular responses by activating a Ca^{2+} sensor, such as calmodulin (CaM), Ca^{2+}-dependent protein kinases (CPKs), or other Ca^{2+} binding proteins [1]. CaM is known to couple Ca^{2+} signals to changes in the activity of downstream target proteins via direct interaction [3]. Therefore, understanding of calmodulin binding proteins (CaMBP) is crucial to determine how Ca^{2+} signals are transduced to downstream events to trigger cellular responses.

Receptor-like protein kinases (RLKs) are a subgroup of protein kinases characterized by an extracellular domain, a transmembrane domain, and an intracellular kinase domain [4]. RLKs are classified according to amino acid sequence motifs in the putative extracellular domain [5]. The largest group is RLKs with leucine-rich repeats (LRRs), which are characterized by at least five tandem repeats of a leucine-rich consensus sequence [5]. LRR–RLKs have been implicated in a diverse range of signaling processes, such as brassinosteroid signaling via BRI1 and BAK1 [6], perception of

Abbreviations: AtCaM, *Arabidopsis* calmodulin; CaM, calmodulin; CaMBD, calmodulin binding domain; CaMBP, calmodulin binding protein; CBRLK1, calmodulin-binding receptor-like kinase; 5-FOA, 5-fluoroorotic acid; GST, glutathione S-transferase; HRP, horseradish peroxidase; N_{ub} and C_{ub}, N- and C-terminal halves of ubiquitin, respectively

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bacterial flagellin by FLS2 [7], and meristem development controlled by CLV1 [8]. The second largest group of RLKs is a family of S-locus receptor kinases (SRKs), which were first identified in Brassica. SRKs have an extracellular S-domain with a high degree of similarity to S-locus glycoproteins and play a role in the self-incompatibility response [9]. The third group is comprised of RLKs that are similar to TNF receptor, EGF receptor, and a lectin-binding domain [10,11]. Putative ligands for SRK [9], CLV1 [8], BRI1 [6], and FLS2 [7] have been identified, and proteins that interact with the kinase domains of RLKs have also been found [12]. However, the biochemical regulations and physiological functions of most plant RLKs have not been studied yet. Previously it was reported that a few RLKs were able to interact with CaM [13,14]. However, the specific interaction with CaM and biochemical characteristics of CaM binding RLKs was poorly studied yet.

Here we isolated an S-locus receptor-like kinase, calmodulin-binding receptor-like protein kinase (CBRLK1) (AGI No. At1g11350), from *Arabidopsis* as a CaM binding protein. The direct interaction of CBRLK1 and CaM was investigated. We also demonstrated the subcellular localization and biochemical properties of CBRLK1.

2. Materials and methods

2.1. Screening of Arabidopsis cDNA expression library

Screening of the *Arabidopsis* cDNA expression library using horseradish peroxidase (HRP)–conjugated *Arabidopsis* calmodulin 2 (AtCaM2) (AtCaM2::HRP) was carried out as previously described [15].

2.2. Expression of recombinant proteins in Escherichia coli and CaM binding assay

All clones were individually introduced into *E. coli* BL21 (DE3) pLysS and expressed. Recombinant proteins were purified on a glutathione-sepharose 4B column (GE healthcare). Expressed glutathione S-transferase (GST)-fusion proteins were detected using a polyclonal GST-specific antiserum. The CaM:HRP overlay assay was carried out as previously described [15].

2.3. CaM mobility shift assay with a synthetic peptide

AtCaM2 (303 pmol) was incubated with increasing concentrations of the 18 amino acids peptide spanning the CBRLK1 calmodulin binding domain (CaMBD) (^{601}Val–^{618}Ile) in binding buffer (100 mM Tris–HCl [pH 7.2], plus 0.1 mM $CaCl_2$ or 2 mM EGTA) at room temperature for 1 h. The bound complexes were resolved by non-denaturing PAGE and visualized by staining with Coomassie brilliant blue.

2.4. Phosphodiesterase competition assay

Bovine heart CaM-deficient PDE (Sigma) activity was assayed as described in [15] with varying concentrations of AtCaM2 (1–200 nM) plus 100 nM synthetic peptide. The following equation was used to calculate dissociation constants [16]: $K_d = ([P_t] + K - [\mathrm{CaM}])K/([\mathrm{CaM}\text{-}] - \mathrm{K})$, where $[P_t]$ is the total concentration of peptide added and [CaM] and K represent the concentrations of CaM required to obtain half-maximal activation of PDE in the presence or absence of peptides, respectively.

