Expression and purification of recombinant *Physcomitrella patens* DEK1-C2L proteins

Analysis of the phospholipid binding capacity of DEK1-C2L

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Contents

1. SUMMARY 4

2. INTRODUCTION 5

2-1. Definition of calpains 5

2-2. The origin and evolution of DEK1 7

2-3. Domain structure of DEK1 10

2-4. The function of DEK1 11

2-5. C2L structure/function 13

2-6. Recombinant protein expression 14

2-7. Aim of study 18

3. MATERIALS AND METHODS 20

3-1. Expression vectors 20

3-2. Cell culturing 20

3-4. Protein refolding and concentration of protein sample 22

3-5. Preparation of liposomes 22

3-6. Assay of protein-phospholipid binding ability 23

3-7. SDS-PAGE 23

3-8. Coomassie blue staining 24

3-9. Western blot and Immunostaining 24

3-10. Measuring protein concentration 24

4. RESULTS 26

4-1. Verification of recombinant protein production 26

4-4. Re-folding and concentration of recombinant His-PpDEK1-C2L protein 31

4-5. Analysis of the liposome binding ability of recombinant PpDEK1-C2L protein 32

5. DISCUSSION AND CONCLUSION 35

5.1 Expression and purification of recombinant DEK1-C2L proteins 35

5.2 Analysis of the phospholipid binding ability of *P. patens* DEK1-C2L domain 38

5-3. Further Studies 40

6. APPENDIX 41

6-1. Primer overview 41

6-2. Vectors Maps 42

7. REFERENCES 45

1. SUMMARY

Calpains are large group of ubiquitous and highly modular calcium dependent cysteine proteases that mainly are located in the cytoplasm of the cells and usually their activity is associated with binding to the phospholipids of intra cellular membranes. Calpains play critical role in regulating important physiologic functions in vast majority of different kinds of organisms, through performing limited cleavages of their substrates. Malfunction or dysfunction of calpains can cause serious disorders. The CysPc domain is the catalytic core domain of the calpain family and all proteins with a CysPc domain are considered as calpains. DEK1 is the only calpain family member that has been discovered in land pant so far. DEK1 structure is highly conserved among land plants through half billion years of evolution. This conservation of DEK1 indicates very important functions for this enzyme in land plants. Though, the exact function of DEK1 is not clear. Researches on DEK1 function have revealed that a functional *dek1* gene is essential for maintaining the epidermal cell layer in developing tissues. Null mutation of *dek1* causes abortion of embryo growth in land plants. Recent studies indicate a critical role of *dek1* the in orientation of the correct cell division plane in the moss *Physcomitrella patens*. DEK1 is composed of a large transmembrane domain at the N-terminus that leads to the catalytic core domain of calpain family (CysPc) through a hydrophilic arm, and a C2L domain at the C-terminus. Limited experimental data concerning DEK1-C2L function is available, but significant level of conservation in amino acid sequences was detected between DEK1-C2L and animal calpain C2L domain indicating functional similarities. The C2L domain of animal calpain regulates enzyme activity via orchestrating Ca^{2+} and membrane binding. The work reported here is divided into two sub-projects. In the first part recombinant *P. patents* DEK1-C2L protein was expressed in *Escherichia coli* BL21 cells using the expression vector pET302 and the protein was purified by affinity chromatography. In the second part of the experiment, the phospholipid binding ability of the *P. patents* DEK1-C2L protein was investigated using size exclusion chromatography spin column technique. The work presented here suggests that the C2L domain of *P. patens* DEK1 bind to liposomes and this ability increases in presence of Ca^{2+}.

2. INTRODUCTION

2-1. Definition of calpains

Calpains are a large and highly modular group of proteases that perform limited proteolysis of their target protein substrates. Calpains have diverse functions and regulate a multitude of intra cellular processes including cell division, proliferation, migration, and cell death (Goll, Thompson, Li, Wei, & Cong, 2002). They have been named for the calcium dependence of the papain-like, cysteine protease activity of the vertebrate m-calpain (Croal & Ersfeld, 2007). Calpins are ubiquitous enzymes and members of this family are present in most eukaryotic organisms in addition to some prokaryotic organisms (Zhao et al., 2012). Calpains are mainly located in the cytoplasm of cells and their activation is often associated with binding to the phospholipids of plasma membrane or membrane of organelles (Tompa, Emori, Sorimachi, Suzuki, & Friedrich, 2001). Calpains do not degrade their target substrates instead they perform limited proteolysis of their targets in respond to calcium signaling and modify either by activation or inactivation the action of their substrate proteins (Sorimachi, Hata, & Ono, 2010). The catalytic core domain, the CysPc domain, contains the active site catalytic triad of cysteine (Cys), histidine (His) and aspargine (Asn). Proteins showing sequence similarity to the CysPc domain of the m-and µ-calpains, which are regarded as founder protein of this family, are classified as calpains. In a recent study, 24 different domains were found in calpains, and 41 different calpain domain architectures were identified across all eukaryotic super groups (Zhao et al., 2012). µ-calpain was the first member of this family discovered in soluble extracts from rat brain and skeletal muscle in 1969 (Gopalakrishna & Barsky, 1986). µ-calpain and m-calpain are different in calcium requirement for half-maximal activity and these enzymes are the most extensively characterized calpains. µ and m- calpains are heterodimers, each comprised of an 80-kDa large catalytic subunit and a 30-kDa small regulatory subunit (Ohno et al., 1984; Tompa et al., 2001). The catalytic subunit harbors four conserved domains (Croall DE, 2007; Macqueen, Delbridge, S., & Johnston, 2010) (Figure 1A); an N-terminal anchor helix (Nter) that is involved in autolytic activation, the catalytic core CysPc domain (comprised of two subdomains PC1 and PC2) harboring two calcium binding sites (Figure 1B), a C2-like (C2L) domain that harbors acidic residues and a penta-EF-hand (PEF(L)) domain; In brief denoted as: Nter-CysPc-C2L-PEF(L). The regulatory subunit harbors two domains (Figure 1B); a glycine and proline-rich domain and a PEF(S) domain that is structurally similar to PEF(L) domain of the catalytic subunit. The crystal

structure of rat μ-calpain has been resolved and is shown in Figure 1A. Some calpains do not harbor the regulatory subunit, including the mammalian p94 calpain (Sorimachi & Ono, 2012), and less is known of how the activity of these calpains are regulated.

