C-terminal extension of calmodulin-like 3 protein from *Oryza sativa* L.: interaction with a high mobility group target protein

Aumnart Chinpongpanich¹, Srivilai Phean-O-Pas¹, Mayura Thongchuang², Li-Jia Qu³,⁴, and Teerapong Buaboocha¹,*

¹Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand, ²Division of Food Safety Management and Technology, Department of Science, Faculty of Science and Technology, Rajamangala University of Technology Krungthep, Bangkok 10120, Thailand, ³National Laboratory for Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China, and ⁴National Plant Gene Research Center (Beijing), Beijing 100101, China

*Correspondence address. Tel: +662-2185436; Fax: +662-2185418; E-mail: teerapong.b@chula.ac.th

Received 3 June 2015; Accepted 28 July 2015

Abstract

A large number of calmodulin-like (CML) proteins are present in plants, but there is little detailed information on the functions of these proteins in rice (*Oryza sativa* L.). Here, the CML3 protein from rice (*OsCML3*) and its truncated form lacking the C-terminal extension (*OsCML3m*) were found to exhibit a Ca²⁺-binding property and subsequent conformational change, but the ability to bind the CaM kinase II peptide was only observed for OsCML3m. Changes in their secondary structure upon Ca²⁺-binding measured by circular dichroism revealed that OsCML3m had a higher helical content than OsCML3. Moreover, OsCML3 was mainly localized in the plasma membrane, whereas OsCML3m was found in the nucleus. The rice high mobility group B1 (OsHMGB1) protein was identified as one of the putative OsCML3 target proteins. Bimolecular fluorescence complementation analysis revealed that OsHMGB1 bound OsCML3, OsCML3m or OsCML3s (cysteine to serine mutation at the prenylation site) in the nucleus presumably through the methionine and phenylalanine-rich hydrophobic patches, confirming that OsHMGB1 is a target protein in planta. The effect of OsCML3 or OsCML3m on the DNA-binding ability of OsHMGB1 was measured using an electrophoretic mobility shift assay. OsCML3m decreased the level of OsHMGB1 binding to pUC19 double-stranded DNA whereas OsCML3 did not. Taken together, OsCML3 probably provides a mechanism for manipulating the DNA-binding ability of OsHMGB1 in the nucleus and its C-terminal extension provides an intracellular Ca²⁺ regulatory switch.

Key words: calcium, calmodulin (CaM), cell signaling, DNA–protein interaction, high mobility group

Introduction

Calcium signals play an important role in the response and adaptation to a variety of stimuli, including light, abiotic and biotic stresses, and hormones [1]. Changes of intracellular calcium levels are transduced through the stimulation of calcium ion (Ca²⁺) sensor proteins, one of which is the small multifunctional protein calmodulin (CaM) that binds Ca²⁺ and alters the activity of a large number of target proteins in eukaryotes including plants. Structurally, CaM has two globular domains that are connected by a flexible α-helix, and each globular domain contains two Ca²⁺-binding sites (EF-hands). Functionally, CaM regulates a variety of target proteins, including kinases, metabolic proteins, cytoskeletal proteins, ion channels and pumps, and transcription...
C-terminal extension of CML3 from *Oryza sativa* L.  

881

Factors [2]. Specific biological functions of plant CaMs are not well known [3], but plants possess a large family of unique CaM-like Ca$^{2+}$-binding proteins (CMLs), most of which contain an EF-hand Ca$^{2+}$-binding motif with no other identifiable functional domains. A large family of 50 CML genes in *Arabidopsis* (AtCML) [4] and 32 CML genes [5] in rice (*OsCML*) have been identified from their annotated genomes. Previously, AtCML24 (one of the first CMLs characterized) was shown to play a role in ion homeostasis, photoperiod-response, and abscisic acid-mediated inhibition of germination and seedling growth [6]. Moreover, AtCML43 and AtCML18 had been implicated in pathogen responses [7] and salinity tolerance [8], respectively. AtCML8 expression was shown to respond to salicylic acid in *A. thaliana* seedlings [9]. AtCML37, AtCML38, and AtCML39 play important roles as sensors in Ca$^{2+}$-mediated developmental and stress response pathways [10]. These reports suggest that CMLs likely have diverse functions to interpret Ca$^{2+}$ signals during development and stress responses.

In rice (*O. sativa*), 3 OsCaM and 32 OsCML proteins have been classified [5]. They are small proteins, consisting of 145–250 amino acid residues, and share an amino acid identity of 30.2–84.6% with OsCaM1. However, there is little information on the functional properties, subcellular localization, and transcriptional expression in different tissues and organelles, although OsCaM and OsCML genes showed differential expression patterns in different rice tissues or osmotic stresses [11]. OsCML31 (alias OsMSR2) was shown to confer enhanced tolerance to salt stress in transgenic *Arabidopsis* [12] and rice [13]. Interestingly, three OsCMLs contain a basic C-terminal extension (CTE) with a putative prenylation CAAX motif (C is cysteine, A is aliphatic, and X is a variety of amino acids) found as CML in OsCML1 and CML in OsCML2 and OsCML3. OsCML1 (alias OsCaM61) [14] was reported to be membrane-associated when it was prenylated and localized in the nucleus when it was un-prenylated [15]. A similar protein, PhCaM53 in petunia was also reported to depend on its prenylation state.