2.5. Yeast split ubiquitin assay

The yeast split ubiquitin assay was performed as previously described [17]. *Saccharomyces cerevisiae* strain JD53 was used for all the experiments. CBRLK1 cDNA was cloned into pMet-Ste14-C_{ub}-RUra3, replacing yeast *Ste14*. AtCaM2 cDNA was cloned into modified versions of the pCup-N_{ub}-Sec62 vector, replacing yeast *Sec62* [18]. Interactions between each pair of proteins were tested on selective medium containing 1 mg/ml 5-fluoroorotic acid (5-FOA) and selective medium lacking uracil. Plates were incubated at 30 °C for 3–5 d, unless specified otherwise.

2.6. Phosphorylation assays

The kinase activity of CBRLK1 was measured as the incorporation of radioactivity from γ-^{32}P-ATP into the CBRLK1 kinase domain (KD) protein (autophosphorylation) or into the substrate proteins. Assays were performed at room temperature for 30 min in a final volume of 20 μl containing 500 ng of GST–CBRLK1 KD fusion protein, 10 μCi of γ-^{32}P-ATP, 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 0.5 mM EDTA, and 0.5 mM EGTA in the different concentrations of divalent cations (Mg^{2+} or Mn^{2+}) and substrate proteins.

2.7. Mass spectrometric analysis of phosphopeptides using TiO_2 microcolumns

GST-fused CBRLK1 KD protein bands autophosphorylated in vitro were in-gel digested [19] by modified trypsin (Promega). The tryptic peptides were dissolved in loading buffer (80% acetonitrile/5% trifluoroacetic acid) and passed through a TiO_2 microcolumn [20,21]. The phosphopeptides were eluted with NH_4OH (pH 10.5), purified by Poros Oligo R3 reversed-phase material (Applied Biosystems), and eluted using 2,5-dihydroxybenzoic acid (DHB; Fluka) solution (20 mg/ml DHB in 50% acetonitrile/0.1% trifluoroacetic acid/1% ortho-phosphoric acid) directly onto the target [22]. MALDI-TOF analysis was performed using a Voyager-DE STR mass spectrometer (PerSeptive Biosystems Inc.). Mass spectra were obtained in the reflectron/delayed extraction mode. Monoisotopic peptide masses were analyzed using the MoverZ software (www.proteometric.com).

2.8. Confocal microscopy

Confocal images were generated using a laser confocal microscope (Olympus, Fluoview 1000) attached to a vertical microscope equipped with a fluorescein filter. An X100, 1.35 Plan Apo objective lens was used to image root tips. The GFP signal was excited with the 488 nm wavelength under confocal laser-scanning microscope with argon ion laser system. These fluorescence images were collected in the green channels.

3. Results

3.1. Isolation of a CaM-binding receptor-like protein kinase (CBRLK1) in Arabidopsis

To identify the molecular components of CaM-mediated defense signaling, an *Arabidopsis* cDNA expression library prepared from seedlings treated with the bacterial pathogen *Pst* DC3000 was screened. The screening was performed using HRP–conjugated CaM as a probe. One of the isolated clones encoded an S-locus receptor-like kinase designated CBRLK1. CBRLK1 consists of 830 amino acids with a calculated molecular mass of 93.2 kDa and possesses the typical structural features of an RLK-type kinase, including a hydrophobic N-terminal putative signal peptide (residues 1–25), an extracellular domain consisting of an S-domain (residues 1–424), a single hydrophobic membrane-spanning domain of 22 amino acids (residues 435–456), and an intracellular C-terminal kinase catalytic domain with all 11 subdomains described for protein

kinases (residues 517–801). A comparison of the deduced amino acid sequences of CBRLK1 with those of several S-domain family members showed that the N-terminal half of CBRLK1 shares 31%, 32%, 35%, 28%, and 27% identities with S-domains of SRK6, SRK910, ARK1, RLK4, and RLK1, respectively (Supplementary Fig. 1A). The putative kinase catalytic domain of CBRLK1 shares high similarity with those of other S-domain RLKs (Supplementary Fig. 1B).

