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Expression, purification and analysis of an *Arabidopsis* recombinant CBL-Interacting Protein Kinase3 (CIPK3) and its constitutively active form

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Abstract

AtCIPK3 is a member of CBL (calcium B-like)-interacting serine-threonine protein kinases which play an important role in various developmental and adaptation processes in *Arabidopsis*. Studies conducted on members of this family such as SOS3, PKS8 and PKS11 have provided insight of how these novel kinases interact with their target substrates in the signal-response coupling process. Since SOS3, PKS8 and PKS11 had low enzymatic activities *in vitro* and their amino acid sequences highly homologous to that of CIPK3 in the conserved region, it was thought that CIPK3 would also have low enzymatic activity. To enhance enzymatic activity of CIPK3, a constitutively active form CIPK3T183D was generated by a Thr¹⁸³ (T) to Asp¹⁸³ (D) substitution in the conserved activation loop. To obtain sufficient protein for analysis, the glutathione S-transferase (GST) fusion protein system was used to express both CIPK3T183D and CIPK3. Although CIPK3 and CIPK3T183D were successfully expressed, they were found mainly in the inclusion bodies with three low molecular mass (LMM) proteins. These LMM proteins that had the same affinity to the GST-Bind resin as GST-CIPK3 or GST-CIPK3T183D, obliterating the one-step affinity purification protocol. Similarly, expression of CIPK3 or CIPK3T183D in His fusion system also led to protein production in inclusion bodies. However, only His-CIPK3 or His-CIPK3T183D was produced. To isolate the fusion protein, we adapted a two step protocol by first extracting the protein from inclusion bodies with sarkosyl followed by precipitating the protein with ammonium sulfate prior to affinity purification. His-CIPK3 or His-CIPK3T183D purified by this method was enzymatically active. In addition, a 9 fold increase in kinase activity was observed in His-CIPK3T183D when compared to His-CIPK3, indicating that Thr¹⁸³ to Asp¹⁸³ substitution had succeeded in increasing the kinase activity. However, the relationship between the amino acid substitution in the conserved activation loop and the

increased kinase activity is currently unknown.

Keywords: CIPK3; Site-directed mutagenesis; Sarkosyl; Affinity purification; Protein kinase; peptide phosphorylation.

Introduction

The *Arabidopsis* CBL (calcineurin B-like)-interacting Ser/Thr protein kinases (AtCIPKs) represent a family of novel kinases that are involved in Ca^{2+} signal transduction pathway in many adaptation and developmental processes [1]. In higher plants, pairing of specific CBL proteins and CIPKs forms a complex signaling network that allows for flexible yet specific coupled signal-response. In these signal-response processes, plants appear to sense their environment and relay these external signals to the interior of the cell by changing the concentration of Ca^{2+} , producing a distinct set of Ca^{2+} mediated changes referred to as Ca^{2+} signatures or codes.

Ca^{2+} signal decoding mechanisms in plants are currently thought to be more complicated than those in other organisms [1]. The CBL-CIPK network is an important Ca^{2+} decoding paradigm that is unique in plants and the progress in establishing this network has been particularly rapid over the last few years. In the CBL-CIPK signaling network, the decoding process starts with the Ca^{2+} sensors (CBL proteins) binding to the Ca^{2+} with high affinity and thereby alter their own structures to allow for further interactions. These structural changes result in triggering interactions with the downstream target proteins (CIPKs), and the interactions activate the kinase activity of CIPKs which modulate the function of other proteins such as transcriptional factors or membrane transporters, and elicit changes in cellular processes such as gene expression or ionic fluxes [2].

Physiological and biochemical studies have been conducted with some CBL-CIPK pairs. The original example of the CBL-CIPK-substrate paradigm is deduced from the salt overly sensitive

(SOS) pathway [3]. The calcineurin B-like calcium sensor SOS3 (AtCBL4) and the CIPK-type kinase SOS2 (AtCIPK24) appear to be part of a Ca^{2+} regulated signaling pathway that specifically mediates salt stress signaling and adaptation by regulating the Na^+/H^+ antiporter SOS1 [3, 4, 5]. CBL-CIPK network also plays an important role in plant nutrient sensing and adaptation. A forward genetic screen for low- K^+ -tolerant mutants identified CIPK23 as a critical K^+ -nutrition determinant in *Arabidopsis* [6]. A systematic reverse genetic analysis of all CIPKs identified CIPK23 crucial for stomatal response and low- K^+ response [7, 8]. Since CIPK23 interacts with a number of CBLs including CBL1 and CBL9, it was speculated that CBL1 and CBL9 functioned in CIPK23-mediated pathways regulating K^+ -nutrition and stomatal responses. More recently, the CBL9-CIPK23 network was identified to involve in nitrate sensing in a breakthrough study conducted by the group of Yi-Fang Tsay [9]. The results from Tsay's group showed that CBL9-CIPK23 complex could phosphorylate T101 of CHL1 (NRT1.1) which was responsible for sensing a wide range of nitrate concentrations in the soil using a dual-affinity binding and phosphorylation/dephosphorylation switch [9].

