# Identification of non-specific lipid transfer protein-1 as a calmodulin-binding protein in *Arabidopsis*

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**Abstract Although non-specific lipid transfer proteins (nsLTPs) are widely present in plants, their functions and regulations have not been fully understood. In this report, *Arabidopsis* nsLTP1 was cloned and expressed to investigate its binding to calmodulin (CaM). Gel overlay assays revealed that recombinant nsLTP1 bound to CaM in a calcium-independent manner. The association of nsLTP1 and CaM was corroborated using CaM–Sepharose beads to specifically isolate recombinant nsLTP1 from crude bacterial lysate. The CaM-binding site was mapped in nsLTP1 to the region of 69–80 amino acids. This region is highly conserved among plant nsLTPs, implicating that nsLTPs are a new family of CaM-binding proteins whose functions may be mediated by CaM signaling.**



*Keywords:* Non-specific lipid transfer protein-1; Calmodulin; Calmodulin-binding protein; CaMBP-10

## 1. Introduction

Lipid transfer proteins (nsLTPs) are characterized by their abilities to transport lipids between membranes in vitro [1]. They are ubiquitous proteins that have been found in animals, plants, and microorganisms [2]. nsLTPs have been isolated and characterized from various plant tissues, including maize seedlings, spinach leaves, castor bean, and wheat. It has been implicated that plant nsLTPs are involved in a variety of biological processes, such as cutin formation, embryogenesis, defense reactions against phytopathogens, symbiosis, and plant adaptation to various environmental conditions [3]. However, it is not clearly understood how nsLTPs exert their effects and how nsLTPs are mediated in these processes [4,5].

As an important second messenger, calcium ($Ca^{2+}$) is involved in diverse physiological processes in plants. Calmodulin (CaM), which is the best characterized $Ca^{2+}$-binding protein, is known to be a primary transducer of intracellular $Ca^{2+}$ signals [6–8]. To initiate signal transduction, CaM interacts with a variety of protein targets, namely calmodulin-binding proteins (CaMBPs). Ultimately, the CaM signaling controls physiological processes [9]. Therefore, the identification and characterization of novel CaMBPs are crucial for better understanding of $Ca^{2+}$-mediated signaling and the molecular basis of CaM actions. In animals, a number of CaMBPs have been identified. They include metabolic enzymes, protein kinases, receptors, ion channel proteins and G-proteins [10]. In plants, a fast growing number of proteins have been characterized in recent years as CaM targets, such as kinesin [11], chloroplast chaperonin [12], chimeric calcium/calmodulin-dependent protein kinase [13], glutamate decarboxylase [14], SAURs [15], and membrane transporter-like protein [16]. More recently, 17 new CaMBPs were identified in *Arabidopsis* by Reddy et al. [17]. To our knowledge, the association of CaM and *Arabidopsis* nsLTPs has not yet been documented.

With the apparent molecular weight of 10 kDa, CaMBP-10 is a novel $Ca^{2+}$-independent CaMBP that was isolated from Chinese cabbage in our research group. It was found that CaMBP-10 binds to CaM to mediate the activities of CaM-stimulated target enzymes phosphodiesterase [18] and NAD kinase [19], and that CaMBP-10 is involved in the control of plant hormone responses [20–22] and photosynthesis [23]. Further, CaMBP-10 exhibits very high homologies with nsLTPs on both DNA and amino acid sequence levels. In addition, they have very similar biochemical and physical properties. Therefore, it was attempted to conclude that CaMBP-10 is a new member of the nsLTP family [24]. To demonstrate nsLTPs as new CaMBPs, we cloned and expressed *Arabidopsis* nsLTP1 for CaM-binding assays. It was revealed from the CaM gel overlay assay and CaM pulled-down assay that nsLTP1 binds to CaM in a $Ca^{2+}$-independent manner, which is in agreement with the CaM-binding result of CaMBP-10. The CaM-binding site was delineated to a conserved region of nsLTPs using truncation and deletion mutants of nsLTP1. Taken together, these results demonstrate that *Arabidopsis* nsLTP1 is a new CaM-binding protein, implying the regulation of nsLTP1 functions by calmodulin signaling.