3.2. Mapping of a CaM-binding domain (CaMBD) in CBRLK1

Comparative analysis of the CaM-binding regions of many CaM-BPs has led to the identification of multiple sequence motifs required for complex formation with CaM [23]. Based on the structural characteristics of known CaMBDs, a putative CaMBD was predicted in the C-terminus of CBRLK1, between Val^{601} and Ile^{618} (Fig. 1A). Within this 18-amino acid stretch, hydrophobic amino acids are present at positions 1 (Val^{601}), 8 (Trp^{608}), and 14 (Ile^{614}). Several basic residues (two lysines and two arginines) are interspersed between these hydrophobic residues, which is a known structural characteristic of Ca^{2+}-dependent CaM-binding motifs. A helical wheel projection of the CBRLK1 CaMBD (18 amino acids, Val^{601} to Ile^{618}) reveals a characteristic segregation of basic and polar residues on one side with hydrophobic amino acids on the other side (Fig. 2A).

To confirm the location of the putative CaMBD, GST-fusion constructs encoding the full-length CBRLK1 (designated D0) and eight serial deletion fragments (D1, D2, D3, D4, D5, D6, D7, and D8) were generated (Fig. 1A). Expression of the GST-fusion proteins was verified by protein blot analysis with an anti-GST polyclonal antibody. Two recombinant proteins that contain the putative CaMBD (D0 and D7) interacted with AtCaM2::HRP conjugate, and GST-fusion proteins lacking the predicted CaMBD (D1, D2, D3, D4, D5, D6, and D8) did not interact with CaM. Moreover, CaM bound to CBRLK1 in the presence of Ca^{2+} but not in the absence of Ca^{2+} (Fig. 1B). These results indicate that CaM binds to a CaMBD located in the C-terminus region of CBRLK1 in a Ca^{2+} -dependent manner.

3.3. Binding of a synthetic peptide to CaM

To further analyze the putative binding of CaM to the 18-amino acid sequence of CBRLK1 from Val^{601} to Ile^{618}, a peptide corresponding to this region was synthesized and employed for a CaM mobility shift assay on non-denaturing polyacrylamide gels [16]. As shown in Fig. 2B, the amount of peptide–CaM complex increased with increasing concentrations of the synthetic peptide in the presence of Ca^{2+} and was undetectable in the presence of EGTA. This result is consistent with data from a previous CaM overlay assay under similar Ca^{2+} and EGTA conditions. At a molar ratio of 1:1 (peptide:CaM), a half of CaM was shifted; whereas, at molar ratio of 3:1 (peptide:CaM), all CaM was shifted.

To confirm binding of the synthetic peptide to CaM, a competition assay with PDE, a Ca^{2+}/CaM-dependent enzyme, was performed. To determine the K_d values of the peptide for activation of PDE by CaM, the CaM-mediated, dose-dependent activation of PDE was monitored in the presence (100 nM) and in the absence of the peptide (Fig. 2C). The activation curves shifted to the right in the presence of the peptide, indicating competition between PDE and the peptide for binding to CaM. Concentrations of AtCaM2 needed to achieve half-maximal activation of PDE activity (K_m) in the absence and presence of the 18-mer peptide were 10.9 nM and 40 nM, respectively, a 4-fold difference. The K_d value of the peptide for the activation of PDE by AtCaM2 is 26.5 nM.

3.4. In vivo interaction between CBRLK1 and CaM in yeast

To examine direct interactions between CaM and CBRLK1 in vivo, a yeast split ubiquitin assay system, based on the reassem-