CIPK3 is responsive to ABA, cold, high salt, wounding and drought and appears to serve multiple functions in the early development of *Arabidopsis*. Further studies showed that CIPK3 functioned as a molecular link between stress-induced and ABA-induced calcium signals which triggered gene expression in plant cells [10]. Despite considerable interest in the physiological and biochemical functions of CIPKs, there were many unanswered questions. The structure-function studies involving enzyme-substrate binding of many CIPKs have not been reported, and detailed mechanisms that allow CIPKs to act as phosphoryating enzymes of the target genes are not fully understood. To facilitate the study of enzyme activity, a highly active CIPK3 and a large

quantity of the enzyme are required as the first step.

In this report, we describe the use of a simple protocol to isolate the two fusion proteins, His-CIPK3 and His-CIPK3T183D, by first extracting the proteins from inclusion bodies with sarkosyl followed by precipitating the proteins with ammonium sulfate prior to affinity purification. The fusion proteins purified remained enzymatically active. In addition, a 9 fold increase in phosphorylation activity was observed in the purified His-CIPK3T183D when compared to His-CIPK3, indicating that the Thr¹⁸³ to Asp¹⁸³ substitution had successfully enhanced the enzymatic activity. However, it was unclear how amino acid substitution had affected the enzymatic activity as no apparent change in the 3-D structure of the activation loop between His fusion proteins CIPK3T183D and His-CIPK3 was detected using the rat MARK2 (1ZMU) chain A subunit as a model template.

Material and methods

Cloning of CIPK3

Total RNA was isolated from 2-week-old wild-type *Arabidopsis* (Col-0 ecotype) plants using an RNAeasy mini kit (Qiagen). The first strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). The full-length *CIPK3* cDNA was amplified using high fidelity AccuPrimer Pfx DNA Polymerase (Invitrogen) with the following specific primers containing *Bam*HI sites (underlined in both forward and reverse primers) at the termini: 5'-CAGGATCCATGTTGATCCCCAACAAAAATT-3' (forward) and 5'-CTGGATCCTCACTTTGCTGTTTCTTTCTTAAC-3' (reverse). The PCR products were gel-

purified, digested, and cloned into a modified pENTR3C vector and the DNA sequence was verified by sequencing. This vector was designated as GT6.

Site-directed mutagenesis

The CIPK3 Thr¹⁸³ to Asp¹⁸³ substitution in the activation loop was conducted according to the QuickChange Site-directed Mutagenesis kit instruction manual (Stratagene). The primers were: CIPK3T183D-forward 5'-AGGGATGATGGACTCTTGCATGACTCGTGTGGAACACCA-3'; CIPK3T183D-reverse 5'-AGTTTGGTGTTCACACGAGTCATGCAAGAGTCCATCAT-3'.

These primers containing the aspartic codon, indicated by underlines, were used to generate the mutated DNA sequences. Mutagenesis reactions were carried out on the GT6 plasmid DNA using AccuPrimer *Pfx* DNA Polymerase with the following PCR condition: 95°C for 30 s, followed by 12 cycles of 95°C for 30 s, 58°C for 1 min, and 68°C for 4 min. The PCR products were gel-purified and treated with *DpnI* (NEB) to digest the methylated template DNA. The digested PCR product was transformed into DH10B competent cells. The resulting vector was designated as GT7. The DNA sequence after mutagenesis was verified by sequencing.

Vector constructions for expression of GST- and His-Tag fusion proteins

For GST-CIPK3 fusion protein vector construction, the ORF of CIPK3 was cut with *Bam*HI from GT6 and ligated to pGEX4T-1. Vector construction for GST-CIPK3T183D was conducted similarly as GST-CIPK3 using the Thr¹⁸³ to Asp¹⁸³ substituted DNA sequence of GT7 for cloning into pGEX4T-1. For His-CIPK3 fusion vector construction, the ORF of CIPK3 was PCR amplified using GT6 as a template with the following primers containing *Nhe*I and *Xho*I sites (both restriction sites were underlined) at the termini: 5'-

CAGCTAGCATGTTGATCCCCAACAAAAAATT-3' (forward) and 5'-
GACTCGAGTCACTTTGCTGTTTCTTTCTTAAC-3' (reverse). The PCR product was cut with *NheI* and *XhoI* and cloned into pET28a. Vector construction for His-CIPK3T183D was conducted in the same manner as that of His-CIPK3 using the Thr¹⁸³ to Asp¹⁸³ substituted DNA sequence of GT7 for cloning into pET28a. All DNA sequences were verified by sequencing before transformation of *E. coli*.