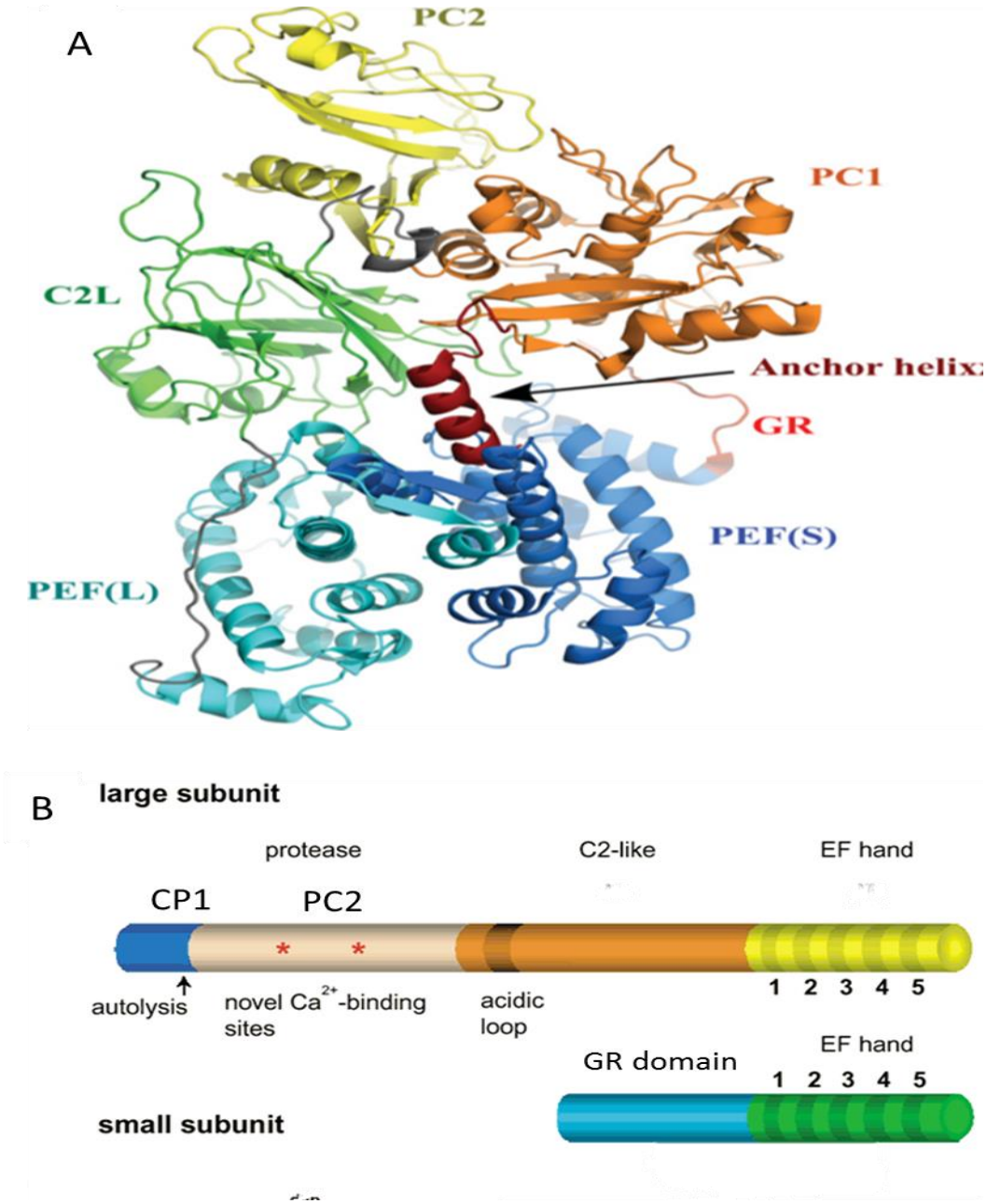


Figure 1: A: Structure of human m-calpain (Campbell & Davies, 2012). The N-terminus of the large subunit begins with the anchor helix (red) and leads into the protease core domains comprised of PC1 (orange) and PC2 (yellow). The C2L domain (green) is attached to PC2 subdomain of the catalytic core domain. The PEF domain (light blue) is at the C-terminus. The PEF domain of the large catalytically subunit is paired with the C-terminal PEF domain of the small regulatory subunit (dark blue).The GR domain is located at the N-terminus of the small subunit. B: Schematic representation of the domain structure of the large and small subunits of m-calpain (Khorchid & Ikura, 2002).

Members of the calpain protein family have been classified into two groups based on domain architecture. Calpain with domain architecture similar to μ and m-calpains are classified as classical calpains. The other group, the non-classical calpains are composed of calpains lacking both the N-ter and the PEF domains. This group of calpains often contain additional domains in combination with CysPc (Zhao et al., 2012). The physiological role of calpains depends on the nature of the substrate. Identifying calpain substrate is an area of intensive research, however difficult because no specific target sequence motifs have been identified (Caberoy, Alvarado, & Li, 2011) and the rules governing the specificity of calpains still remain unclear. So far various types of proteins including transcription factors, calmodulin-binding proteins, components of receptor-mediated signal transduction and cytoskeletal proteins have been experimentally identified as calpain substrates (Wang, Villalobo, & Roufogalis, 1989; Weber, Hühns, Lüthen, & Jonas, 2009). Because of the wide range of identified calpain substrates it is clear that this group of enzymes plays important roles in different physiological cell activities in nature such as cell proliferation, apoptosis, differentiation, and signal transduction. Also it has been reported that calpains are involve in endocytosis, exocytosis, and intracellular membrane fusion (Sato et al, 1995). Disfunction or malfunction of calpains may cause life threaten consequences. In human defects in calpain function are associated with serious diseases, for instance, muscular dystrophies, gastropathy, tumorigenesis and neurogenesis disorders (Branca, 2004). In mice and plants defects in calpain activity causes embryo-lethality and disfunction causes loss of sex determination in nematodes (Branca, 2004). Because of the clearly important function of calpains in many different organisms, it is important to have a comprehensive understanding of calpains structure and functions.

2-2. The origin and evolution of DEK1

The *dek1* gene was first encountered and analyzed in *Zea maize* and the gene was named after the aberrant phenotype (defective kernel 1) caused by null mutation of this gene in *Z. mays*. More than 65 complete genome sequences are currently available form land plants, and analysis of these genomes have revealed that DEK1 is the only calpain variant in land plants (Zhao et al., 2012; Liang et al., 2013). The DEK1 calpain has a large N-terminal transmembrane domain (TML), and these TML calpain can be trace 1.5 billion year back in time (Zhao et al., 2012). Recent studies suggest that the common ancestor of the green lineage

possessed both cytosolic and transmembrane calpains (Liang et al., 2013). Based on recent unpublished results (pers. med. Johansen W; manuscript submitted for publication.) it appears that after divergence of the *Chlorophyta* and *Charophyta* algae (Figure 2), *Chlorophyte* organisms lost transmembrane calpains only retaining cytosolic calpains, and *charophyte* algae, the predecessor of land plants, retained both cytosolic and transmembrane calpains. The cytosolic calpains were subsequently lost during evolution retaining only the transmembrane calpain DEK1 in land plants. At what stage of and land plant evolution cytosolic calpains were lost is currently unknown (pers. med. Johansen W.). Phylogenetic analyses of available transmembrane calpains using CysPc alignments demonstrate that transmembran calpains are divided into two distinct clades (Liang et al., 2013). One of them is the *Streptophyta* clade comprised of the *charophytes* and *embryophytes*. The other group is the non-land plant clade. In the *Streptophyta* clade, the calpain of the unicellular *charophyte* alga *Mesostigma viride* forms a separate sub-clade (Figure 3) (Liang et al., 2013) suggesting divergence between the *dek1*-like gene of *M. viride* and the land plants calpain *dek1*, further supporting that a major evolutionary shift in DEK1 function occurred during evolution of *Charophycea* through *Zygnematophyceae* and *Coleochaetophyceae* to land plants (Liang et al., 2013).

