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

Ho Soo Kim\(^{a,b}\), Mi Soon Jung\(^a\), Kyunghhee Lee\(^{a,b}\), Kyung Eun Kim\(^a\), Jae Hyuk Yoo\(^b\), Min Chul Kim\(^a\), Doh Hoon Kim\(^c\), Moo Je Cho\(^a\), Woo Sik Chung\(^{a,b,*}\)

\(^a\) Division of Applied Life Science (BK21 Program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, 900 Gajwa, Room No. 6-320, Jinju 660-701, Republic of Korea
\(^b\) Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea
\(^c\) Faculty of Plant Biotechnology, Dong-A University, Busan 604-714, Republic of Korea

**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.

**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 (CaMBPs) 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...
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 horse-radish 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) (Val458le) in binding buffer (100 mM Tris-HCl [pH 7.2], plus 0.1 mM CaCl2 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]: 

\[ K_d = \frac{[P]}{[CaM]} \times K \]

where \([P]\) 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-Cub-RUra3, replacing yeast Ste14. AtCaM2 cDNA was cloned into modified versions of the pCup-NUb-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 \(\gamma^{32P}\)-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 \(\gamma^{32P}\)-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 (Mg²⁺ or Mn²⁺) and substrate proteins.

2.7. Mass spectrometric analysis of phosphopeptides using TiO₂ 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% trifluoracetic acid) and passed through a TiO₂ microcolumn [20,21]. The phosphopeptides were eluted with NH₄OH (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% trifluoracetic 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 Val601 and Ile618 (Fig. 1A). Within this 18-amino acid stretch, hydrophobic amino acids are present at positions 1 (Val601), 8 (Trp608), and 14 (Ile614). Several basic residues (two lysines and two arginines) are interspersed between these hydrophobic residues, which is a known structural characteristic of Ca2+-dependent CaM-binding motifs. A helical wheel projection of the CBRLK1 CaMBD (18 amino acids, Val601 to Ile618) 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 Ca2+ but not in the absence of Ca2+ (Fig. 1B). These results indicate that CaM binds to a CaMBD located in the C-terminus region of CBRLK1 in a Ca2+-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 Val601 to Ile618, 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 Ca2+ and was undetectable in the presence of EGTA. This result is consistent with data from a previous CaM overlay assay under similar Ca2+ 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 Ca2+/CaM-dependent enzyme, was performed. To determine the Kd 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 (Km) in the absence and presence of the 18-mer peptide were 10.9 nM and 40 nM, respectively, a 4-fold difference. The Kd 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-
bly of N- and C-terminal halves (Nub and Cub) of ubiquitin (Ub), was used [17,18]. CaM and full-length CBRLK1 were fused to the C-terminus of Nub and the N-terminus of Cub, respectively. The Cub of ubiquitin was linked to an N-terminally modified Ura3p reporter containing Arg at position 1 (RUra3p). When CaM interacts with the CBRLK1 protein, Nub and Cub 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-Cub-RUra3p and Nub-CaM are unable to grow on plates lacking uracil but are able to grow on plates containing 5-FOA [15]. No interactions were observed between Cub-RUra3p and Nub-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 MgCl2 and MnCl2 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 MgCl2, followed by a plateau of the autophosphorylation at more than 10 mM MgCl2. MnCl2 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 IIIa). All proteins were clearly phosphorylated by the CBRLK1 KD (Fig. 4B). To confirm the residue specificity of CBRLK1 autophosphorylation, phosphoamino acid
assays were performed by using an autophosphorylated CBRLK1 KD fusion protein. In these assays, serine and threonine residues 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, TiO2 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 TiO2 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 CBRLK1-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, TiO2 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 TiO2 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.

### Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Amino acids</th>
<th>Number of phosphate groups</th>
<th>Expected</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(M+H)+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PSQVREFPYHRYV</td>
<td>545–562</td>
<td>1</td>
<td>2000.96</td>
<td>2001.00</td>
</tr>
<tr>
<td>2</td>
<td>DLKANLLDKLRPF</td>
<td>637–652</td>
<td>1</td>
<td>1876.94</td>
<td>1876.99</td>
</tr>
<tr>
<td>3</td>
<td>LQGGLHR</td>
<td>653–660</td>
<td>1</td>
<td>958.44</td>
<td>958.46</td>
</tr>
<tr>
<td>4</td>
<td>TPQKEVVR</td>
<td>661–673</td>
<td>1</td>
<td>1573.69</td>
<td>1573.75</td>
</tr>
<tr>
<td>5</td>
<td>PQQKKEVVR</td>
<td>661–673</td>
<td>2</td>
<td>1653.66</td>
<td>1653.71</td>
</tr>
<tr>
<td>6</td>
<td>RSGGTVQKPLQY</td>
<td>712–731</td>
<td>1</td>
<td>2412.04</td>
<td>2412.04</td>
</tr>
<tr>
<td>7</td>
<td>NGQFQKPLQY</td>
<td>713–731</td>
<td>1</td>
<td>2255.94</td>
<td>2255.96</td>
</tr>
<tr>
<td>8</td>
<td>SRTGVQKPLQY</td>
<td>802–816</td>
<td>1</td>
<td>1671.70</td>
<td>1671.79</td>
</tr>
<tr>
<td>9</td>
<td>GSNVQKPLQY</td>
<td>817–826</td>
<td>1</td>
<td>1126.55</td>
<td>1126.59</td>
</tr>
<tr>
<td>10</td>
<td>ANIMNQKPLQY</td>
<td>817–830</td>
<td>1</td>
<td>1553.81</td>
<td>1553.88</td>
</tr>
<tr>
<td>11</td>
<td>ANIMNQKPLQY</td>
<td>817–830</td>
<td>2</td>
<td>1633.77</td>
<td>1633.83</td>
</tr>
</tbody>
</table>

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 Brassica oleracea, CLV1 and AtCaMRLK from Arabidopsis, were reported [13,14]. The CaMBD of SRK was identified in the subdomain Va 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 Ca2+-dependent CaMBD of CBRLK1 was located in subdomain Va of the kinase domain. This finding was confirmed by three different CaM binding analyses. First, the Ca2+-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 Val601 to His619) (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 EGF [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 been 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.

Acknowledgments

This work was supported by a Grant (FP060303-01) from the PDRC of the 21st Century Frontier Research Program and a BK-21 program funded by the Rural Development Administration. H.S.K., M.S.J. and K.E.K. were supported by scholarships from the BK21 program of the Ministry of Education, Science and Technology. H.S.K. was partially supported by the Seoul Science Fellowship Fund.

Appendix A. Supplementary data


References