Expression and purification of GST fusion proteins

GST-CIPK3 and GST-CIPK3T183D vectors were used to transform *E. coli* Rosetta 2 cells (Novagen) for fusion protein productions. An aliquot of 5 ml overnight culture in Lauria-Bertani (LB) medium was transferred to a fresh 200 ml LB medium and grown at 37 °C to an absorbance of 0.8 at OD₆₀₀. Recombinant protein expression was induced by 0.25 mM isopropyl β-d-thiogalactopyranoside (IPTG) for 4 h. The cells were harvested by centrifugation and stored at -80°C until use. Protein purification procedure was conducted according to Frangioni et al [11] with minor modifications. Briefly, the bacteria pellets were resuspended in BugBuster protein extraction reagent (Novagen) containing 25U/ml Benzonase Nuclease (Novagen) that was added immediately to the extract buffer before use. The suspension was incubated at room temperature for 15 min. The lysate was clarified by centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatant was transferred to a fresh tube and the pellet containing inclusion bodies was saved for protein extraction. The inclusion bodies were washed with 1:10 diluted BugBuster reagent and resuspended in ice-cold STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) with 5 mM dithiothreitol (DTT). The inclusion bodies were solubilized by the addition of sarkosyl (N-laurylsarcosine) to a final concentration of 0.5% (v/v) in STE and shaken gently at 4 °C for 30

min. The lysate was clarified by centrifugation for 5 min at 13,000 rpm and the supernatant was transferred to a new tube. Triton X-100 was added to a final concentration of 0.7% (v/v) in STE and the lysate was vortexed for 5 seconds. The protein samples were added to the washed GST Bind resin (Novagen) and incubated at 4 °C on an Orbitron Rotator for 30 min. The mixture was then loaded onto a column (Novagen) and washed three times with 10 volumes of ice-cold wash buffer (8.4 mM Na₂HPO₄, 1.9 mM NaH₂PO₄, pH 7.4, 150 mM NaCl). Purified protein was eluted with 3 volumes of elution buffer (50mM Tris-HCl, pH8.0, and 10 mM reduced glutathione) and stored at -80 °C.

Expression and purification of His fusion proteins

Expression and induction of His-tagged proteins was conducted in a similar manner as the GST-tagged proteins described earlier. Bacterial pellet containing the His-tagged protein was solubilized with BugBuster reagent and the inclusion bodies were resuspended in ice-cold STE buffer containing 5 mM DTT, and sarkosyl was added to a final concentration 0.5% (v/v). The suspension was gently mixed at 4 °C for 30 min. After centrifugation for 5 min at 13,000 rpm, protein was precipitated by adding 50% (v/v) saturated ammonium sulphate drop-wise to the supernatant. The precipitate was isolated by centrifugation and resuspended in His-Binding buffer (300 mM NaCl, 20 mM Tris-HCl pH 7.9, 2.5 mM imidazole) containing 0.05% (v/v) of sarkosyl. The presence of sarkosyl in the His-Bind buffer was required to facilitate elution of the fusion protein which had a tendency to bind too tightly to the His-Bind resin to allow elution. The use of 0.05% sarkosyl was determined in an earlier experiment by examining optimal protein recovery from the affinity column against a range of sarkosyl concentrations (see Results). The protein in His-Bind buffer containing sarkosyl was clarified by centrifugation and

the supernatant was added to the washed His-Bind resin (Novagen) and incubated at 4 °C on an Orbitron Rotator for 30 min. The mixture was loaded onto a column and washed three times with 10 volumes of ice-cold wash buffer (300 mM NaCl, 20 mM Tris-HCl pH 7.9, 25 mM imidazole). Protein was eluted three times each with 3 volumes of elution buffer (300 mM NaCl, 20 mM Tris-HCl pH 7.9, 300 mM imidazole). The eluted protein was dialyzed against storage buffer (20mM Tris-HCl pH 7.2, 0.5 mM CaCl₂, 2 mM DTT, 10% glycerol) using Spectro/Por dialysis membrane (MW cut-off at 12-14 kDa, Spectrum Medical Industries Inc.) and centrifuged to remove any precipitate formed during dialysis and stored at -80°C until use.