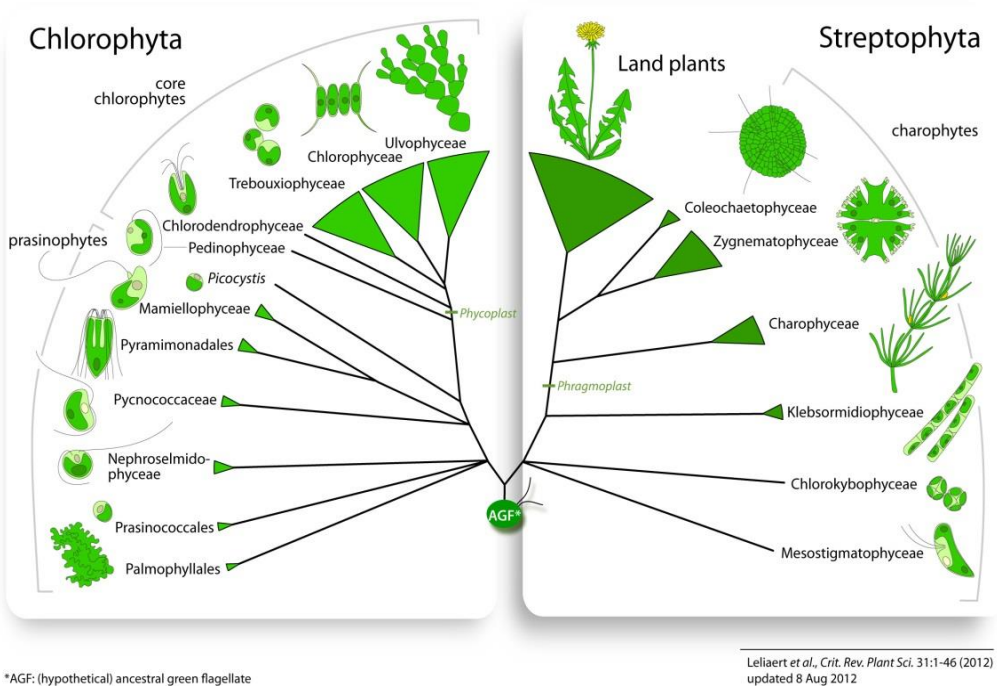


Figure2: Phylogenetic tree of *Viridiplantae* (Leliaert et al., 2012). The common ancestor of the green linage was divided in two main clades, the *Chlorophyta* and *Charophyta* algae. Land plants evolved from the *Charophyta* algae.

Sequence data from higher plants demonstrate high degree of sequence conservation in DEK1. The amino acid sequence of *Arabidopsis thaliana* DEK1 is 70 % identical to DEK1 of *Z. maize.* This sequence conservation is raised to 80 % when the catalytic core domains (CysPc) of *A. thaliana* and *Z. maize* DEK1 are analyzed. The level of conservation of the amino acid sequences of catalytic core domain is also high, 89 %, between the DEK1 proteins of *Physcomitrella patens* and *A. thaliana,* that are separated by ~500 million years of evolution (Liang et al., 2013; Lid et al., 2002). Complementation assays using the CysPc-C2L domain of the moss *P. patens* to rescue the *A. thaliana dek1* embryo lethal mutant phenotype indicates that the CysPc-C2L domain of DEK1 enzyme is also conserved functionally during hundred million years of evolution (Liang et al., 2013)**.** Structural and functional conservation of DEK1 in land plants through hundreds of millions years indicates the indispensability of DEK1 for an operative physiology of land plants.

Figure3: Phylogenetic analyses of *streptophyte* TML-calpains, using CysPc–C2L alignments by neighbor-joining and maximum-likelihood methods. Classical animal calpains has been used as an out-group (Liang et al., 2013).

2-3. Domain structure of DEK1

The DEK1 gene encodes a 240 kDa protein that in *Z. mays* consists of 2,159-amino acids (Lid et al., 2002). Analyses of calpain sequences have revealed that all calpains in land plants have identical and highly conserved domain architecture (Figure 4). The DEK1 enzymes are composed of an N-terminal transmembrane (TML) domain consisting of 23 transmembrane segments only interrupted by an approximately 280 amino acid Loop. The localization of this loop is contradictive as it has been predicted to both be extracellular (Lid et al., 2002) and intracellular (Kumar et al., 2010).

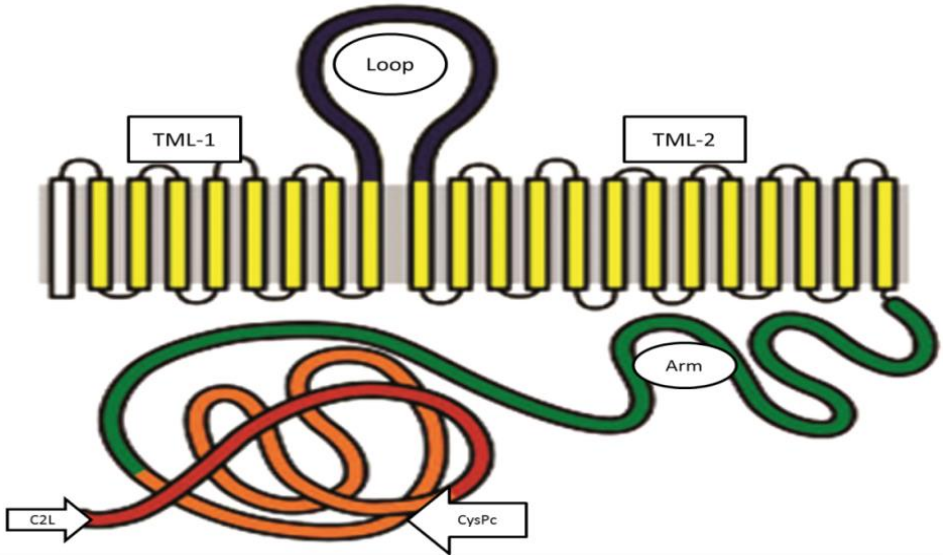


Figure 4: Schematic model of overall DEK1 structure (modified Figure from Lid et al, 2002).

The CysPc-C2L domain of DEK1 is connected to the TML domain by a hydrophilic Arm domain of unknown function. The CysPc domain of land plants harbors the catalytic active site residues of calpains (Liang et al., 2013; Wang et al., 2003). Prediction of DEK1 CysPc structure shows that the overall structure is similar to animal CysPc domains, including the two CysPc subdomains PC1 and PC2 (Wang et al., 2003, Liang et al., 2013). Functional analysis in A. thaliana have showed that the four conserved amino acid residues involved in CysPc Ca2+ binding in animal calpains are conserved in DEK1 and functionally essential (Liang et al., 2013). The active site residues Cys is located in subdomain PC1 (Figure 5& 6), and PC2 subdomain harborsHis and Asn. These three amino acids together comprise the catalytic active site of the DEK1-CysPc domain (Figure 5). Also in animal calpain there are calcium-binding sites on PC1 and PC2 subdomain essential for activation of the animal

calpains (Hanna, Campbell, & Davies, 2008). The C-terminal end of DEK1 harbors a C2-like (C2L) domain (for more information see page13 paragraph 2.5). This domain is structurally related to C2L domain of animal m-calpain (Wang et al., 2003).