As mentioned, there have been many CML-binding proteins identified in plants [17], but no report of CML-binding proteins in rice is available. Here, the difference in biophysical characteristics and subcellular localization of OsCML3 and a truncated version of OsCML3 lacking the CTE (OsCML3m) were examined to investigate the effect of the CTE. To investigate the function of OsCML3, the rice high mobility group B1 (OsHMGB1), as one of the OsCML3-binding proteins, was identified and the interaction was verified using bimolecular fluorescence complementation (BiFC) assays. HMGB1 is a DNA-binding protein that contains a non-specific sequence DNA-binding domain, which can bind to four-way junctions, cisplatin-modified DNA and DNA minicircles [18]. HMGB1 functions as an architectural factor to facilitate the assembly of nucleoprotein complexes, and other DNA-dependent processes, which are involved in the regulation of transcription, recombination, and DNA repair [19,20–23]. Electrophoretic mobility shift assay (EMSA) was used to examine the DNA-binding ability of OsHMGB1 in the presence of OsCML3 or OsCML3m. A possible mechanism of the regulation of DNA-binding ability of OsHMGB1 via the action of OsCML3 was proposed.

**Materials and Methods**

Sequence retrieval and analysis

Nucleotide and amino acid sequences were obtained from the GenBank database via the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) [24], and the rice databases, including the MSU Rice Genome Annotation Project database [25] and the Rice Annotation Project Database [26]. Subcellular localization prediction was performed using the predicting plant protein subcellular location (Plant-PLoc) [27–30], Subnuclear Compartments Prediction System (Version 2.0) [31,32], and WoLF PSORT [33,34] programs.

Cloning, expression, and purification of recombinant OsCML3 and OsCML3m proteins

For OsCML3, the previously reported expression clone (OsCML3) [35] was used. The truncated clone of OsCML3 that lacked the sequence encoding the CTE, termed OsCML3m, was constructed by polymerase chain reaction (PCR) using the cDNA clone of the OsCML3 gene (AK111834) obtained from the DNA Bank of NIAS (Nagasaki Institute of Applied Science, Nagasaki, Japan) as the template and the OsCML3m primers (Table 1). PCR was performed using Phusion DNA polymerase (New England Biolabs, Ipswitch, USA) with thermal cycling for 30 cycles of 94°C for 5 min, 63°C for 1 min and 72°C for 1 min, and then a final 72°C for 10 min. The PCR product was cleaved and cloned into pET21a (Novagen, Darmstadt, Germany) using the selected restriction enzymes (Ndel and XhoI). The resulting selected clone was confirmed by DNA sequencing. Induction of recombinant protein production was performed in *Escherichia coli* BL21 (DE3) for 4 h by isopropyl β-D-thiogalactoside (IPTG) with a final concentration of 0.3 mM. The cells were then harvested, resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid (EDTA), sonicated and then centrifuged (27,000 × g for 30 min at 4°C). Protein purification was performed using hydrophobic chromatography on C-terminal extension of CML3 from *Oryza sativa* L.

**Table 1. Primers used for PCR in this study**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5′→3′)</th>
<th>Reverse (5′→3′)</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsCML3m</td>
<td>CATATGGACCACTCTGACAAAA</td>
<td>ATTCGAGTCTCATCGCTTTGCC</td>
<td>Protein expression</td>
</tr>
<tr>
<td>OsHMGB1</td>
<td>CACCATGAAAGGGGGCAATCCC</td>
<td>TCAGAGATGGTACATGGAGG</td>
<td>BiFC assay</td>
</tr>
<tr>
<td>OsCML3</td>
<td>CACCATGACCAACTGACAAAAAGG</td>
<td>TCAGAGATGGTACATGGAGG</td>
<td>BiFC assay</td>
</tr>
<tr>
<td>OsCML3s</td>
<td>CACCATGACCACTCTGACAAAAAGG</td>
<td>TCAGAGATGGTACATGGAGG</td>
<td>BiFC assay</td>
</tr>
<tr>
<td>OsHMGB1s</td>
<td>GGAATTCATATGAAGGGGGGCAATCCC</td>
<td>CGGTCTGGTCATCTGCTGCATC</td>
<td>EMSA</td>
</tr>
<tr>
<td>GFP</td>
<td>CATATGCTAGATCTGAAGTAAGGA</td>
<td>GGTCAACATACCTGCTGGTGGTGG</td>
<td>Subcellular localization</td>
</tr>
<tr>
<td>OsCML1</td>
<td>CTCAGGAGGTGGTGCTGGCACTGAGG</td>
<td>GGTCAACATACCTGCTGGTGGTGG</td>
<td>Subcellular localization</td>
</tr>
<tr>
<td>OsCML1m</td>
<td>CTCAGGAGGTGGTGCTGGCACTGAGG</td>
<td>GGTCAACATACCTGCTGGTGGTGG</td>
<td>Subcellular localization</td>
</tr>
<tr>
<td>OsCML3</td>
<td>CTCAGGGGGGGGAGGAAATGGACC</td>
<td>GGTCAACATACCTGCTGGTGGTGG</td>
<td>Subcellular localization</td>
</tr>
</tbody>
</table>
phenyl-Sepharose (Amersham, Little Chalfont, UK). Binding and washing were performed and proteins were eluted as previously reported [35]. All proteins samples were analyzed by 12% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) and then stained with Coomassie blue.