Protein assay and SDS-polyacrylamide electrophoresis.

Protein concentration was determined by the method as described by Bradford (Bio-Rad) with bovine serum albumin as a standard [12]. SDS-PAGE was conducted with Mini-Protean II Cell (Bio-Rad). For SDS-PAGE analysis, the acrylamide concentrations used in the 1-mm-thick gels were 10% (w/v) for the resolving gel and 4% (w/v) for the stacking gel. Gels were stained with Coomassie brilliant blue R-250 and destained in boiling water for 5 min. Prestained proteins (Bio-Rad) were used for molecular weight standards.

Protein kinase activity assays

In vitro phosphorylation assays were performed as described by Hastie et al. [13] with minor modifications. Three synthetic peptides (Selleck Chemicals LLC, USA) p1 (LRRASLG, also called kemptide), p2 (VRKRTLRL) and p3 (ALARAASAAALARRR) were used as substrates. Phosphorylation was measured as the amount of radioactivity from [γ -³²P]ATP (PerkinElmer Life Sciences) incorporated into the synthetic peptide substrate. Reaction mixtures without the

synthetic peptide or kinase proteins were used as controls to account for the background radioactivity. Unless otherwise stated, the reaction mixture of kinase assay contained 20 mM Tris-HCl, pH 7.2, 2.5 mM MnCl₂, 0.5 mM CaCl₂, 2 mM DTT, 150 μM p3 peptide substrate and 10 μg protein kinase. Reactions were initiated by adding 3 μl of 1mM [γ -³²P]ATP (diluted from the original concentration by 1mM cold ATP to produce a specific radioactivity of 1x10⁵ to 1x10⁶ cpm per nmol) and reaction mixtures, each containing a total volume of 30 μl, were transferred to temperature block at 30 °C for 30 min. At the end of incubation period, the entire reaction mixture of 30 μl was spotted onto the center of a 2.1-cm square of P-81 phosphocellulose paper (Whatman) which was immediately immersed in a beaker containing 75 mM phosphoric acid to terminate the reaction. The P-81 paper was then washed 3 times in cold 75mM phosphoric acid, rinsed briefly with acetone to remove the phosphoric acid and then air-dried. P-81 papers were transferred to scintillation vials (VWR) containing 4 ml of liquid scintillation cocktail (Beckman Coulter), and phosphorylation was quantified by LS6500 Multi-purpose Scintillation Counter (Beckman Coulter). Protein kinase activity was determined according to Hastie et al [13].

To study the effects of protein kinase concentration in the phosphorylation reactions, CIPK3T183D protein in a range of 0.01 μg to 10 μg was added to the reaction cocktail with fixed concentrations of 2.5 mM MnCl₂ as cofactor and 150 μM p3 as substrate. The reaction cocktail consisted of 20 mM Tris-HCl, pH 7.2, 0.5 mM CaCl₂ and 2 mM DTT. For determination of effects of the cofactor, a range of 0-10 mM MnCl₂ or MgCl₂ was added to the reaction cocktails with fixed concentration of CIPK3T183D as an enzyme (10 μg) and p3 as a substrate (150 μM). For substrate specificity assays, peptides p1, p2 and p3 at 150 μM were tested individually in the reaction cocktail containing fixed concentrations of MnCl₂ (2.5 mM) as cofactor and

CIPK3T183D as enzyme (10 µg). For comparison of kinase activity, 10 µg of either CIPK3 or CIPK3T183D were added to the reaction cocktail with fixed concentrations of MnCl₂ (2.5 mM) as cofactor and p3 as substrate (150 µM).

Structural modelling of CIPK3 and CIPK3T183D

For structural modelling, the DNA sequences of CIPK3 and CIPK3T183D were used as input information into the protein structure prediction web server ESyPred3D (<http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/>) [14], using the crystallized rat MARK2 (1ZMU) protein chain A as a template [15]. The simulated 3-D structure were viewed by using ICM-pro v3.4 (MolSoft L.L.C., La Jolla, CA, USA).