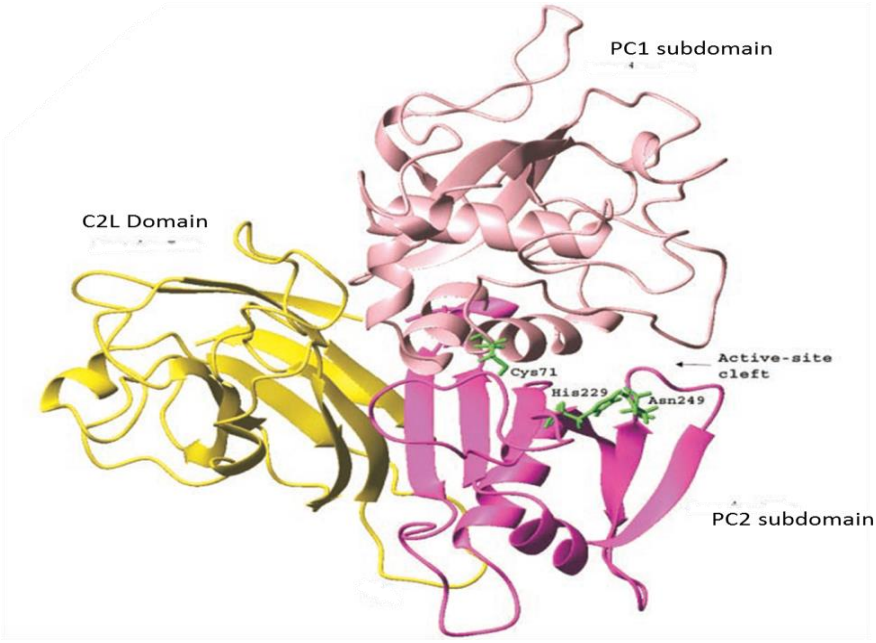


Figure 5: Three dimensional modeling of DEK1 calpain prepared by MOLMOL program (Wang et al., 2003). In this modeled structure of DEK1 CysPc-C2L active site residues are shown. Cysteine (Cys 71) is located in the α-helix of PC1 subdomain and histidine (His 229) and asparagine (Asn 249) are located on subdomain PC2.

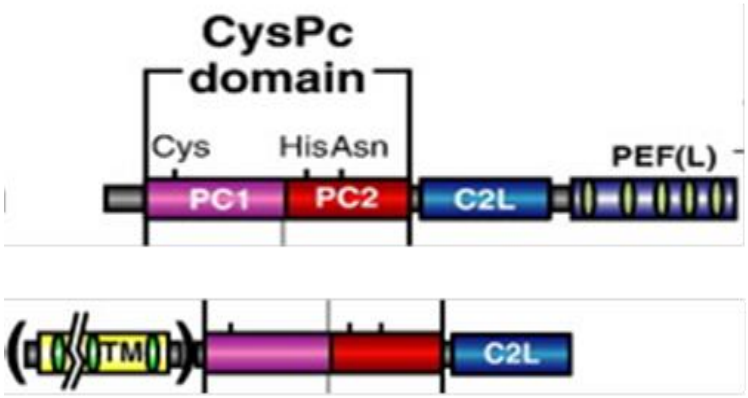


Figure6:DEK1 domain architecture (down) in comparison with classical calpain domain architecture (up)

2-4. The function of DEK1

DEK1 physiologic function is not completely understood, but structural and functional conservation of DEK1 in land plants through hundred millions of years indicates an important role for this protein in land plants. Studies in maize have revealed that a functional *dek1* gene

is essential for developing and maintaining aleurone cell fat. In maize seeds with mutant *dek1* gene the auleron cell layer is missing (Lid et al., 2002). The aleurone layer of maize plant seed is a one cell layer thick and covers the endosperm. The alueron contains lipids and oils and secrets enzymes that lyse stored nutrition in developing seed, providing nutrients during seedling growth. DEK1 transcripts can be found in all cell types of developing maize endosperm, suggesting, that DEK1 regulation occur post- transcriptionally (Lid et al., 2002). Maize with mutation in *dek1* gene fail in embryogenesis, and maize plants with weak *dek1* allele show defects in embryo formation and cell specification (Becraft, Li, Dey, & Asuncion-Crabb, 2002). Following studies recently revealed another interesting role for DEK1 enzyme in land plants. This enzyme is essential for correct orientation of the cell division plane during initial bud development and is essential for building three-dimensional body structure in the moss *P. patens* (Perroud et al., 2014). It has been demonstrated that the transition from the *protonema* stage to the *gametophores* stage in *P. patens ⊿ dek1* mutants fails, resulting in early bud development arrest (Perroud et al., 2014). Based on accumulated evidence it has been suggested that in developing seeds DEK1 senses positional signals and react to this cues that results is specification of epidermal layer (Perroud et al., 2014). However the biochemical pathway of perception of spatial information and orientation of cell division, that results in three dimensional development, is not completely understood. It is proposed that in higher plants DEK1 transmembrane domain, which is interrupted by a loop, act as a sensor and senses environmental cues. Also it is suggested, that the cytosolic calpain cysteine proteinase work as an effector and cleaves an unidentified substrate proteins in respond to positional signals (Tian et al., 2007). As it is mentioned previously (see the paragraph 2-3) DEK1CysPc domain function is conserved among land plants for ~500 million years (Liang et al., 2013). This functional conservation of CysPc domain of DEK1 suggests that the substrate of this catalytic core also may be conserved (Liang et al., 2013). Research concerning cellular targets of μ-and m-calpain has revealed that calpains among other substrates cleave microtubule-associated proteins (Billger, Wallin, & Karlsson, 1988). Microtubule-associated proteins belonging to the MAP65 and CLASP families control orientation of cell division and epidermal cell fate specification in *A. thaliana* roots (Dhonukshe, Weits, & Cruz-Ramirez, 2013). Considering this information it is tempting to speculate that DEK1 cleaves cellular components that control cell specification and orientation of cell divisions in land plants (Liang et al., 2013). This hypothesis can explain the aberrant cell division and plane positioning in the *P. patens dek1* mutant phenotype. Despite the high structural conservation between animal and land plant CysPc-C2L domains, DEK1 of

maize is not dependent of calcium ions for *in vitro* activity. In a study performed by Cunxi Wang (2003), recombinant DEK1-CysPc-C2L protein was incubated with purified casein. In this assay casein was degraded after 9 hours even in the absence of Ca^{2+}. However, presence of Ca^{2+} increases the activity of the enzyme *in vitro*. It has been suggested that the difference in Ca^{2+} requirement for activity between DEK1 and animal calpain is a result of differences in amino acid residues arrangement in CysPc-C2L domains of these two calpains (Johnson, Faulkner, Jeffree, & Ingram, 2008; Wang et al., 2003). Animal calpain harbors an acidic loop in the C2L domain that interacts with acidic residues in the subdomain PC2 of animal CysPc. This electrostatic interaction causes a repulsive force that rearranges the calpain's subdomains into an active conformation. Animal CysPc domain harbors in addition two binding sites for Ca^{2+} (Hanna et al., 2008; Strobl, Fernandez-Catalan, & Braun, 2000). When Ca^{2+} bind to these sites, the repulsive force that keeps two subdomains apart is abolish and realign the catalytic cleft into an active conformation (Fernandez-Montalvan et al., 2004). The DEK1-C2L domain lacks the acidic residues and these are also absent in the DEK1 PC2 subdomain (Liang et al 2013, Wang et al. 2003), suggesting that the DEK1 protein is not activated by Ca^{+2} in the same way as animal calpains. However DEK1 CysPc-C2L caseinolytic activity increases in the presence of Ca^{2+} (Wang et al., 2003). It has been suggested that this increase in activity of recombinat protein is due to a slight repositioning of PC1 and PC2 subdomains which optimize the catalytic triad configuration (Wang et al., 2003). Despite caseinolytic activity in the absence of Ca^{+2}, mutation in the putative calcium binding sites of the *A. thaliana* DEK1-CysPc cause inactivity of the calpain in the complementation assay (Liang et al., 2013). Further investigations are needed to investigated whether DEK1-CysPc with mutation in calcium binding sits is inactive because of it needs for calcium ions *in vivo*, or it is because of change in DEK1-CysPc 3D structure (Liang et al., 2013).