## 2. Materials and methods

### 2.1. Materials

*Arabidopsis thaliana* was grown at 22 °C in a green house. The plasmid pET-32a(+) and *Escherichia coli* BL21(DE3) $trxB^-$ were purchased from Novagen. Bovine brain CaM and colloidal gold-labeling of CaM were prepared as previously described [25,27]. Monoclonal antibody against *Arabidopsis* nsLTP1 and colloidal gold-labeling of

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*Abbreviations:* nsLTP1, non-specific lipid transfer protein-1; CaM, calmodulin; BAA, amphiphilic α-helix; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenyl methylsulfonyl fluoride; RT, room temperature

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the antibody were prepared as described in [26,27]. CaM–Sepharose beads were prepared as described in [28]. All other reagents were purchased from Sigma.

### 2.2. Preparation of total RNA and RT-PCR

Total RNA was isolated from *Arabidopsis* leaves by the modified method of acid guanidium thiocyanate–phenol–chloroform RNA extraction [29]. Isolated RNA was treated with RNase-free DNase I (Promega) to remove contaminated genomic DNA. RNA quality was examined by electrophoresis in 1% agarose gels.

In order to clone nsLTP1 by PCR, *Arabidopsis* cDNA was prepared from the extracted RNA using oligo(dT) as the primer. To remove the signal peptide (1–26 amino acids), mature nsLTP1 primers were designed according to its coding sequence (GenBank Accession No. AY049296):

5′-CCATGG (*Nco*I) CGCTAAGCTGTGGCTCAGTTAAC-3′ and

5′-CTCGAG (*Xho*I) TCATCACCTCACGGTTTTGCA-3′.

*Nco*I/*Xho*I restriction sites in pET-32a(+) were used for cloning.

### 2.3. Construction of nsLTP1 fragments

Full-length of mature nsLTP1 and three truncation or deletion mutants were constructed as follows: FT, for the full length of mature nsLTP1; nsLTP1(1–68), for the fragment of mature nsLTP1 1–68 amino acids; nsLTP1(1–80), for the fragment of mature nsLTP1(1–80) amino acids; and nsLTP1(ΔBAA), for the nsLTP1 construct with 45–58 amino acids deleted. The region of 45–58 amino acids was predicted to be a potential CaM-binding domain by sequence alignment with known amphiphilic α-helices (BAA) CaM-binding motifs. nsLTP1(1–68) and nsLTP1(1–80) were generated by PCR and subcloned into sites in pET-32a(+) for protein expression in bacteria. Deletion of 45–58 amino acids was performed using the overlapping extension PCR technique as described in [30] with the primers of ΔBAA forward 5′-GGCTCTGGTCTCAACGCAGGCCGTGCAGC-3′ and ΔBAA reverse 5′-GCGTTGAGACCAGAGCCACGGTCTGGGGTCGTCTTGG-3′. The sequences were cloned in the vector pET-32a(+) and the constructs were verified by DNA sequencing.

### 2.4. Expression and purification of recombinant proteins

For nsLTP1 protein expression, plasmids were introduced into *E. coli* BL21(DE3) trxB$^-$. After the induction of protein expression by 1 mM IPTG for 3 h at 37 °C, bacteria were harvested and then resuspended in the cold binding buffer (100 mM Tris–HCl, pH 7.9, 50 mM imidazole, 2.5 mM NaCl, and 0.05 mM phenyl methylsulfonyl fluoride, PMSF). The bacterial suspension was sonicated on ice until it was no longer viscous and then centrifuged at $10000 \times g$ for 25 min at 4 °C to remove cell debris. The supernatant containing soluble fusion proteins Trx-nsLTP was applied to a $Ni^{2+}$-chelating Sepharose fast flow column (Novagen) prewashed with the binding buffer. Protein purification on the $Ni^{2+}$-chelating Sepharose fast flow column was carried out according to the manufacture's protocol. Protein eluates were collected and dialyzed against the enterokinase buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, and 2 mM $CaCl_2$). To remove the thioredoxin fusion tag, the fusion proteins were treated with enterokinase for 16 h at room temperature (RT). The resulting protein mixtures were dialyzed against binding buffer and then loaded onto the $Ni^{2+}$-chelating Sepharose fast flow column to collect Trx-free nsLTP1 proteins in the flow-through. Collected proteins were dialyzed against deionized water before lyophilization.