Results and discussion

Substitution of threonine with aspartic acid residue in the activation loop by site-directed mutagenesis

Amino acid sequence alignment with SOS2 and PKS11 shows that CIPK3 contains a putative “activation loop” in the N-terminal SNF1-like kinase catalytic domain and a conserved FISL motif in the C-terminal regulatory domain (Fig. 1). CIPK3 contains an open reading frame of 1,356 bp encoding a protein of 451 amino acids with an estimated molecular mass of 51.8 kDa. The predicted isoelectric point is 7.98. Previously, studies have shown that GST fusion proteins of SOS2, PKS11 and PKS18 were difficult to assay *in vitro* because of their low enzyme activity [4, 16-19]. However, the enzymatic activity of these three CIPK genes was enhanced by a threonine to aspartic substitution in the activation loop. Given that the deduced amino acid sequence of CIPK3 was highly homologous to those of SOS2 and PKS11, low kinase activity of

the CIPK3 was anticipated. Thus, it would be desirable to generate a highly active form of CIPK3 by modifying the amino acid sequence so that enzyme activity could be studied with ease. To achieve our objective, we constructed a constitutively active form of CIPK3 by substituting the Thr¹⁸³ with Asp¹⁸³ in the activation loop using site-directed mutagenesis. The resulting constitutively active is designated as CIPK3T183D.

GST-CIPK3 and GST-CIPK3T183D fusion proteins were only expressed in the inclusion bodies

The pGEX glutathione S-transferase (GST) fusion protein system is used extensively for production of fusion protein which can be isolated from bacterial and eukaryotic cell lysates by one-step affinity purification. Several members of *Arabidopsis* CIPK family, such as SOS2, PKS11, PKS18, have been successfully expressed and purified from the soluble fraction of *E. coli* cell lysate. Based on these studies, we constructed a fusion protein of CIPK3 with a GST-tag and cloned it into the pGEX4T-1 vector for expression. Since there were many rare codons in the CIPK3 DNA sequence, we used the Rosetta 2 bacterial strain which supplied tRNAs for seven rare condons (AUA, AGC, AGA, CUA, CCC, CGG and GGA) designed to enhance the expression of eukaryotic proteins containing codons rarely used in *E. coli*.

As detected by SDS-PAGE gel, large quantity of proteins were produced from bacterial cultures expressing GST-CIPK3 (Fig. 2, lane 3) and GST-CIPK3T183D (Fig.2, lane 4) after IPTG induction. Since both GST-CIPK3 and GST-CIPK3T183D had an estimated molecular mass (MM) of 78.1 kDa (51.8kDa for CIPK3 (or CIPK3T183D) + 26.31kDa for GST-tag), the protein bands marked with asterisks were thought to be the target fusion proteins (Fig. 2, lanes 3 and 4). However, further analysis indicated both GST-CIPK3 and GST-CIPK3T183D were expressed as

insoluble proteins in the pellets (Fig. 2, lanes 11 and 12). This was contrary to the observation made by Smith and Johnson who reported the production of GST fusion proteins in the soluble fractions [20].

Reduction in growth temperature has been reported by Saluta and Bell [21] to facilitate production of fusion proteins in the supernatant. However, attempts in generating CIPK3 or CIPK3T183D fusion proteins at 20 °C, 23 °C and 28 °C (data not shown) in addition to 37 °C did not meet with any success. Similarly, reductions in IPTG concentration and induction times, and the use of alternative bacterial hosts such as BL21 DE3, Origami2 DE3 and Rosetta-gami2 DE3 for fusion protein production also failed to produce either fusion proteins in the soluble fraction (data not shown).

GST fusion proteins purified from inclusion bodies included additional truncated GST fusion proteins

Since both GST-CIPK3 and GST-CIPK3T183D fusion proteins are mainly present in the pellet (Fig. 2, lanes 11 and 12), ionic detergent sarkosyl in STE buffer was used to extract the fusion proteins from the inclusion bodies according to the method described by Frangioni [11]. As indicated by CIPK3 fusion protein (depicted by an asterisk) in Fig. 2 lane 15, 0.5% sarkosyl in STE buffer was used successfully to extract the fusion protein from inclusion bodies. However, three lower molecular mass (LMM) proteins, depicted by “V” (Fig. 2 lane 15), were also extracted from the inclusion bodies together with the CIPK3 fusion protein.

After sarkosyl extraction, Triton X-100 at 0.7% (approximately in a 2:1 molar ratio of sarkosyl to

Triton X-100) was added to the STE buffer to facilitate binding of fusion protein to the GST-Bind resin [11]. The protein-resin mixture was loaded on a column which was washed with ice cold wash buffer and eluted using the elution buffer (see Materials and Methods). Unfortunately, affinity purification did not succeed in eliminating the three LMM proteins from the desired fusion protein as they appeared to have same affinity as GST-CIPK3 (Fig. 3, lane 9) to the resin. Since these LMM proteins were closely associated with GST-CIPK3 in the inclusion bodies, they were thought to be truncated GST fusion proteins resulted from premature termination of translation processes. The fusion protein mixture after affinity purification (Fig.3, lane 9) showed GST activity with glutathione (GSH, Sigma) and 1-Chloro-2, 4-dinitrobenzene (CDNB, Sigma) as substrates [11], indicating that the mixture containing the GST-CIPK3 fusion protein was enzymatically active (data not shown). Similar results were obtained from CIPK3T183D fusion protein (data not shown).