2-5. C2L structure/function

The C2L or C2 like domain of the calpain super family, also known as domain III has been named after it's structural similarity to C2 domain of the original protein kinase C that regulate enzyme activity via orchestrating Ca^{2+} and membrane binding (Tompa et al., 2001). Animal C2L domains also bind calcium ions through its acidic loop residues (Strobl et al., 2000). This ability increases in the presence of phospholipids, and in the same way the presence of Ca^{2+} increases phospholipid binding ability of the C2L domain (Tompa et al., 2001). It has been suggested that the C2L domain of calpains regulate catalytic activity of the

enzyme by binding to calcium ions and binding to membrane phospholipids (Tompa, 2001). So it can be inferred that C2L domain is indispensable for an active calpain. When it comes to the DEK1-C2L domain limited information is available. In the same way, the structure-function relationship between DEK1 CysPc and C2L domain is not clear (Wang, 2003). Three dimensional modeling of DEK1-C2L domain shows high similarity to animal C2L domain structure. Also amino acid sequence alignment between animal calpain and DEK1 reveals high conservation between animal C2L and DEK1-C2L (Wang, 2003). Because of high similarity in spatial structure and amino acid sequence alignment similarity in function of animal and DEK1 C2L domain is expected. Despite structural and sequence similarity there is a large difference between animal and DEK1 C2L domains. Animal C2L domain has a very acidic loop consisting of ten negatively charged residues that tend to bind Ca^{2+}. There is only one conserved negatively charged amino acid in DEK1-C2L domain (Asp-347) (Wang, 2003). So it is unlikely that DEK1-C2L domain has regulatory role in DEK1 enzyme via calcium binding in the same way as animal calpains. But lack of sepal giant cell in *A. thaliana* with a single point mutation in a highly conserved residue of DEK1-C2L domain (*dek1-4*) suggests a regulatory role for DEK1-C2L domain in a specific cellular context within the epidermis (Roeder, Cunha, Ohno, & Meyerowitz, 2012). Nevertheless as animal calpain, C2L domain is indispensable for proteolytic activity of DEK1. In an experiment performed by Wang et al. in 2003, recombinant-DEK1-CysPc protein was incubated with casein to investigate if the CysPc domain alone has caseinolytic activity in the absence of the C2L domain. Activity measurement showed that DEK1-CysPc is unable to degraded casein in absence of C2L domain. This finding clearly indicates the necessity of C2L domain for *in vitro* activation of calpain activity.

2-6. Recombinant protein expression

Life is absolutely dependent on proteins, and all living organisms provide a large amount of different proteins that together determine the characteristics of the organism. This makes necessary to study proteins structurally and functionally for life since researchers in order to have better understanding of life on earth (Cornvik, 2006). Before 1970s the only way to study polypeptides was to extract proteins from natural sources. But some proteins are very low abundant in the cell making extraction of sufficient amounts of proteins to study very challenging. Beside, extraction from natural sources of some kind of proteins such as insulin, interferon and different kind of hormones can be too expensive (Clark, 2001). In mid 1970s a

restriction enzyme was purified for first time from *Hemophilus influenzae*. This achievement along with the discovery of the enzyme ligases allowed scientists to construct the first artificial plasmid which were used as a vector to transfer genetic materials between organisms. This was the first step in producing recombinant proteins (Cornvik, 2006). Recombinant protein is a polypeptide that has been produced by experimental manipulation of genetic materials using genetic engineering and recombinant DNA technology. Now a days different kind of expression systems are used to produce recombinant proteins in bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture. Each of these expression systems has its own advantages and disadvantages (Baneyx, 1999). One of the most favorable host cells for expression of recombinant protein is the gram-negative bacterium *Escherichia coli* (Sørensen & Mortensen, 2005). This bacterium has a relatively simple structure compared with other available expression systems. The genetics of *E. coli* has been characterized better than other host cells and there is a well-established understanding of transcription, translation, and protein folding process in *E. coli*. Also there are a lot of improved vectors that are adopted to insert recombinant DNA of complex eukaryotic proteins into *E. coli*. Another advantage of this bacterium is its ability to grow rapidly and at high density on inexpensive substrates. One of the most commonly used *E.coli* strains for recombinant protein expression is *E.coli* BL21. In this strain, two genes encoding the protease enzymes, ompT and lon have been disabled to prevent interference in the process of production and isolation of recombinant proteins (Sørensen & Mortensen, 2005). The lon protease is an intracellular protease that *E. coli* makes to degrade abnormal proteins such as recombinant proteins. The OmpT protease is a protease that *E. coli* makes to degrade extracellular proteins. It degrades the protein after bacterial cells are lysed. Despite of all these advantages some problems can be faced. One of the obstacles is that in rapid, large-scale production of recombinant proteins using *E. coli* as expression system, expressed recombinant proteins do not always fold properly in their native state. Frequently, heterologous proteins that have been produce in *E. coli* accumulate in the form of large insoluble aggregations within the bacterium (Cornvik, 2006). These insoluble aggregations are known as inclusion bodies (IBs).There is not much information about structure and mechanism of IBs formation. It is proposed that aggregation of expressed recombinant protein into inclusion body is a response to stresses that cause due to expression of proteins at high rate in a short time. Some researchers have suggested that when the concentration of expressed recombinant proteins reach 200-300 mg/ml in the cytoplasm of *E.coli* proteins start to fold into a compact form rather than into their native state (Sørensen & Mortensen, 2005) (Figure 7). In the process of

producing recombinant protein using *E.coli* formation of IBs can be minimized by controlling the incubation temperature or through the control of expression rate. Another method to decrease formation of inclusion bodies is engineering target protein by using solubility tag–technology (Jonasson, Liljeqvist, Nygren, & Ståhl, 2002).

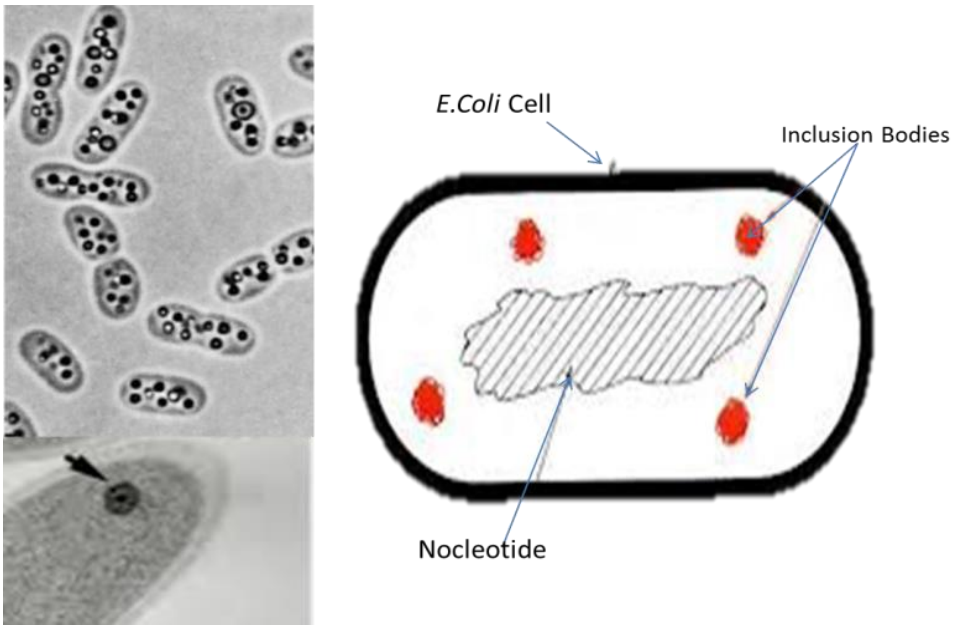


Figure 7: Inclusion bodies (IBs) in *E.coli* cell. Inclusion bodies usually are protein aggregates in nuclear or cytoplasmic of a cell (Cruts et al., 2006). Figure at the right side shows a picture of IBs in the *E.coli* cells, taken by light microscope. The Figure at the left side demonstrate a schematic representation of IBs in *E.coli* cell