In CaM overlay assay, bacteria expressing nsLTP1 proteins were lysed in the enterokinase buffer by sonication. After clarification by centrifugation, the lysate was treated with enterokinase to cleave off the Trx tag from the expressed fusion proteins. Upon completion of the cleavage, the lysate containing 40 μg proteins was applied to SDS–PAGE (12.5% gels) for the CaM overlay assay. The expressed nsLTP1 proteins were visualized by Coomassie blue staining. Protein quantification was performed with the Bradford method using γ-globulin as the standard.

### 2.5. CaM gel overlay assay

10 μg of recombinant nsLTP1 proteins was resolved by 12.5% SDS–PAGE and then electrotransferred onto nitrocellulose membranes. After being blocked for 1 h in TBS (20 mM Tris–HCl, pH 7.4 and 150 mM NaCl) containing 0.05% Tween 20 and 1% fat-free milk powder, the membranes were incubated with colloidal gold-labeled CaM ($A_{520} = 0.40$) in the same buffer supplemented with either 1 mM $CaCl_2$ or 5 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) for 30 min. The membranes were rigorously washed with deionized water before they were stained in the silver staining solution for 1 min.

### 2.6. CaM–Sepharose pull-down assay

CaM–Sepharose pull-down assays were carried out essentially as described in [31]. Bacterial lysates containing recombinant nsLTP1 proteins were treated with enterokinase for 16 h to remove the Trx tag. After dialysis in the CaM–Sepharose binding buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 6 M urea, 1 mM $CaCl_2$ or 5 mM EGTA), 300 μl of the lysates were mixed with 150 μl CaM–Sepharose beads and the beads suspesions were incubated at RT for 1 h with shaking. The beads were collected by brief centrifugation, washed for three times with the binding buffer, and then boiled for 5 min in 60 μl of the Laemmli sample buffer. Proteins from the boiled samples were analyzed on 12.5% SDS–PAGE to detect nsLTP1 using the monoclonal nsLTP1 antibody.

## 3. Results and discussion

### 3.1. Cloning of nsLTP1

To characterize the CaM binding of plant nsLTP1, we designed a pair of specific primers for PCR amplification according to the mature *Arabidopsis* nsLTP1 sequence. As expected, a PCR product of 282 bp corresponding to amino acids 27 to the end of nsLTP1 (signal peptide 1–26 amino acids were omitted) was obtained and cloned into the plasmid vector pET-32a(+). The recombinant plasimd was subjected to *Nco*I/*Xho*I restriction analysis. The length of the inset was in agreement with that of the PCR product. The pET-32a(+)-nsLTP1 construction was confirmed by DNA sequencing (Fig. 1).

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cta agc tgt ggc tca gtt aac agc aac ttg gca gcg tgc att ggc tac gtg ctc caa ggt ggt   63
 L  S  C  G  S  V  N  S  N  L  A  A  C  I  G  Y  V  L  Q  G  G
gtc att ccc cca gcg tgt tgc tcc ggc gtt aaa aac ctc aac agc ata gcc aag acg acc cca  126
 V  I  P  P  A  C  C  S  G  V  K  N  L  N  S  I  A  K  T  T  P
gac cgt cag caa gct tgc aat tgc att caa ggt gcc gct aga gcc tta ggc tct ggt ctc aac  189
 D  R  Q  Q  A  C  N  C  I  Q  G  A  A  R  A  L  G  S  G  L  N
gct ggc cgt gca gct gga att cct aag gca tgt gga gtc aat att cct tac aaa atc agc acc  252
 A  G  R  A  A  G  I  P  K  A  C  G  V  N  I  P  Y  K  I  S  T
agc acc aac tgc aaa acc gtg agg tga tga                                            282
 S  T  N  C  K  T  V  R
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Fig. 1. The sequencing result is in agreement with the nucleotide sequence of nsLTP1 (GenBank Accession No. AY049296). The primers used to specifically amplify nsLTP1 are denoted by single lines. The predicted BAA domain is denoted by double line. The putative CaM-binding site is shaded.