Isolation of His-tag fusion protein from inclusion bodies

With the problem encountered in GST-tag fusion protein system, we turned to His-tag fusion protein system as an alternative. With the His-tag fusion protein system, the fusion protein of His-CIPK3 or His-CIPK3T183D was cloned into pET28a vector and expressed in Rosetta 2 cells. Similar to the GST-tag fusion protein system, either His-CIPK3 or His-CIPK3T183D was produced in the inclusion bodies. However, the desired fusion protein was found as a single protein band on the SDS-PAGE gel without the presence of LMM proteins (Fig. 4, lanes 16 and 18). Based on our experience with the GST-fusion proteins, sarkosyl was used to solubilize either His-CIPK3 or His-CIPK3T183D from inclusion bodies. However, sarkosyl greater than 0.3% in STE buffer appeared to interfere with binding to the affinity resin and its removal was necessary

to allow protein binding to the His-Bind resin. Similarly, removal of EDTA in STE buffer [22] was necessary as it would chelate the nickel ions from the NTA groups of the His-binding resin, preventing the His-tag fusion proteins from binding to the resin. To remove both sarkosyl and EDTA from the extracted His fusion proteins, 50% (v/v) saturated ammonium sulfate solution was used to precipitate the fusion protein. The precipitated fusion proteins were then solubilized in His binding buffer prior to column loading. When applied to the His-binding resin, the fusion proteins bound very tightly to the resin and could not be eluted without the presence of sarkosyl. Consequently, a range of sarkosyl from 0.005%, 0.01%, 0.05%, and 0.1% was added to binding, wash and elution buffers for assessing optimal protein recovery (Fig. 5).

Using the results of protein recovery obtained from His-CIPK3T183D, 0.05% of sarkosyl in binding buffer, wash buffer and elution buffer was found to achieve optimal protein production (Fig. 5). With concentrations of sarkosyl higher than 0.1%, loss of the bound fusion protein from the affinity column occurred via in flow-through and during column washes (Fig. 5, 0.1% sarkosyl). Similarly, with sarkosyl concentrations lower than 0.05%, recovery of the eluted protein was not optimal (Fig. 5, 0.01 to 0% sarkosyl). The protein eluted from the His-Bind resin was dialyzed against storage buffer using Spectro/Por dialysis membrane. After dialysis, the fusion proteins were then centrifuged for 5 min at 13,000 rpm to remove any precipitate formed. The centrifugation step also removed the precipitate formed from traces of sarkosyl and divalent cation (Ca^{2+}) during the dialysis process. Similar results were obtained from His-CIPK3 (data not shown).

The effects of Thr¹⁸³ to Asp¹⁸³ substitution in the activation loop of CIPK3

Since fusion proteins of SOS2, PKS18 and PKS11 showed low kinase activity [4, 16-19], the purified constitutively active form of His-CIPK3T183D was used for analyses of enzymatic activity, cofactor requirement and peptide substrate preference.

To measure the enzyme activity, a range of 0.01 to 10 μ g of HisCIPK3T183D was tested in the reaction buffer containing 20 mM Tris-HCl (pH 7.2), 0.5mM CaCl_2 and 2 mM DTT and 2.5 mM MnCl_2 , using 150 μ M of p3 as a substrate. Details of the enzyme assay were described in Material and Methods Section and were similar to those used for measuring the enzymatic activities of SOS2, PKS18 and PKS11 [4, 16, 19]. As shown in Fig. 6a, the kinase activity of His-CIPK3T183D increased with increasing concentrations of the fusion protein. Based on these results, 10 μ g of fusion protein of His-CIPK3T183D were used for all subsequent studies involving cofactor preference, peptide substrate specificity and comparison of enzyme activity between His-CIPK3 and His-CIPK3T183D.