Proteins in the form of inclusion body are not active biochemically and need to refold into their native state. Refolding of recombinant proteins from inclusion bodies into an active conformation can be challenging. This step is challenging because refolding of IBs is not a straightforward process and often requiring an extensive trial-and-error approach. Refolding of recombinant proteins into native state is affected by several factors including denaturant solution that is used to solubilize the inclusion bodies, removing of the denaturant and using small additive molecules to assist refolding process. Also there are different methods to refold protein into its native state from IBs. Some of most common methods include dialysis, dilution, high pressure treatment and chromatographic refolding. One of the most commonly used methods is dialysis. Dialysis is a technique to separate macromolecules from unwanted components by passive diffusion of the solution through a semi-permeable membrane. To refold recombinant protein in native state using dialysis method, IBs are first isolated by high speed centrifugation of crude extract (disrupted cells). Secondly, the obtained pellets are washed by detergents to remove cell debris. Then IBs are dissolved in 6 M guanidine

hydrochloride or 8 M urea that cause complete denaturation of the proteins in IBs. Solubilized IBs can be contaminated with host proteins. These proteins can trigger co-aggregation and result in decreased of renaturation yields. Thus, in some cases processing of IBs include a purification step such as ion exchange, size exclusion, reverse phase chromatography or metal affinity prior to dialysis (Cardamone, 1995). Finally denatured recombinant proteins are dialyzed into a buffer with neutral PH (aqueous solvent). Ideally dialysis should results in refolding of recombinant proteins into native state (De Bernardez Clark, 1998). This goal may be achieved by decreasing denaturant solvent concentration against aqueous solvent concentration. Proteins in the high concentration of denaturant are well solved, unfolded and flexible. Transfer of proteins by changing concentration from denaturant buffer into aqueous buffer force them to collapse into folded state. Usually, sudden changes in concentration cause miss folding and aggregation. The key to successful refolding by dialysis is an intermediate state of denaturant concentration that is achieved by changing concentration from denaturant to aqueous slowly. In the intermediate state, concentration of denaturant is low enough to force proteins to collapse. At the same time denaturant concentration is high enough to allow protein molecules to stay solved and flexible to recognize their structure (De Bernardez Clark, 1998). Desired protein molecules are retained, because of their large size, in the dialysis tube made of semi-permeable membrane, but molecules of urea or guanidine are small enough to pass though the membrane. Decrease in the concentration of urea or guanidine in dialysate, causes to reduce the concentration of urea or guanidine molecules in the dialysis tube (Figure 8). Exchanging diluted denaturing buffer (dialysate) with an aqueous solvent buffer, removes the urea or guanidine molecules that are no longer in the sample. This method decreases concentration of urea or guanidine molecules to negligible levels (Tsumoto, Ejima, Kumagai, & Arakawa, 2003).

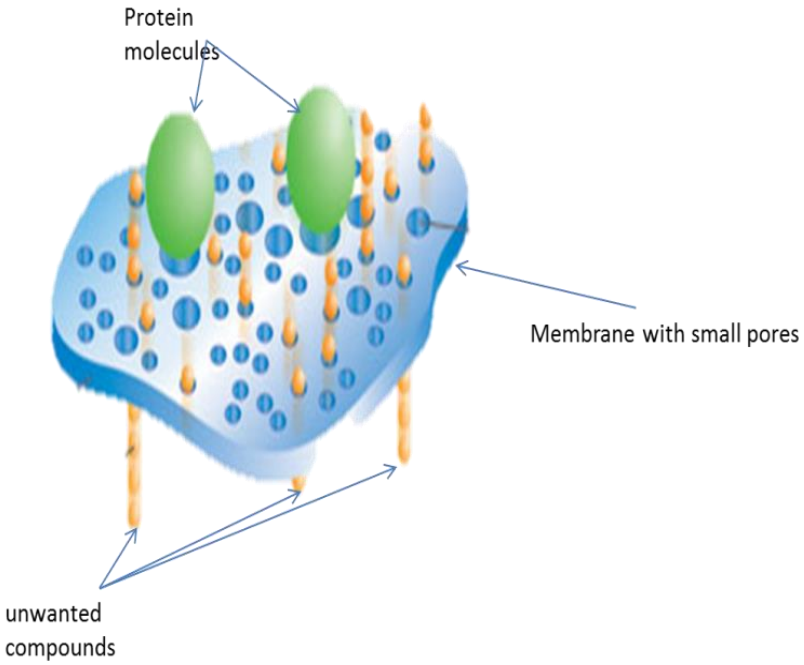


Figure 8: Dialysis membrane. A dialysis membrane is a semi-permeable tube with small sized pores that lets contaminant molecules to pass through freely, but keeps desired protein molecules, that are too large to pass through pores, in to the dialysis sample (www.piercenet.com).

2-7. Aim of study

Animal calpains have phospholipid binding property and it has been suggested that C2L domain is the primary phospholipid binding site (Tompa, 2001). Further it has been suggested that the C2L domain of animal calpains controls calpain activity through its Ca^{2+} and phospholipid binding ability. Also it has been suggested that binding of the CysPc-C2L domains to the membrane phospholipids trigger enzyme activity (Leloup et al., 2010). Despite of high degree of conservation between animal calpain and DEK1 calpain, plant calpain does not have the acidic and negatively charged amino acids, implicated in phospholipid and Ca^{2+} binding in animal calpains, suggesting that plant calpains does not possess regulatory effect through calcium binding property (Wang, 2003). Nevertheless, according to Roeder et al. (2012) the C2L domain of DEK1 plays a regulatory role in specific cellular context In addition, it has been demonstrated that the DEK1-CysPc domain is not functional in the absence of the C2L domain in *in vitro* studies. Altogether, important role of the DEK1-C2L in the physiology of plant makes it necessary to investigate characteristics of this domain. The main objective of this study is to investigate the phospholipid binding properties of

recombinant *P. patens* DEK1-C2L proteins. The study can be subdivided into two sub-projects:

1) Expression and purification of recombinant *P. patens* DEK1-C2L proteins. For this purpose recombinant *P. patens*-DEK1-C2L proteins were expressed in *E. coli*. Recombinant protein was purified by affinity chromatography and refolded into the native state by dialysis.

2) Development of assay to measure the phospholipid binding capacity of DEK1-C2L. For this purpose, refolded and concentrated *P. patens* DEK1-C2L proteins were applied to size exclusion chromatography spin column with or without liposomes.