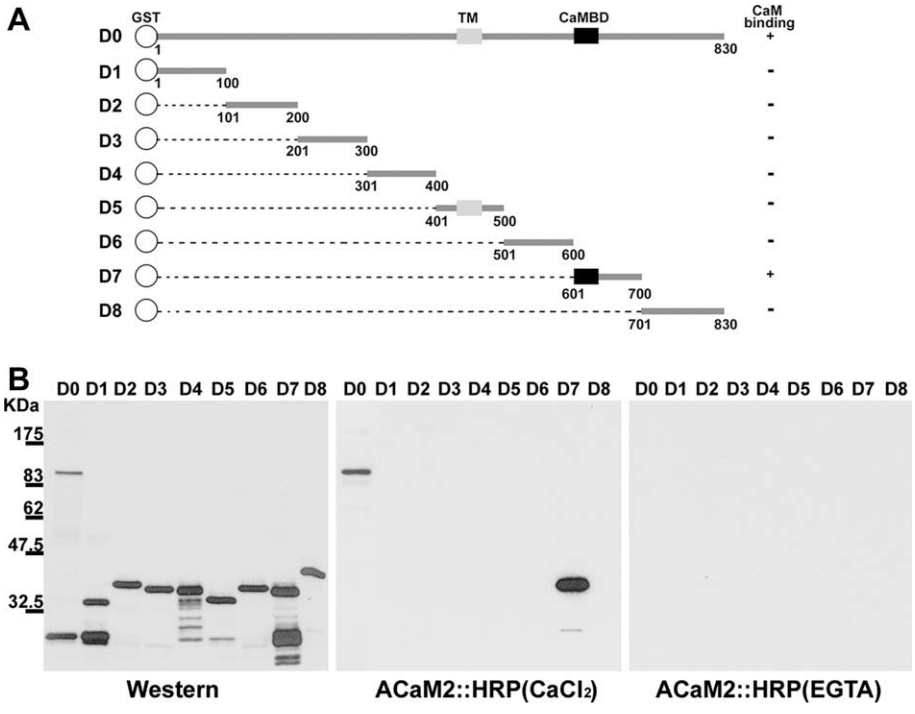

Fig. 1. Identification of the CaMBD of CBRLK1. (A) Schematic representation of GST-fused CBRLK1 full-length and serial deletion fragments. The designations D0 and D1–D8 represent GST-fused protein constructs containing full-length clone or indicated clone fragments, respectively. A putative CaMBD and a putative single transmembrane domain (TM) are designated by a black and gray box, respectively. Amino acid positions of each serial fragment are indicated by numbers. D0–D8 represent GST-fusion constructs containing full-length CBRLK1 or the specified fragment. CaM-binding ability is specified as + (CaM binding) or – (no CaM binding). (B) CaM binding overlay analysis. GST and GST-fusion proteins of full-length (D0) and serial fragment mutants (D1–D8) of CBRLK1 were expressed in *E. coli*. Expressed recombinant proteins were analyzed by protein blotting with an anti-GST antibody. The CaM::HRP overlay assay was performed using AtCaM2::HRP in the presence of 0.1 mM $CaCl_2$ and of 2 mM EGTA.