**Electrophoretic shift analysis**
To analyze the recombinant proteins by electrophoresis shift assay, a final concentration of 1 mM CaCl₂ or 3 mM ethyleneglycoltetraacetic acid (EGTA) was added to each protein solution (500 pmole), mixed and then resolved by SDS-PAGE with a 12.5% (w/v) acrylamide resolving gel. Proteins were then detected by Coomassie blue staining. To examine their peptide-binding ability, each protein (200 pmole) was mixed with the peptide derived from CaMKII (Sigma, St Louis, USA) at different molar equivalents and then analyzed as previously described [35].

**Circular dichroism (CD) spectroscopy**
CD spectroscopy was performed at 25°C in a J-715 Spectropolarimeter (Jasco, Easton, USA) with constant N₂ flushing. The far-ultraviolet (UV) CD spectrum was measured from 190 to 250 nm in 1 mM Tris–HCl (pH 7.5) and 1 mM KCl in the presence of 1 mM CaCl₂ or 1 mM EGTA. The final protein concentration was 10 μM. All measurements were performed within 30 min after sample preparation, using a 1-mm-path-length quartz cell with a 1 s response time, 50 mdeg sensitivity, 50 nm/min scan speed, and a 2.0 nm spectral bandwidth. The average of three scans was taken. The secondary structure of the protein was estimated using K2D3 method (http://k2d3.ogc.ca/index.html) [37].

**Fluorescence measurement**
Measurement of the fluorescence emission spectra of 8-anilino-1-naphthalenesulphonate (ANS; Sigma) was performed on an LS55 Luminescence Spectrometer (PerkinElmer, Waltham, USA) at 25°C. Fluorescence emission spectra were monitored with an excitation wavelength of 370 nm and emission spectra in the range 400–650 nm were scanned. All measurements were performed using 1 μM of protein in 1 mM Tris–HCl (pH 7.5)/1 mM KCl with ANS at a final concentration of 100 μM in the presence of 1 mM CaCl₂ or 1 mM EGTA.

**Screening of rice cDNA expression library**
The ³⁵S-labeled purified rOsCML3 and rOsCML3m proteins were prepared as probes to screen a rice cDNA expression library. To construct the library, polyadenylated RNA was purified from the ‘KDML105’ rice total RNA using the GenElute mRNA Miniprep kit (Sigma) and then used as template for cDNA synthesis using the cDNA synthesis kit (Stratagene, La Jolla, USA). The Uni-ZAP XR Vector kit (Stratagene) was used to ligate the prepared rice cDNA to the lambda vector, resulting in the primary library. Primary, secondary, and tertiary screenings of the amplified library were performed. Single clones were excised and analyzed with the PstI restriction enzyme. All unique pBluescript SK(+) plasmids obtained from the single-clone excision were sequenced. The obtained sequences were BLAST-searched against the Rice Genome Annotation Project and Rice Annotation Project databases to identify the cloned cDNA inserts.

**Subcellular localization**
To construct the pCAMBIA1302 containing either OsCML3 or OsCML3m fused with green fluorescent protein (GFP) at the N-terminal end, the coding region of OsCML3, OsCML3m, and GFP were amplified using the primers shown in Table 1. All PCR reactions were performed using Vent DNA polymerase (New England Biolabs) with 30 cycles of 94°C for 1 min, 59.3°C for 1 min and 72°C for 1 min and 30 s, and then followed by a final 72°C for 10 min. The resulting amplicons were cloned into the T&A cloning vector (RBC Bioscience Co., Taiwan, China) to give pTA-OsCML3, pTA-OsCML3m, and pTA-OsGFP, respectively. The gene fragments of OsCML3 and OsCML3m were individually inserted into pTA-OsGFP via the XhoI and BstEII sites at the 3’ end of the GFP coding sequence, resulting in pTA-GFP-OsCML3 and pTA-GFP-OsCML3m, respectively. The fragments of GFP-OsCML3 and GFP-OsCML3m were sub-cloned into pCAMBIA1302 using NcoI and BstEII sites, resulting in pCAMBIA-GFP-OsCML3 and pCAMBIA-GFP-OsCML3m, respectively. The pCAMBIA1302 plasmids containing GFP-OsCML1 or GFP-OsCML1m were also constructed and used for comparison. Each recombinant plasmid was introduced into Agrobacterium tumefaciens strain GV3101 by heat shock. The solution of Agrobacterium was then infiltrated into the leaf of tobacco plants and the plants were grown in the dark for 16 h followed by 48 h in the growth chamber. Confocal microscopy was performed with a Leica SPE microscope (Leica, Solms, Germany) using an excitation wavelength of 488 nm.