### 3.2. CaM binds to nsLTP1 in the presence and absence of $Ca^{2+}$

Recombinant Trx-nsLTP1 was expressed from the plasmid pET-32a(+)-nsLTP1 in *E. coli* BL21 (DE3) $trxB^{-}$. The protein was highly expressed upon the IPTG induction (Fig. 2A, lane 3) and affinity purified by a $Ni^{2+}$-chelating Sepharose fast flow column. After the cleavage of the fusion protein by enterokinase, the Trx tag was bound and removed by the $Ni^{2+}$-chelating Sepharose fast flow column, unbound nsLTP1 was retrieved from the flow-through. Purified nsLTP1 migrated at about 10 kDa on SDS–PAGE (Fig. 2A, lane 6).

To probe CaM binding, 10 μg nsLTP1 was resolved on SDS–PAGE to perform gel overlay assays with gold-labeled CaM. Results indicated that nsLTP1 bound to CaM in both the presence and absence of $Ca^{2+}$, which were the addition of 1 mM $Ca^{2+}$ and 5 mM EGTA, respectively (Fig. 2B, lanes 3 and 4), even though the binding was relatively weak at the EGTA condition. This $Ca^{2+}$-independent CaM-binding mode of nsLTP1 is in agreement with that of CaMBP-10 [18].

### 3.3. Recombinant nsLTP1 is pulled-down by CaM–Sepharose beads

To confirm the binding of *Arabidopsis* nsLTP1 to CaM, CaM–Sepharose beads were used to pull-down nsLTP1 from the nsLTP1-expressing bacterial lysate. Fig. 3A shows that a 10 kDa protein was pulled-down specifically from the bacterial extract in both the presence and absence of $Ca^{2+}$ (lanes 3 and 4). Further, this protein band was revealed as nsLTP1 on the anti-nsLTP1 Western blot (Fig. 3B, lanes 2 and 3). This result strongly support the notion that nsLTP1 binds to CaM.

Based on the results of CaM gel overlay assay and the pull-down assay, we attempt to conclude that the nsLTP1 is a new CaMBP that binds to CaM in a distinct $Ca^{2+}$-independent mechanism, even though in the presence of EGTA, the binding is relatively weak.

### 3.4. Mapping of the CaM-binding region within nsLTP1

Since CaMBPs does not have any consensus CaM-binding sequence motif, it is difficult to predict the CaM-binding region at the amino acid sequence level. CaM is often found

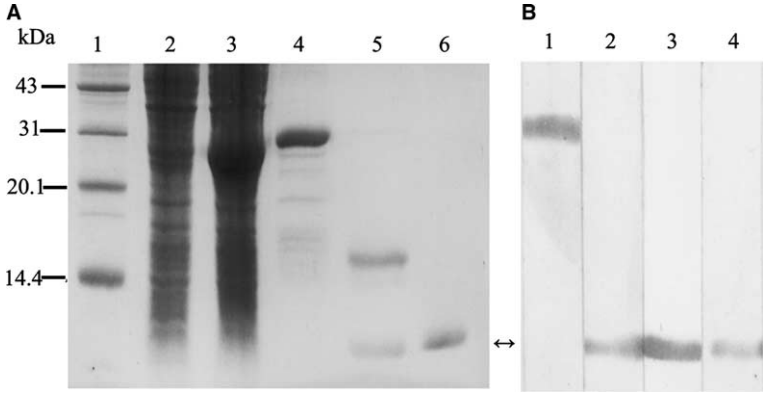


Fig. 2. Protein expression and CaM gel overlay assay. (A) Expression and purification of recombinant nsLTP1. Proteins were analyzed by 12.5% SDS–PAGE: lane 1, standard protein markers; lane 2, bacterial lysate without induction; lane 3, bacterial lysate after induction; lane 4, purified fusion protein; lane 5, enterokinase digested fusion protein; lane 6, purified recombinant nsLTP1. (B) CaM gel overlay assay: lane 1, purified fusion protein bound to CaM in the presence of 1 mM $Ca^{2+}$; lane 2, after digestion by enterokinase, only the interest protein bound to CaM in the presence of 1 mM $Ca^{2+}$; lanes 3 and 4, 10 μg purified recombinant nsLTP1 bound to CaM in the presence of either 1 mM $Ca^{2+}$ or 5 mM EGTA, respectively.