3. Materials and Methods

3-1. Expression vectors

In this experiment four expression constructions were initially tested and used to express DEK1-C2L recombinant proteins. These vectors were constructed by ligation of the *A. thaliana* and *P. patens* DEK1-C2L cDNA into two different expression vectors: pET302_NT-His, with an N-terminal 6xHis-tag and a T7 promoter; and pETM-41_MBP, with N-terminal MBP and 6xHis tags, controlled by T7 promoter (Figure 9).

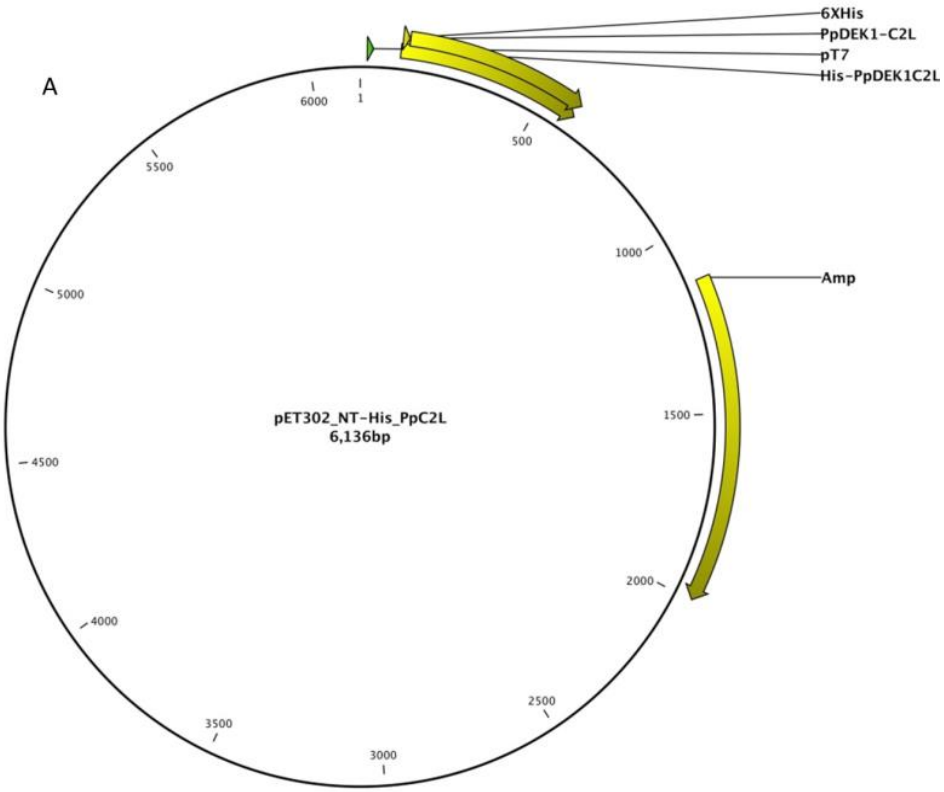


Figure 9: Vector maps of the pET302_NT-His_PpC2L plasmid, was used in this study (for other vectors map see Appendix).

3-2. Cell culturing

E. coli BL21 harboring sequenced and verified pET302_NT-His_PpC2L, pET302_NT-His_AtC2L, pETM-41_MBP-PpC2L and pETM-41_MBP-AtC2L plasmids were streaked on LB-agar medium (10 g tryptone, 5 g yeast extract and 10 g sodium chloride per 1000 mL of

dH2O ; pH 7.0 and containing 1.5 % agar) using standard aseptic protocol of microbiology. Proper antibiotics (50 µg/mL kanamycin for pETM-41_MBP vectors and 50 µg/mL ampicillin for pET302_NT-His vectors) were added to the LB-agar medium after cooling to 55 ^{o}C. Inoculated LB medium were incubated overnight (O/N) at 37 °C. Liquid cultures were initiated from single well isolated colonies from the agar plates. For small-scale protein production, cells were grown in 2 mL culture volume. For large scale protein production, 100 mL culture volume was used. Appropriate antibiotics were added to a final concentration of 100 µg/mL. Cultures were grown at 37 °C at 225 RPM O/N, and then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM and the culture incubated for additional 4 hours at 37 °C and 225 RPM.

3.3 Recombinant protein isolation and purification

Cells were harvested at 14000 rpm at 4 °C for 5 minutes. The pellet was re-suspended in B-PER (Thermo Scientific) solution (4 mL per gram of cell pellet) containing 0.1 mg/mL lysozyme and 5U/mL DNaseI. Lyses of the cells were achieved by incubating the samples for 15 minutes at room temperature with occasional mixing. The resulting crude extract was centrifuged at 14,000 rpm and 4 °C for 10 minutes, supernatant (soluble fraction) was discarded and the pellets (insoluble fraction) were re-suspended in B-PER (1 g in 1 mL) and analyzed by SDS-PAGE and western blotting techniques. Purification was performed using HisPur^{TM} Cobalt Spin Columns (Thermo Scientific). These spin columns contain cobalt-charged tetradentate chelator immobilized onto 6 % cross-linked agarose. Purification was performed at room temperature. The pellet (after centrifugation of the crude cell extract) containing the insoluble protein fraction was dissolved and solubilized in 8 mL of Equilibration/Wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 8 M urea, 10 mM imidazole; pH 7.4). Before applying the protein extract to the spin column the, the bottom tab was removed from the column then the column was placed onto a 15 mL Falcon tube and centrifuged at 700 × g for 2 minutes to remove the storage buffer. The column was equilibrated with 6 mL Equilibration/Wash buffer and buffer was removed by centrifugation (700 × g for 2 minutes). The protein extract (8 mL) was added to the spin column and the column was placed on an orbital shaker for 30 minutes. The spin column was centrifuged at 700 × g for 2 minutes and the flow-through was collected. The resin was washed 3 times using 6 mL Equilibration/Wash buffer each time by centrifugation at 700 × g for 2 minutes and fractions collected in three separate tubes. His tagged protein was finally eluted from the

column resin by adding 3mL Elution Buffer (50 mM sodium phosphate, 300 mM sodium chloride, 8 M urea, 150 mM imidazole; pH 7.4) and centrifuged at 700 × g for 2 minutes. This step was repeated two additional times and fractions were collected in separate tubes. All collected fractions were monitored by measuring the absorbance at 280 nm then analyzed by SDS-PAGE and Western blotting.

3-4. Protein refolding and concentration of protein sample

Purified and denatured protein samples were subjected to dialysis to refold proteins. Denatured protein sample (4 mL) was transferred into a dialysis tube (Snakeskin Dialysis Tubing, Thermo Scientific, molecular weight cut off: 50 KDa). The tube was floated in 250 ml 8 M urea in a beaker and steered for 6 hours at 4 °C. Every 8-12 hours 80 mL 25 mM Tris-HCl (pH 7.5) was added into the beaker, gently and under continues steering. When the volume of the dialysis solution reached 1000 mL, the solution was replaced with 500 mL of 25 mM Tris-HCl and 150 mM NaCl (pH 7.5) and steered for additional 6 hours at 4 °C. Refolded proteins were concentrated using Vivaspin 4 ml concentration column (Sartorius Company) according to the manufactures instructions. The columns concentrated the samples approximately 5x folded. Concentrated protein was pipetted out from the column and stored at 4 °C until used.