**BIFC assay**
The gene fragments of OsHMGB1 (AK062226), OsCML3, OsCML3m, and OsCML3s (serine-to-cysteine mutation at the prenylation site) were amplified by PCR using individual cDNA clones from the DNA Bank of NIAS (Nagasaki Institute of Applied Science) and the respective primer pairs (Table 1). All PCR reactions were performed using KOD DNA polymerase (Toyobo, Tokyo, Japan) with 35 cycles of 94°C for 1 min, 59°C for 45 s and 68°C for 1 min, and then followed by a final 68°C for 10 min. PCR products were ligated into the pENTR vector (Invitrogen, Carlsbad, USA) via a TOPO reaction, resulting in pENTR-HMGB1, pENTR-CML3, pENTR-CML3m, and pENTR-CML3s, respectively. Then, the pcCFPwG construct [38] was used to generate pcCFP-CML3, pcCFP-CML3m, and pcCFP-CML3s by LR Clonase™ II enzyme mix (LR recombination reactions) between pcCFPwG and pENTR-CML3 or pENTR-CML3m or pENTR-CML3s, respectively, and prepared as above. The pcYFPwG construct [38] was also used to construct pcYFP-HMGB1 by a similar reaction between pcYFPwG and pENTR-HMGB1. Each pair of plasmids of pcCFP-CML3 and pcYFP-HMGB1, or pcCFP-CML3m and pcYFP-HMGB1, or pcCFP-CML3s and pcYFP-HMGB1 was then co-transformed into Agrobacterium strain GV3101. The mixtures of the two Agrobacterium strains: GV3101 (OD₆₀₀ = 0.5) and p19 (OD₆₀₀ = 0.3) were co-infiltrated into the leaf of 6-week-old tobacco plants. The treated plants were grown in the dark for 16 h followed by 48 h in the growth chamber [38]. Confocal microscopy was performed with a Fluoview FV10i (Olympus, Tokyo, Japan) using an excitation wavelength of 488 nm.

**Electrophoretic mobility shift assay (EMSA)**
To generate the recombinant plasmid encoding HMGB1 fused with a His₅-Tag at the N-terminal end, PCR amplification by Phusion DNA polymerase (New England Biolabs) was performed using the cDNA clone for OsHMGB1 as the template and the primer pair shown in Table 1. The PCR consisted of 30 cycles of 98°C for 7 s, 53°C for
Results

The 35-amino-acid CTE of OsCML3 interferes with its target protein binding

When the deduced amino acid sequences of *Oryza sativa* CaM-Like 3 (OsCML3) and its CTE-truncated form (OsCML3m) were compared by multiple sequence alignment with that of the rice CaM, OsCaM1, they were found to be highly similar (Fig. 1). All these proteins have four EF-hand domains and OsCML3 and OsCML3m shared 55.4% and 68.5% amino acid sequence identities with OsCaM1, respectively. In addition, OsCML3 contains a basic CTE domain with a putative prenylation CaaX (C is cysteine, A is aliphatic, and X is a variety of amino acids) motif (CTIL in OsCML3) [41].

To examine the role of the CTE, the rOsCML3m was produced and purified by Ca²⁺-dependent hydrophobic chromatography using phenyl-Sepharose as shown in Fig. 2A. The apparent molecular weight (Mₚ) derived from SDS-PAGE resolution, corresponded to the predicted Mₚ from the deduced amino acid sequence. One of the characteristics of a typical CaM is its ability to bind Ca²⁺ in the presence of SDS, which increases its electrophoretic mobility relative to that in the absence of Ca²⁺. The rOsCML3m displayed this characteristic mobility shift when incubated prior to electrophoresis with 1 mM CaCl₂ compared with that with 3 mM EGTA (Fig. 2C). The degree of mobility shift of rOsCML3m appeared to be similar to that of the full-length OsCML3 as shown in Fig. 2B. When incubated with the CaM kinase II (CaMKII) peptide, a CaM-binding protein, rOsCML3m displayed a peptide–protein complex in the presence of Ca²⁺ (Fig. 2E), whereas rOsCML3 did not (Fig. 2D). In the absence of Ca²⁺, both proteins showed no binding ability to the peptide (data not shown). These results suggest that rOsCML3 has structural regions for binding the CaMKII peptide similar to those in CaM, but that the CTE domain appeared to obstruct this interaction.