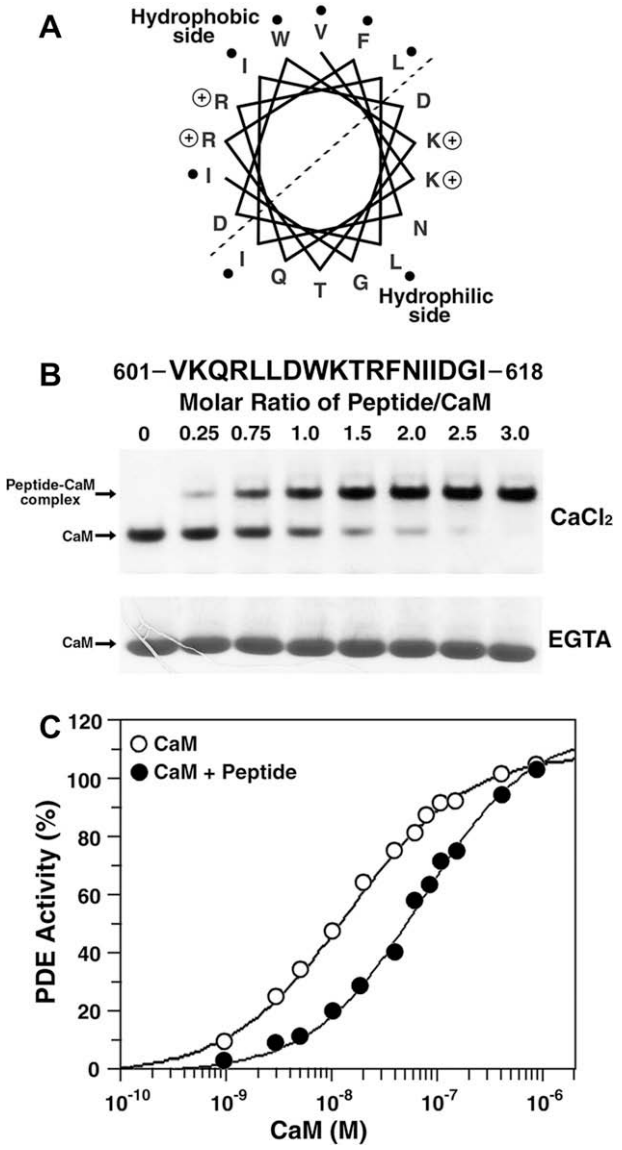

Fig. 2. Characterization of CaMBD in CBRLK1. (A) Helical wheel projection of the putative CaM binding motif of CBRLK1. Hydrophobic and basic amino acid residues are marked with ● and +, respectively. The dashed line indicates that the helix is amphipathic, one side being hydrophobic and the other side hydrophilic. (B) Gel mobility shift assay. CaMBD peptide (18-mer corresponding to amino acids 601–618) is depicted at the top. AtCaM2 (303 pmol) was incubated with increasing amounts of peptide (peptide/CaM molar ratios are indicated) in the presence of 0.1 mM $CaCl_2$ (upper panel) or 2 mM EGTA (lower panel). Samples were separated by non-denaturing PAGE and stained with Coomassie brilliant blue. Arrows indicate the positions of free CaM and the peptide–CaM complex. (C) Effect of synthetic CBRLK1 CaMBD peptide on the activation of PDE by CaM. PDE activity was measured in the presence of varying concentrations of AtCaM2, either in the presence or absence of a fixed concentration (100 nM) of synthetic CBRLK1 CaMBD peptide.

bly of N- and C-terminal halves (N_{ub} and C_{ub}) of ubiquitin (Ub), was used [17,18]. CaM and full-length CBRLK1 were fused to the C-terminus of N_{ub} and the N-terminus of C_{ub}, respectively. The C_{ub} of ubiquitin was linked to an N-terminally modified Ura3p reporter containing Arg at position 1 (RUra3p). When CaM interacts with the CBRLK1 protein, N_{ub} and C_{ub} reassemble into native-like Ub. This is followed by cleavage of RUra3p by ubiquitin-specific proteases (UBP) and rapid degradation of the released RUra3p through the N-end rule pathway of protein degradation. Consequently, cells coexpressing CBRLK1-C_{ub}-RUra3p and N_{ub}-CaM are unable to grow on plates lacking uracil but are able to grow on plates containing 5-FOA, which is converted into toxic 5-fluorouracil by RUra3p. In the opposite cases, yeast cells are uracil prototrophs and 5-FOA sensitive.

Cells co-expressing CBRLK1-C_{ub}-RUra3p and N_{ub}-CaM were unable to grow on plates lacking uracil but successfully grew on plates containing 5-FOA, indicating that CBRLK1 effectively forms stable complexes with CaM in vivo (Fig. 3). Cells co-expressing DsPTP1-C_{ub}-RUra3p and N_{ub}-CaM used for a positive control were unable to grow on plates lacking uracil but grew on plates containing 5-FOA [15]. No interactions were observed between C_{ub}-RUra3p and N_{ub}-CaM, which was used as a negative control.

3.5. The kinase activity of CBRLK1

To examine the kinase activity of CBRLK1, the GST-fused CBRLK1 KD (residues 457–830) was expressed in *E. coli* and purified by affinity chromatography. To know the requirement of divalent cations for kinase activity of CBRLK1, different amounts of $MgCl_2$ and $MnCl_2$ were used in the kinase assay. A linear increase in the enzymatic activity of the CBRLK1 KD was observed in the range of 0.5–10 mM $MgCl_2$, followed by a plateau of the autophosphorylation at more than 10 mM $MgCl_2$. $MnCl_2$ conferred the highest enzymatic activity of the CBRLK1 KD at 2 mM (Fig. 4A). The ability of the CBRLK1 protein to phosphorylate other proteins was tested with general substrates such as myelin basic protein (MBP), casein, and histone (Type IIIS). All proteins were clearly phosphorylated by the CBRLK1 KD (Fig. 4B). To confirm the residue specificity of CBRLK1 autophosphorylation, phosphoamino acid