To study the cofactor preference, kinase activity of His-CIPK3T183D was studied in the presence of varying concentrations of two divalent cations, Mg^{2+} or Mn^{2+} . As shown in Fig. 6b, substrate phosphorylation involving the incorporation of radioactivity of radioactive ATP increased with increasing concentrations of Mn^{2+} (from 0 to 1 mM) or Mg^{2+} (from 0 to 2.5 mM). Interestingly, Mn^{2+} appeared to be a much more effective than Mg^{2+} as a cofactor to induce a higher activity of CIPK3T181D. Optimal activation of Mn^{2+} was observed at 1 mM, and concentrations above 1 mM became inhibitory to the enzyme activity. Similarly, 2.5 mM of Mn^{2+} was found to activate substrate phosphorylation of PKS11T161D in the study reported by Gong et al. [19]. It is unclear whether Mn^{2+} have played a role in regulating CIPK3 activity under

physiological condition. In contrast to Mn^{2+} , the effect of Mg^{2+} on the activity of CIPK3T183D remained relatively unchanged in the range of 1 to 10 mM.

Based on the study reported in SOS2 [4], three synthetic peptides, p1, p2 and p3 were made for measuring the enzymatic activity of His-CIPK3T183D. As shown in Fig. 6c, CIPK3T183D shows enzymatic activity with all three peptide substrates but the enzymatic activity generated with p3 is higher than those with p1 and p2 as substrates. Similar observations have been made with SOS2, PKS18 and PKS11 using p1, p2 and p3 as substrates [4, 16, 19].

To compare kinase activity of CIPK3 and CIPK3T183D, we used 10 μ g of each purified fusion protein as the enzyme and 150 μ M of p3 as the substrate in a 30 μ l reaction mix. As shown in Fig. 6d, the results indicated that kinase activity of CIPK3T183D was 9 fold higher than that of CIPK3. Thus, substitution of Thr¹⁸³ to Asp¹⁸³ in the activation loop of CIPK3 has succeeded in enhancing the kinase activity of the enzyme.

Structural modelling of CIPK3 and CIPK3T183D

Changes of enzyme activity are often indicative of in the topo-morphology change in catalytic proteins. In order to determine whether Thr¹⁸³ to Asp¹⁸³ substitution of CIPK3 has changed the topography of the protein, 3-D structures of CIPK3 and CIPK3T183D were generated and compared using the homology modelling web-service, ESyPred3D [14]. Since structural determination by X-ray crystallography with plant CIPK proteins has not been conducted, the closest aligned protein kinase that can be used for comparison is the rat MARK2 (1ZMU) chain A [15]. The rat MARK2 (1ZMU) chain A subunit shared a sequence identity of 28.2% with

CIPK3 (Fig. 7). Based on the amino acid sequence information of the N-terminal, the 3-D structures generated showed that both the predicted CIPK3 and CIPK3T183D proteins have structural features similar to 1ZMU chain A (Fig. 7). The Thr¹⁸³ to Asp¹⁸³ substitution in the activation loop did not reveal any changes in the 3-D structure of CIPK3T183D when compared to CIPK3 to account for the increased of the enzyme activity as shown in Fig. 6d. However, considering the 28.2% amino acid sequence identity between CIPK3 and its template, the predicted structure of the CIPK3 and CIPK3T183D might not be sufficiently accurate for structural analysis of these two proteins. To facilitate an accurate structural determination and biochemical analysis of the CIPKs, it is necessary to crystallize and analyze a representative member of this family by X-ray to predict the 3-D structure such that it can be used as a template for analysis.

Conclusion

We have successfully expressed and purified the His fusion proteins of CIPK3 and its constitutively active form CIPK3T183D. During this study, we adapted a protocol that can be used to purify His fusion proteins from inclusion bodies. The proteins purified by this method retain their activities as indicated by CIPK3 and CIPK3T183D His fusion proteins. This method is simple and reliable for obtaining abundance of highly purified fusion proteins in a one step protocol without expensive equipment such as FPLC or HPLC. As with many protein purification procedures, this method may not work for every His fusion protein. However, we hope that this method will facilitate purification of some His fusion proteins required for cellular and molecular studies.

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FIGURE LEGENDS

Fig. 1. Amino acid sequence alignment of CIPK3, SOS2 and PKS11.

Boxes in the amino acid sequences indicate the putative activation loop in the N-terminal SFN1-like kinase domain and the conserved FISL motif in the C-terminal regulatory domain. T-shading depicts the conserved Thr residue in the activation loop. Dashes are used to provide the maximum alignment of the three sequences.

Fig. 2. Induction and solubility analysis of GST fusion proteins of CIPK3 and CIPK3T183D expressed in *E. coli* Rosetta 2 cells.