The helical content upon Ca²⁺ binding of OsCML3 is affected by CTE

Far-UV CD spectrum was used to study whether secondary structure changes of OsCML3m occurred upon Ca²⁺ binding. Previously, major conformational changes, including an increase in α-helix upon Ca²⁺-binding, have been documented for CaM [42,43]. The far-UV CD spectra of OsCML3 (Fig. 3A) and OsCML3m (Fig. 3B) in the presence of 1 mM CaCl₂ or 1 mM EGTA had two minima near 208 and 222 nm indicating that both proteins contain substantial α-helical secondary structure. The molar ellipticity per residue for n amino acid residues [θ]ₙ at 222 nm of rOsCML3m from the spectra in the presence of 1 mM CaCl₂ or 1 mM EGTA with their calculated alpha helicity [37] and changes upon Ca²⁺ addition in comparison with that of rOsCML3 are summarized in Table 2, where Δ(θ)₂₂₂ and Δ(θ)₂₀₈ are the absolute and percentage difference in [θ]ₙ at 222 nm between the presence and absence of Ca²⁺. Upon Ca²⁺ addition, an increase in [θ]ₙ at 222 nm was clearly observed for rOsCML3m with a 68.7% change while rOsCML3 showed a much smaller change in [θ]ₙ (10.5%). These results indicate that the helical content is highly increased in rOsCML3m protein upon Ca²⁺ binding.

In this study, ANS was used to measure the Ca²⁺-induced exposure of hydrophobic patches in the globular domains because its fluorescence spectrum is changed and can be monitored when it binds to the accessible hydrophobic surface of the proteins. The emission spectra of ANS when mixed with rOsCML3 [35] or rOsCML3m in the presence of Ca²⁺ or EGTA are shown in Fig. 3C,D, respectively. Table 3 summarizes the changes in ANS fluorescence in the presence of rOsCML3m upon Ca²⁺ addition in comparison with those of rOsCML3. When mixed with each protein in the presence of EGTA, ANS displayed a relatively weak fluorescence with a maximum

---

**Figure 1. Comparison of the amino acid sequences of OsCML3 and OsCML3m with that of OsCaM1 by multiple sequence alignment**  The sequences are compared with OsCaM1 as a standard; identical residues are indicated by a dash (−), and a gap introduced for alignment purposes is indicated by a dot (.). Residues serving as Ca²⁺-binding ligands in the EF-hand motifs (EF-hand 1–4) are marked with an asterisk (*). Methionine (M) and Phenylalanine (F) residues and the polybasic C-terminal extension (CTE) are shown in bold and italic, respectively. The CaaX box of OsCML3 for prenylation is underlined.
wavelength near 520 nm, which was almost identical to that of ANS alone. In the presence of Ca²⁺, a significant blue shift (46 and 47 nm for rOsCML3 or rOsCML3m, respectively) in the maximum emission wavelength was observed. Similar large increases in the fluorescence intensity of rOsCML3 (4.75-fold) and rOsCML3m (5.31-fold) were observed, suggesting that the 35-amino-acid CTE does not impede the exposure of its hydrophobic surface upon Ca²⁺ binding. However, in agreement with its smaller change in the increased helical content...
OsCML3 is localized to the plasma membrane via the CTE
OsCML1 (alias OsCaM61), the CML protein most similar to OsCML3 [14], was reported to be membrane-associated when prenylated and localized in the nucleus when unprenylated [15]. OsCML3 also has a potential prenylation site in the CTE domain, as described above, which may function in membrane association similar to OsCML1. To test whether the basic CTE domain containing the prenylation site played such a role in OsCML3, the cellular localization of the CTE-truncated OsCML3 protein compared with the full-length protein (OsCML3) was determined. By bioinformatics analysis using PlantLoc, WoLF PSORT, and Plant-mPLoc, OsCML3 and OsCML3m were predicted to localize in several compartments (data not shown), but the actual localization of OsCML3 and OsCML3m could not be concluded from these predictions. To examine the localization of OsCML3 and OsCML3m in planta, the pCAMBIA-GFP-OsCML3 and pCAMBIA-GFP-OsCML3m fusion constructs were individually introduced into A. tumefaciens strain GV3101, and then into tobacco leaf cells. The green fluorescence signal of GFP-CML3, which contained the CTE and putative prenylation site, was mostly observed in the plasma membrane of tobacco cells, while the GFP signal of GFP-CML3m (lacking the CTE and predicted prenylation site) was found in both the cytoplasm and the nucleus (Fig. 4).