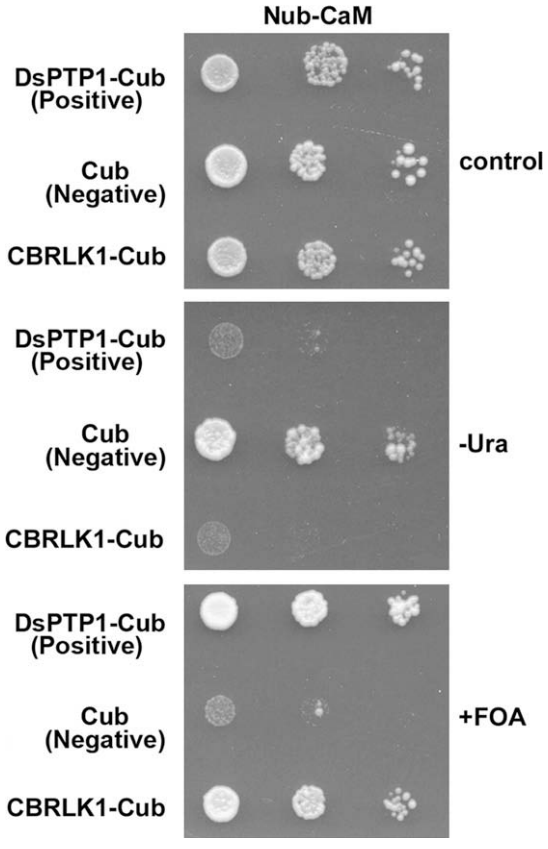

Fig. 3. Split ubiquitin assay to monitor interaction between CaM and CBRLK1 in vivo. Shown are serial 10-fold dilutions of cells co-expressing C_{ub} or CBRLK1-C_{ub}-RUra3 fusion together with CaM (N_{ub}-AtCaM2) on plates lacking tryptophan and histidine (Control), as well as lacking uracil (−Ura) or containing 5-FOA (+FOA). Cells co-expressing DsPTP1-C_{ub}-RUra3p and N_{ub}-AtCaM2 were used for a positive control. All proteins were expressed from single-copy vectors.

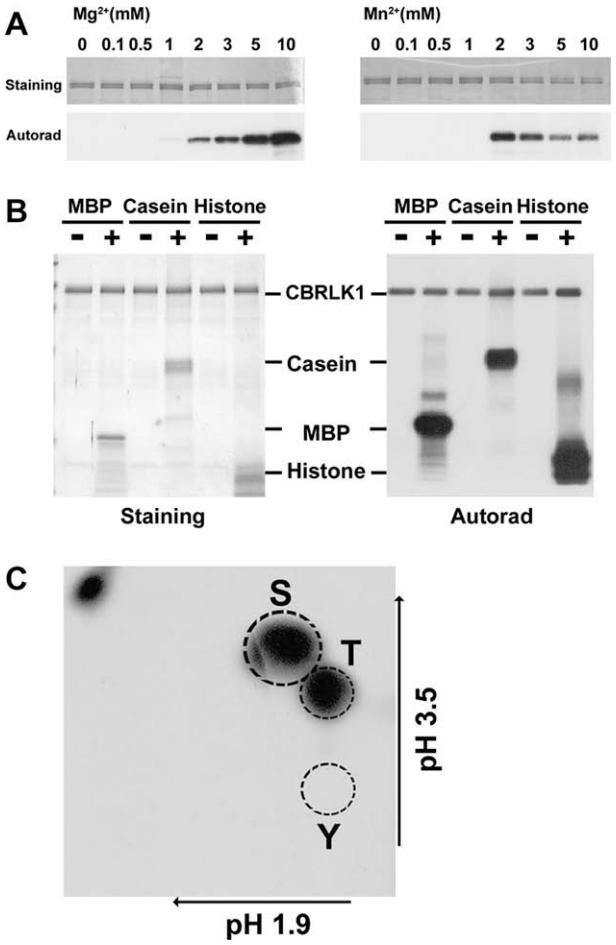

Fig. 4. Enzymatic properties of CBRLK1. (A) Autophosphorylation activity of CBRLK1 KD dependent on divalent cations. Autophosphorylation assay was performed in kinase buffer containing different concentrations of $MgCl_2$ and $MnCl_2$. Samples were then resolved by 10% SDS–PAGE. Both the Coomassie brilliant blue stained gel (upper, Staining) and autoradiogram (lower, Autorad) of the same gel are shown. (B) Kinase activity of CBRLK1 KD against general substrates. Myelin basic protein (MBP), casein, and histone were used to kinase assays containing CBRLK1 KD. +, – indicate the presence or absence of substrates, respectively. Coomassie brilliant blue staining (left, Staining) and autoradiogram (right, Autorad) of the same gel are shown. (C) Phosphoamino acid analysis of CBRLK1 KD autophosphorylation. The autoradiogram spots indicate positions of phosphorylated serine and threonine residues. Dashed circles indicate positions of standard phosphorylated serine (S), threonine (T), and tyrosine (Y).