Lanes 1-4 show total GST tag fusion proteins produced by *E. coli*. Lane 1 shows the total proteins produced by the bacterial culture harboring the unmodified pGEX4T-1 (control). Lane 2 shows proteins produced by the bacterial culture expressing unmodified pGEX4T-1. Lanes 3 and 4 show total proteins produced by the bacterial cultures expressing fusion proteins GST-CIPK3 and GST-CIPK3T183D, respectively, after induction with 0.25 mM IPTG at 37 °C for 4 hr. Lanes 5, 6, 7 and 8 show soluble protein fractions in supernatant from various bacterial cultures as indicated by labels above the lanes. Lanes 9, 10, 11 and 12 show insoluble proteins in pellets from various bacterial cultures as indicated by labels above the lanes. Lanes 13-16 show the proteins in supernatant (Lane 13), pellet (Lane 14) and in pellet extracted with 0.5% sarkosyl (Lane 15) and after sarkosyl extraction (Lane 16) in the bacterial cultures expressing GST-CIPK3 after IPTG induction. Similar results to those of GST-CIPK3 (Lanes 13-16) were also obtained from GST-CIPK3T183D using 0.5% sarkosyl for protein extraction from the pellet (data not shown). M, protein molecular weight standards. Asterisk (*) depicts the target proteins, GST-CIPK3 or GST-CIPK3T183D, and "V" depicts the variant proteins.

Fig. 3. Purification of GST-CIPK3 fusion protein from inclusion bodies by GST Bind resin.

Lanes 1-3 show total proteins (Lane 1), supernatant (Lane 2) and pellet (Lane 3) of GST-CIPK3 after IPTG induction. Lane 4 shows proteins extracted from inclusion bodies in STE buffer containing 0.5% sarkosyl. Lane 5 shows proteins in inclusion bodies after 0.5% sarkosyl STE buffer extraction. Lane 6 shows solubilized proteins from inclusion bodies by 0.5 sarkosyl before loading to the GST-binding resin affinity column, Lane 7 shows proteins from column flow-through, Lane 8 shows proteins from column wash, Lane 9 shows eluted proteins of GST-

CIPK3. M, protein molecular weight standards. See Fig.2 for the GST-CIPK3 and GST-CIPK3T183D fusion proteins and the variant proteins.

Fig. 4. Expression and solubility analysis of His-CIPK3 and His-CIPK3T183D expressed in *E. coli* Rosetta 2 cells. Lanes 1-6 show total proteins, Lanes 7-12 show proteins in supernatant and Lanes 13-18 show proteins from pellet from various bacterial cultures as indicated by the labels above the lanes. M, protein molecular weight standards.

Fig. 5. Solubility of fusion protein CIPK3T183D in sarkosyl, column flow through, column washes and elutions of protein from Ni-NTA His-binding resin. Lane 1 shows solubilized proteins from inclusion bodies in varying concentrations of sarkosyl prior to column loading, Lane 2 shows unbound column flow-through, Lane 3- 5 show sequential column washes (3x) and Lanes 6-8 shows sequential elutions (3x) of His-CIPK3T183D. Amount of sarkosyl used are shown as percentage (v/v) of the total volume of bind, wash and elution buffers. Note that the overall protein yield of His-CIPK3T183D is optimized at 0.05% of sarkosyl for purifying the target protein from inclusion bodies.

Fig. 6. His-CIPK3T183D and His-CIPK3 protein kinase activity Assay.

(a) Kinase activities of purified His-CIPK3T183D were measured using 0.01 µg to 10 µg of enzyme and peptide p3 as substrate. (b) Dependence of His-CIPK3T183D on divalent cation Mn^{2+} / Mg^{2+} for kinase activities using peptide p3 as a substrate. (c) Kinase activities of His-CIPK3T183D using peptides p1, p2, and p3 as substrates. (d) Differences in kinase activity of His-CIPK3 and His-CIPK3T183D. Kinase activities were measured using 150 µM p3 as substrate in a, b and d as described in “Materials and Methods”. Results represent the means $\pm$ S.D. from three experiments.

Fig. 7. Structural modelling of the *Arabidopsis* CIPK3 and CIPK3T183D using rat MARK2 (1ZMU) subunit A as a template, and amino acid alignment of CIPK3 and rat MARK2 (1ZMU) subunit A.

(a) 3D-structure of rat MARK2 subunit A is used as a template for CIPK3 and CIPK3T183D structural modeling. (b) and (c) show predicted 3D structures of CIPK3 (b) and CIPK3T183D

(c). Dark blue lines of the models from a to c represent activation loop of MARK2, CIPK3 and CIPK3T183D, and yellow lines represent the amino acid residue T183 in b and D183 in c. (d) Amino acid sequence alignment of the CIPK3 and rat MARK2 subunit A. The red box indicates part of the activation loop of CIPK3. Red shading in the red box depicts the amino acid residue Thr in CIPK3 and is substituted by Asp residue in CIPK3T183D.