OsCML3 interacts with OsHMGB1, a novel target for a Ca2+-binding protein, in the nucleus
To identify target proteins of OsCML3, cDNA expression libraries were prepared from the leaf of Oryza sativa L. ‘Khoa Dok Ma Li 105’, and used for screening against rOsCML3m and rOsCML3 as described above. The results revealed that one of the ten putative novel OsCML3m-binding proteins was OsHMGB1. However, when the full-length rOsCML3 was used as the probe, only two target proteins were identified and these did not include OsHMGB1. Since chromosomal HMGB1 proteins are generally considered to be nuclear proteins [44,45]. Although there is no experimental evidence on the subcellular localization of OsHMGB1, the theoretical predictions using three subcellular localization prediction programs similarly indicate that the OsHMGB1 protein is mainly localized in the nucleus.

The interaction between OsCML3 and its putative target, OsHMGB1 in planta, was evaluated using BifC [46]. The N-terminal fragment of yellow fluorescent protein (YFP) was fused with the N-terminal end of OsHMGB1 (YFP-HMGB1), while the C-terminal fragment of cyan fluorescent protein (CFP) was fused with the N-terminal end of OsCML3, OsCML3m or OsCML3s to yield CFP-CML3 or CFP-CML3m or CFP-CML3s, respectively. A green fluorescence signal was clearly observed in all combinations (OsHMGB1 with OsCML3, OsCML3m or OsCML3s) in the nucleus (Fig. 5), confirming the interaction between OsCML3 and OsHMGB1 in planta. The interaction in the nucleus possibly occurred through the nuclear localization signal (NLS) of OsHMGB1. The N-terminal fragment of YFP or the C-terminal fragment of CFP, used as a negative control, yielded no fluorescent signal in leaf cells co-infiltrated with YFP and CFP-CML3, CFP-CML3m or CFP-CML3s, nor with YFP-HMGB1 and CFP.

OsCML3m but not OsCML3 inhibits OsHMGB1 binding to supercoiled DNA
The supercoiled DNA-binding property of rOsHMGB1 was examined by incubating supercoiled pUC19 plasmid (100 ng) with increasing concentrations of rOsHMGB1 (0–3 μM) in the presence of Ca2+. The use of supercoiled DNA was to mimic the structure of DNA within the cells, which is packed and required for DNA/RNA synthesis. The pUC19 DNA interacted with rOsHMGB1 was shown by the resolved DNA bands of lower electrophoretic mobility of the protein–DNA complex compared with that of the free DNA (Fig. 6A). The effect of rOsCML3 or rOsCML3m upon the DNA-binding ability of rOsHMGB1 was evaluated by incubation of 1.0 μM of rOsHMGB1 and 100 ng of pUC19 at room temperature for 10 min and then adding increasing concentrations of rOsCML3 or rOsCML3m (0–2 μM) in the presence of Ca2+. With increasing concentrations of rOsCML3, no effect on

---

### Table 2. Far-UV CD measurements of OsCML3 and OsCML3m

<table>
<thead>
<tr>
<th>Protein</th>
<th>[θ]_{222} \times 10^{3} (deg. cm^{2}/dmole. number of residues)</th>
<th>Δ[θ]_{222}</th>
<th>%Δ[θ]_{222}</th>
<th>Alpha helicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Ca^{2+}</td>
<td>+EGTA</td>
<td>+Ca^{2+}</td>
<td>+EGTA</td>
<td></td>
</tr>
<tr>
<td>OsCML3</td>
<td>16.31</td>
<td>−14.76</td>
<td>10.5</td>
<td>49.08</td>
</tr>
<tr>
<td>OsCML3m</td>
<td>22.73</td>
<td>−13.47</td>
<td>9.26</td>
<td>69.38</td>
</tr>
</tbody>
</table>

*aThe mean residue ellipticity at 222 nm.
*bData obtained from Chinpongpanich et al. [35] for comparison.

### Table 3. ANS fluorescence measurements of OsCML3 and OsCML3m

<table>
<thead>
<tr>
<th>Protein</th>
<th>Emission maximum (nm)</th>
<th>ΔI_{max}</th>
<th>I_{max} (+Ca^{2+})/I_{max} (+EGTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsCML3</td>
<td>468</td>
<td>46</td>
<td>87.27</td>
</tr>
<tr>
<td>OsCML3m</td>
<td>467</td>
<td>47</td>
<td>101.37</td>
</tr>
</tbody>
</table>

*The difference in the maximum emission of ANS in the presence of Ca^{2+} and in the presence of EGTA.
*bThe ratio of the maximum fluorescence intensity of ANS in the presence of Ca^{2+} to that in the presence of EGTA.
*cData obtained from Chinpongpanich et al. [35] for comparison.