assays were performed by using an autophosphorylated CBRLK1 KD fusion protein. In these assays, serine and threonine residues

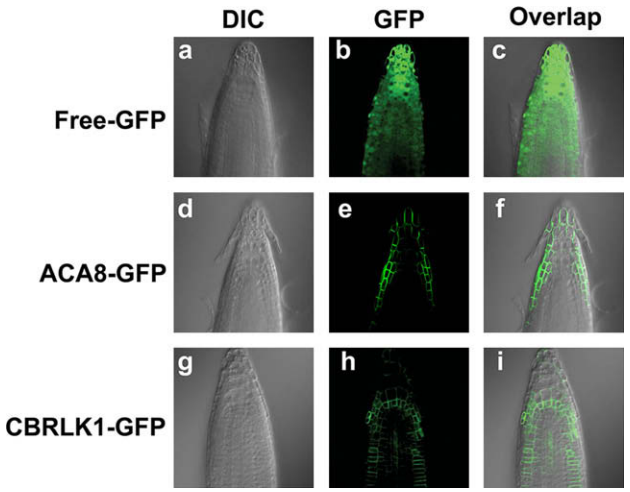

Fig. 5. Subcellular localization of CBRLK1 in *Arabidopsis* roots. Confocal images of transgenic *Arabidopsis* roots that express free-GFP, ACA8-GFP, or CBRLK1–GFP fusion proteins under the control of the 35S promoter. GFP and ACA8-GFP were used as cytosol and plasma membrane marker proteins, respectively. DIC (a, d, g), differential interference contrast; GFP (b, e, h), green fluorescent protein; Overlap (c, f, i), overlap image of DIC and GFP.

were phosphorylated, and no phosphorylated tyrosine residues were detected (Fig. 4C). These results strongly indicate that CBRLK1 is a functional Ser/Thr protein kinase. To map the autophosphorylation sites in CBRLK1, TiO_2 chromatography in combination with MALDI-TOF mass spectrometry was used to selectively enrich phosphopeptides. The GST-fused CBRLK1 KD protein was autophosphorylated in vitro, separated by SDS–PAGE, and digested with trypsin. After TiO_2 purification and R3 desalting, a total of nine serine/threonine residues originating from 11 phosphopeptide peaks were autophosphorylated in vitro (Table 1 and Supplementary Fig. 2). Consequently seven autophosphorylation sites are located in several kinase subdomains and two autophosphorylation sites in behind kinase domain, but not in the CaMBD of CBRLK1. These results indicate that multiple serine/threonine residues on CBRLK1 are autophosphorylated.

3.6. CBRLK1 is localized in the plasma membrane

To determine the subcellular localization of CBRLK1, the fluorescence pattern of green fluorescent protein (GFP) was monitored in transgenic *Arabidopsis* plants that constitutively expressed CBRLK1–GFP fusion proteins using confocal microscopy. The fluorescence of GFP was detected in the living root cells of 1-week-old seedlings grown on an agar-solidified medium. Free-GFP and

Table 1
Phosphopeptides isolated from tryptic digestion of CBRLK1 KD autophosphorylated in vitro.

Peptide	Sequence	Amino acids	Number of phosphate groups	$(M+H)^+$	
				Expected	Measured
1	*TS*GQGVEEFVNEVVVI*S*K	545–562	1	2000.96	2001.00
2	DLKA*S*NILLDENLNPK	637–652	1	1876.94	1876.99
3	I*S*DFGLAR	653–660	1	958.44	958.46
4	IFQGNEDEV*ST*VR	661–673	1	1573.69	1573.75
5	IFQGNEDEV*ST*VR	661–673	2	1653.66	1653.71
6	RN*SS*FYNDGQNPNL*S*AYAWK	712–731	1	2412.04	2412.04
7	N*SS*FYNDGQNPNL*S*AYAWK	713–731	1	2255.94	2255.96
8	RGT*S*EVE*SS*GQ*S*DPR	802–816	1	1671.70	1671.79
9	A*S*INNV*S*L*T*K	817–826	1	1126.55	1126.59
10	A*S*INNV*S*L*T*KI*T*GR	817–830	1	1553.81	1553.88
11	A*S*INNV*S*L*T*KI*T*GR	817–830	2	1633.77	1633.83

Amino acid residues that could potentially be phosphorylated are in italics and underlined.