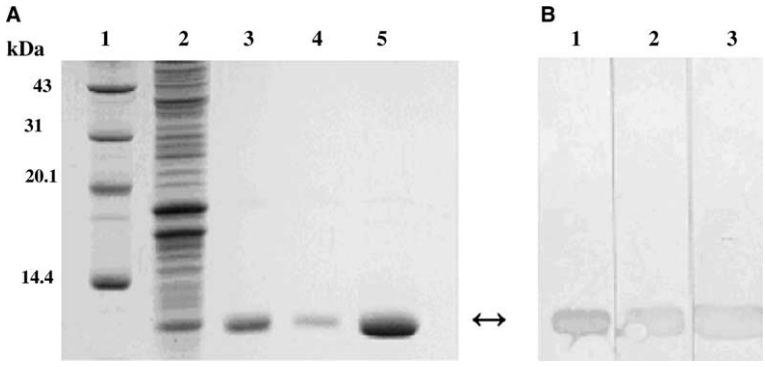


Fig. 3. Detection of recombinant nsLTP1 from pull-down assay with nsLTP1-specific monoclonal antibody. The bacterial lysate was extracted and digested by enterokinase as described in Section 2. The pulled-down proteins were resolved on 12.5% SDS–PAGE. (A) Gel stained with Coomassie blue: lane 1, protein markers; lane 2, bacterial lysate digested with enterokinase; lanes 3 and 4, proteins pulled-down by CaM–Sepharose beads in the presence of either 1 mM $Ca^{2+}$ or 5 mM EGTA, respectively; lane 5, the purified nsLTP1 as control. (B) Immunoblotting: lane 1, purified nsLTP1 as control; lanes 2 and 3, proteins pulled-down by CaM–Sepharose beads in the presence of either 1 mM $Ca^{2+}$ or 5 mM EGTA, respectively.

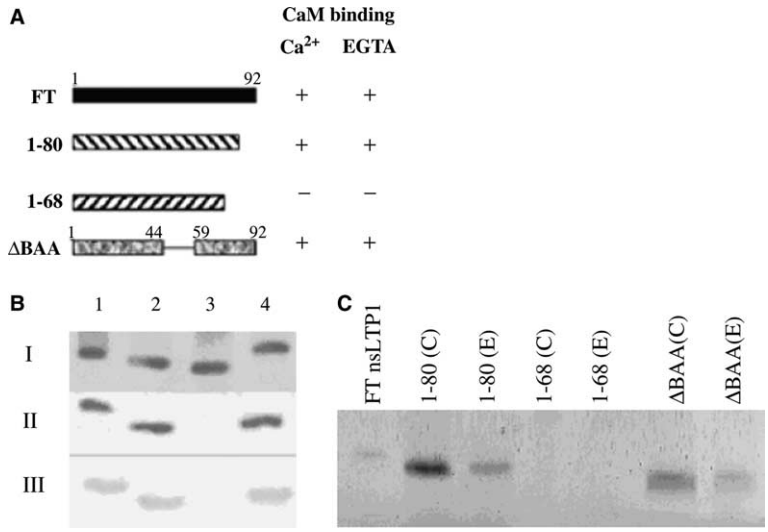


Fig. 4. Identification of the CaM-binding domain of nsLTP1. (A) Schematic representation of nsLTP1 and three truncation or deletion constructs. CaM binding ability is indicated as + (CaM binding) or − (no CaM binding). (B) CaM-binding analysis. Bacterial lysate digested by enterokinase were resolved on 12.5% SDS–PAGE and transferred onto nitrocellulose membranes. The blots were stained with amino black 10B (B, I) or overlaid with gold-labeled CaM in the presence of 1 mM $Ca^{2+}$ (B, II) or 5 mM EGTA (B, III). Lane 1, wild-type nsLTP1; lane 2, 1–80 mutant; lane 3, 1–68 mutant; lane 4, BAA. (C) The 1–80, 1–68 and ΔBAA were subjected to CaM–Sepharose pull-down assay. The pulled-down proteins were resolved on 12.5% SDS–PAGE and stained with Coomassie blue. The letters C and E in parentheses indicate that the proteins were pulled-down at the condition of $Ca^{2+}$ or EGTA, respectively. The FT nsLTP1 was used as control.