---

Figure 4. Subcellular localization of GFP-OsCML3 and GFP-OsCML3m in tobacco leaf cells. The green fluorescence, bright field, and overlay images (60 x magnification) observed in tobacco leaf cells expressing the fusion proteins. Scale bar = 50 μm. Images shown represent those seen from at least 100 such fields of view per sample and five independent samples. Nucleus was indicated with white arrow.
rOsHMGB1 binding to pUC19 was detected, as a similar rOsHMGB1 mobility shift as that without the addition of rOsCML3 (Fig. 6B). In contrast, the electrophoretic mobility of the rOsHMGB1-pUC19 complex increased in a dose-dependent manner in the presence of rOsCML3m from 0.1 µM (Fig. 6C). Thus, only the CTE-truncated rOsCML3m affected the DNA-binding ability of rOsHMGB1. The inhibitory effect of the rOsCML3 was not observed in the absence of Ca²⁺ (data not shown). In addition, due to the high amino acid sequence identity shared with rOsCML3m, rOsCaM1 was tested under the same conditions to examine if rOsCaM1 could also interfere with the supercoiled DNA-binding ability of rOsHMGB1. However, like rOsCML3, rOsCaM1 had no effect on the rOsHMGB1 binding to supercoiled pUC19 (Fig. 6D), indicating that the truncated (CTE-free) rOsCML3m specifically affected the supercoiled DNA-binding ability of rOsHMGB1. Without rOsHMGB1, rOsCML3, rOsCML3m, and rOsCaM1 caused no mobility shift in the supercoiled pUC19 DNA (Fig. 6E–G).

**Discussion**

The use of Ca²⁺-dependent phenyl-Sepharose hydrophobic chromatography to successfully purify rOsCML3 [35] and rOsCML3m was based upon the presence of several methionines and other hydrophobic amino acids, which constitute the hydrophobic patches that are exposed in the Ca²⁺-bound form. Both OsCML3 and OsCML3m
C-terminal extension of CML3 from *Oryza sativa* L.

exhibited the characteristic electrophoretic gel mobility shift and similar changes in the ANS fluorescence spectra in the presence of Ca\(^{2+}\). These results suggest that the polybasic CTE had no apparent effect on the ability of OsCML3 to bind Ca\(^{2+}\) and to expose the hydrophobic regions.

Examination of the OsCML3 CD spectra showed a relatively small change in the \([\theta]_0\) at 208 and 222 nm upon Ca\(^{2+}\) binding, similar to that previously observed with OsCML1 [35]. In contrast, OsCML3m displayed a significant change, indicating a large increase in the helical content of OsCML3m upon Ca\(^{2+}\) binding. Within the CTE, the apo form of OsCML1 (alias OsCaM61) was previously reported to have an extended final helix compared with the holo form, but the Ca\(^{2+}\)-bound form seemed to have a much shorter final helix that became flexible in the Ca\(^{2+}\)-saturated protein [47]. OsCML3 was predicted to have an extended final helix as well (data not shown), but it was shorter than that of OsCML1 suggesting that, if both proteins behave similarly upon Ca\(^{2+}\) binding, the increased helical content of OsCML3 likely comes from other parts of the protein. Supported from previous reports, the increased helical content may be resulted from the central \(\alpha\)-helix becoming more structured upon Ca\(^{2+}\) binding [48], which may be inhibited by the presence of the CTE, thus the smaller change observed in the \([\theta]_0\) from full-length OsCML3. These results suggest that the CTE of OsCML3 has regulatory effects on Ca\(^{2+}\)-induced conformational changes, and in turn upon the specific binding of OsCML3 to its targets, in which the hydrophobic patches and the helices surrounding the Ca\(^{2+}\)-binding loops are very important [49–51].

Interestingly, OsCML3m interacted with the CaM-binding peptide derived from CaMKII while the full-length OsCML3 did not. Thus, the CaM-like region of OsCML3 could interact with the CaMKII peptide upon a Ca\(^{2+}\)-induced conformational change similar to typical CaMs, but the CTE appeared to obstruct this interaction suggesting that the CTE interacts with the rest of the protein. A previous nuclear magnetic resonance study indicated that the CTE of OsCML1 indeed interacted with the rest of the protein, leading to a decreased flexibility of this region [47]. The truncated OsCML1 without its CTE was shown to possess a higher ability to activate MLCK and CaMKII compared with that of the full-length OsCML1, supporting the inhibitory effect of the CTE [15].

For other target proteins, the CTE of OsCML1 has been reported to decrease the activation of phosphodiesterase [2]. In contrast, OsCML1 was reported to activate the CaM-binding protein kinases (OsCBKs) in a Ca\(^{2+}\)-dependent manner, while the CTE domain was not required for this effect [52]. These results suggest a differential activity of the CTE domain upon the binding ability of these CML proteins to different target proteins. Nonetheless, the CTE likely plays regulatory roles in the Ca\(^{2+}\)-modulated activity of these CML proteins. For OsCML3, to overcome the interfering effect of its CTE in vivo, we hypothesized that other proteins or mechanisms may be involved in the target binding of OsCML3, or that there are post-translational modifications of either OsCML3 or its target protein or both proteins to facilitate their interaction.