ACA8-GFP were used as cytosol and plasma membrane marker proteins, respectively [24,25]. The fluorescent signal of free-GFP was dispersed throughout the whole cell and that of ACA8-GFP was clearly detected in the plasma membrane. The fluorescent signal of CBRLK1–GFP was mainly detected in the plasma membrane (Fig. 5). Compared to the GFP fluorescence in ACA8-GFP, the diffused GFP signal around plasma membrane in CBRLK1–GFP was partly detected. It is possible that the diffused GFP signal might come from intracellular vesicles because receptor kinases have been known to be internalized for the regulation [26]. To verify the expression of the intact full-length protein in transgenic plants, we performed Western blotting using an anti-GFP antibody. All GFP fused CBRLK1 were detected at their expected sizes (data not shown).

4. Discussion

RLKs are known to serve as receivers and transducers of external and internal stimuli. Various input signals are transmitted through phosphorylation/dephosphorylation cascades, which lead to changes in gene expression patterns. Although RLK signaling pathways in plants have been extensively studied, mechanisms and components of these pathways are not well known. Recently the interactions of CaM with plant RLKs, including SRK from *brassicca*, CLV1 and AtCaMRLK from *Arabidopsis*, were reported [13,14]. The CaMBD of SRK was identified in the subdomain VIa of the kinase domain, but the CaMBD of AtCaMRLK is located in a cytoplasmic domain close to the transmembrane segment. Interestingly, the CaMBD of CRCK1, a member of cytoplasmic RLKs, is located around subdomain II of the kinase domain, which contains a conserved lysine residue for ATP binding. The kinase activity of CRCK1 is stimulated upon CaM binding to the CaM-binding region [27]. In this report, we showed that the Ca^{2+}-dependent CaMBD of CBRLK1 was located in subdomain VIa of the kinase domain. This finding was confirmed by three different CaM binding analyses. First, the Ca^{2+}-dependent CaMBD of CBRLK1 was mapped using a CaM overlay assay of bacterially expressed CBRLK1 full-length and serial fragments (Fig. 1). Second, the CaMBD of CBRLK1 was further confirmed by CaM mobility shift and by a PDE enzyme competition assay with a synthetic peptide corresponding to an 18-amino acid stretch (from Val^{601} to Ile^{618}) (Fig. 2). Third, in vivo interaction between CaM and CBRLK1 was confirmed using the split ubiquitin assay in yeast (Fig. 3).

We showed that the kinase domain of CBRLK1 (CBRLK1 KD) could strongly phosphorylate three general substrates such as MBP, casein, histone in vitro without specificity (Fig. 4B). CBRLK1 KD might be constitutively active because it was absent of a transmembrane domain and a putative regulatory domain or hyperactivated in that condition. It may be possible that the full-length of CBRLK1 in plasma membrane can phosphorylate in vivo unknown substrates with strict specificity through the membrane localization or specific docking domains.

Although SRK and AtCaMRLK were reported to interact with CaM, no effect of CaM on the SRK and AtCaMRLK kinase activity has been observed until now. We also observed no significant changes in the autophosphorylation and substrate phosphorylation of CBRLK1 by CaM (data not shown). These results suggest that members of the plant RLK family can interact with CaM either in the kinase domain or in the vicinity of the catalytic domain but the interactions are not involved in the regulation of the kinase activities of RLKs. In animal cells, CaM has been shown to interact with two receptor kinases, the EGF receptor and the insulin receptor [28]. It has recently been proposed that CaM plays a role in intracellular trafficking of the EGFR [29]. Internalization and trafficking of transmembrane receptors in animal cells is thought to contribute to receptor degradation and recycling [30]. Because there is a report that internalization of RLKs also occurs in plant [26], there is a possibility that the interaction of CaM with RLKs are involved the desensitization or turnover and recycling of RLKs in plants. It may also be possible that CaM can make an effect on the kinase activity of CBRLK1 on in vivo substrate which has not identified yet although CaM did not affect on the autophosphorylation of CBRLK1. Further molecular and genetic studies can provide new information about the functions and action modes of CBRLK1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.11.046.

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