to interact with CaMBPs in the region comprising basic BAA or other sequence motifs, such as IQ motif, 1-8-14 motif and 1-5-10 motif [33,34]. In the BAA structure, hydrophobic residues are segregated from hydrophilic residues along the helix [32]. Online sequence analysis utilizing the Predict Protein program predicted a putative BAA structure between 45 and 58 amino acids of nsLTP1 (Reference predict_h15853), and no other sequence motif was found.

To map the binding region of nsLTP1, nsLTP1 and three truncation or deletion constructs (1–68, 1–80 and ΔBAA) were generated (Fig. 4A) as described under Method. Bacterially expressed proteins were used for CaM gel overlay assays and CaM–Sepharose pull-down assay in both the presence and the absence of $Ca^{2+}$. Results are shown in Fig. 4B and C. nsLTP(1–80) and ΔBAA mutants exhibited CaM binding with similar affinities to the full-length nsLTP1 (Fig. 4B, lanes 1, 2, and 4), whereas, the 1–68 mutant could not bind to CaM (Fig. 4B, lane 3) in both the $Ca^{2+}$ and EGTA conditions. Similarly, nsLTP(1–80) and ΔBAA mutants could be specifically pulled-down by CaM–Sepharose beads from the bacterial extract (Fig. 4C, 1–80, ΔBAA), and the 1–68 mutant could not be pulled-down by the beads (Fig. 4C, 1–68) in either condition (letters C and E in parentheses indicate $Ca^{2+}$ and EGTA, respectively). It is worthy to note that in both experiments the binding was relatively weak in the presence of EGTA. CaM did not bind to any protein from the control bacterial lysate in which the empty pET-32a(+) vector was used instead of the pET-32a(+)-nsLTP1 plasmid (data not shown).

Sequence analysis of nsLTP1 revealed that there are six hydrophobic residues, one charged residue, three polar residues and two glycine residues in the region of 69–80 amino acids. Comparing the nsLTP1 sequence to other plant nsLTPs, we found that nsLTPs have remarkable homology in this region, especially the hydrophobic residues Ile-70,

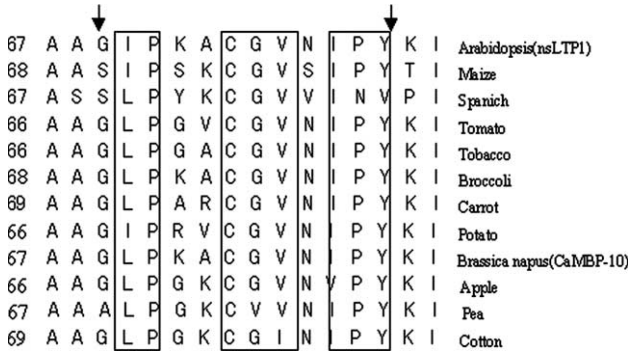


Fig. 5. Comparison of the putative CaM-binding site of nsLTP1 with other plant nsLTPs. The binding site of *Arabidopsis* nsLTP1 is indicated by arrows, the highly conserved amino acid residues are boxed.

Pro-71, Val-76, Ile-78, and the polar residue Cys-74 are conserved among nsLTPs (Fig. 5). As CaM binds to its targets via the hydrophobic interaction, the conserved hydrophobic residues in this region may be involved in the CaM interaction in addition to their proposed function in the transport of fatty acids [5].

In summary, we have acquired evidence to indicate nsLTP1 as a CaMBP and the CaM-binding region is conserved among nsLTPs. It is reasonable to extrapolate the results that plant nsLTPs are CaMBPs even though other nsLTPs were not tested in the CaM binding. Furthermore, the CaM-association of nsLTP1 was partially affected but not completely blocked by the absence of $Ca^{2+}$, implying a complex mechanism of the $Ca^{2+}$/CaM action on nsLTPs. Further mechanistic investigation of this action will facilitate the elucidation of the involvement of $Ca^{2+}$/CaM signaling in nsLTPs' functions.

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