OsHMGB1 was found to be one of the putative targets of OsCML3m by screening a cDNA expression library with OsCML3m as the probe. Screening with the full-length OsCML3 identified only a few targets and not OsHMGB1, which conforms to the notion that the CTE interferes with target binding of OsCML3. The BiFC assay showed that OsHMGB1–OsCML3, OsHMGB1–OsCML3m, and OsHMGB1–OsCML3s interactions all occurred in the nucleus. The NLS of OsHMGB1 likely facilitated the movement of these protein complexes to the nucleus. Possibly, either unknown proteins or mechanisms are involved in exposing specific regions of OsCML3 for binding to OsHMGB1, or that post-translational modification of OsHMGB1 or OsCML3 facilitates the interaction. The latter scenario has been observed from the phosphorylation of maize HMGB1 by protein kinase CK2 that abolishes its interaction with transcription factor DoF2 and the stimulation of DoF2 DNA binding [53] or the methylation and phosphorylation of CaM, which affect its activity for binding to target proteins [54–56].

In general, HMGB proteins are known to play a role in the regulation of transcription and other DNA-dependent processes [19,21,45], and OsHMGB was observed to accumulate in cold-treated rice seedlings [57] while over-expression of AtHMGB2 reduced seed germination under dehydration conditions in *Arabidopsis* [58]. These results indicated that HMGB proteins likely play a role in the responses to various stresses, possibly through DNA-binding dependent processes. Here, the examination of the effect of OsCML3 or OsCML3m on the binding of OsHMGB1 to supercoiled DNA revealed that OsCML3m decreased the binding of OsHMGB1 and supercoiled pUC19 DNA in the presence of Ca\(^{2+}\), while OsCML3 did not. These findings indicated that in vitro the CTE of OsCML3 inhibits its binding ability to OsHMGB1. Since the interaction between OsCML3 and OsHMGB1 was observed in the nucleus, we speculate that, probably with the help of other proteins or unknown mechanisms, the inhibitory effect of the CTE is released leading to the interaction of OsCML3 with OsHMGB1 and the transport of the complex into the nucleus, where OsCML3 might function to regulate the DNA binding of OsHMGB1 in the nucleus and subsequently affect transcription and other DNA-dependent processes [19,21,45].

Overall, OsCML3 and OsCML3m both possessed Ca\(^{2+}\)-binding ability, but exhibited differences in their changes in the CD-spectra upon Ca\(^{2+}\) binding, where OsCML3m showed a larger increase in the helical content. It is suggested that the CTE affects the Ca\(^{2+}\)-induced conformational change of OsCML3. Exposure of the hydrophobic patches was observed for both OsCML3 and OsCML3m; however, the CTE of OsCML3 appeared to influence the Ca\(^{2+}\)-induced conformational change in such a way that OsCML3 cannot interact with OsHMGB1 (identified here as a novel target for Ca\(^{2+}\) sensor proteins). In planta, OsHMGB1 interacted with OsCML3m as well as with full-length OsCML3 in the nucleus, suggesting a mechanism for releasing the inhibitory effect of the CTE exists in the cytosol. OsCML3 is then likely brought into the nucleus through the NLS of OsHMGB1. Moreover, the supercoiled DNA-binding ability of OsHMGB1 was interfered by the presence of OsCML3m in the presence of Ca\(^{2+}\). Taken together, OsCML3 may provide a mechanism for manipulating the DNA-binding ability of OsHMGB1 in the nucleus with the CTE providing an intracellular Ca\(^{2+}\) regulatory switch.

Acknowledgements

We would like to thank Assistant Professor Dr Kuakarun Krusong, Department of Biochemistry, Faculty of Science, Chulalongkorn University for advice and Dr Robert Bucher, PCU, Faculty of Science, Chulalongkorn University, for useful comments, and English proof-reading and editing.

Funding

This work was supported by Thailand Research Fund (No. BRG5680019) to T.B. A.C. was supported by the Royal Golden Jubilee Ph.D. program-RGJ (No. 4.C/53.G.1/P.XX) from the
References

8. Yamaguchi T, Aharon GS, Sottosanto JB, Blumwald E. Vacuolar Na+/H+ pump pot Endowment Fund, Chulalongkorn University and Thailand Academic Development Project. Additional support was provided to T.B.
9. Yamaguchi T, Aharon GS, Sottosanto JB, Blumwald E. Vacuolar Na+/H+ pump Endowment Fund, Chulalongkorn University and Thailand Academic Development Project. Additional support was provided to T.B.