3-5. Preparation of liposomes

5 mg of phosphoinositides from a crude bovine brain extract (Sigma P6023), containing 20-40 % di- and triphosphoinositide (a minimum of 5–10 % of each) and 60–80 % phosphatidylinositol and phosphatidylserine, were dissolved in 1250 µL 1:4 methanol:chloroform solution. The solution was divided in aliquots of 25 µL (containing 100 µg phosphoinositides), then dried using a Savant Speedvac® concentrator. Dried samples were kept at -80 °C until used. Liposomes were prepared by re-suspending the dried sample in 200 µL buffer A (50 mM Tris_HCl, pH 7.5, 150 mM NaCl), vortexed for 5 min and then sonication for 5 × 15 s at 7 dBs. Finally the liposome solution was centrifuged at 15,000 RPM for 1 min to remove large aggregates.

3-6. Assay of protein-phospholipid binding ability

The phospholipid binding ability of recombinant His-tagged *P. patents* DEK1-C2L protein was assayed by spin-column size exclusion chromatography on MicroSpin S200 HR columns (Thermo Scientific). This spin column separates solutes by size; small proteins will be retained more efficiently in the column then larger protein aggregates. This means that binding of liposomes to the proteins of interest promotes elution of the proteins from the column. Wet bed height in the column was reduced to about 4 mm (170 µL) to prevent retentions of proteins completely, irrespective of the presence of liposomes. Columns were centrifuged at 2800 rpm (530 g) for 20 second to wash or elute samples using a microcentrifuge. Before applying the samples, columns were equilibrated with 2 × 200 µL buffer D (buffer A + 1.0 M NaCl) and 2 × 100 µL buffer D with liposomes and EGTA (5 mM) or Ca^{2+} (5 mM). Proteins at a final concentration of approximately 0.1 - 0.3 mg/mL were applied in 50 µL sample volume with or without 50 µg/mL liposome and EGTA or Ca^{2+} at concentrations of 5 mM. The elutes were collected and analyzed by SDS–PAGE.

3-7. SDS-PAGE

SDS-PAGE gels were casted in the thickness of 1.0 mm using Laemmli system. Separation gels were made by mixing the following ingredients: 4.5 mL acrylamide, 3.75 mL 1.5 M Tris-HCl buffer (pH 8.8), 6.5 mL dH_2O, 150 µL 10 % SDS, 150 µL APS and 15 µL TEMED, and stacking gel was cast on top of the separation gel, by mixing the following ingredients: 375 µL acrylamide 1.25 mL of 0.5 M Tris-HCl (pH 6.8), 4.5 mL dH_2O, 50 µL 10 % SDS, 50 µL 10 % APS and 5 µL TEMED. 40 µL protein sample was mixed with 5X sample buffer (0.6 g SDS; 3.75 mL of 1 M Tris-HCl solution pH 6.8; 9 mg bromphenol blue; 2.4 mL β-mercaptoethanol; 4.5 mL glycerol and filled up to 15 mL with dH_2O). Samples were incubated at 95 °C for 5 minutes then transferred to ice. The content of the tubes were collected by brief centrifugation and samples homogenized by gentle pipetting before loading onto the gel. The gels were run in a 1x running buffer (0.025 M Tris; 0.2 M glycine and 0.0035 M SDS) at 200 V for 45 minutes.

3-8. Coomassie blue staining

SDS-PAGE gels were stained in a mixture of 400 mL ethanol, 100 mL acetic acid, 500 mL dH_2O and 2.5 grams of brilliant blue R-250. Gels were incubated in this solution overnight with gentle shacking. Distaining of the gel was performed by immersing the gel in dH_2O and microwaving it at 700 W for several minutes until background distaining was completed.

3-9. Western blot and Immunostaining

Western blotting was performed to detect recombinant proteins (PpDEK1-C2L). Proteins were separated on 12 % SDS-PAGE gel and then transferred to a PVDF membrane at 320 mAmp for 100 min using standard techniques. After blotting the membrane was washed in TBS (Tris Buffered Saline: 25 mM Tris-HCl; 0.15M NaCl; pH 7.2) and immersed in blocking solution (TBS with 5 % milk) for 1 hour. Next, the membrane was washed in TBST (TBS with 0.05 % Tween-20) and incubated with the primary antibody solution (TBST with 3 % milk and 1:5000 Rabbit anti-C2L antibody) and incubated for 2 hours at room temperature or overnight at 4 °C. Afterwards, the membrane was washed with TBST and incubated with the secondary antibody solution (TBST with 3 % milk and 1:4000 Goat anti Rabbit alkaline phosphatase-labeled antibody) for 1 hour. After washing, 15 minutes in TBST, 5 minutes in TBS and at least 5 minutes in water, the detection step was carried out using substrate solution containing 20 mL AP buffer (100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, PH 9.5), 200 µL NBT (30 mg Nitro blue Tetrazolium solubilized in 700 µL Dimethyl formamid (DMF) and 300 µL H_20). The substrate solution was added to the membrane and the membrane incubated until dark purple bands appeared and finally the membrane was rinsed with water.

3-10. Measuring protein concentration

After purification and refolding steps concentration of protein in solution was measured using Quant-iT™ Protein Assay Kit. The Quant-iT™ Working Solution was prepared by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ buffer. For this purpose 199 × 3 µL of working solution was mixed with 3 µl of Quant-iT™ reagent. Standard #1 (Blank) was prepared by mixing 10 µL of 0 ng/µL standard solutions in 190 µL of Quant-iT™ Working Solution in a

thin wall PCR tube. Standard #2 (10 ng/µL) was prepared by mixing 10 µL of 200 ng/µL standard solutions in 190 µL of Quant-iT™ Working Solution in a thin wall PCR tube and Standard #3 was prepared by mixing 10 µL of 400 ng/µL standard solutions in 190 µL of Quant-iT™ Working Solution in a thin wall PCR tube. Protein sample was prepared by adding 20 µL of refolded protein solution in 180 µL of Quant-iT™ Working Solution in a thin wall PCR tube. Tubes were inserted into the Qubit® fluorometer and samples measured at 280 nm. The reading was multiplied by the dilution factor to determine concentration of sample.

4. Results

4-1. Verification of recombinant protein production

Small-scale protein expression experiment was performed to assess recombinant protein production and solubility of the produced recombinant proteins of the four different expression constructions (Material and Methods). The constructs were designed to express the DEK1-C2L domain of *Arabidopsis thaliana (At)* and *Physcomitrella patens (Pp)* as N-terminal MBP- and His-tagged fusion proteins. Cultures were initiated from well isolated *E. coli* BL21 (DE3) colonies harboring sequenced and verified DEK1-C2L expression construct (prepared by Wenche Johansen) and heterologous protein production was induced by the addition of IPTG as described in Material and Method. To investigate the solubility of produced protein, crude cell extracts from induced and un-induced cultures were centrifuged to separate soluble and insoluble material, and the resulting samples were analyzed using the SDS-PAGE assay. The results of these experiments are shown in Figure 10 (insoluble proteins) and Figure 11 (soluble proteins). Strong protein bands corresponding to the expected size of the His-tagged AtDEK1-C2L and PpDEK1-C2L proteins (18.2 and 18.1 kDa, respectively) and MPB-tagged AtDEK1-C2L and PpDEK1-C2L proteins (61.6 and 61.5 kDa, respectively) were observed in the insoluble fraction (Figure 10), surprisingly both in the IPTG-induced (lane 1, 3, 5 and 7) and un-induced samples (lane 2, 4, 6 and 8). Accumulation of proteins of the same size was not observed in the soluble fraction (Figure 11), indicating that the recombinant fusion proteins were produced predominately as inclusion bodies at the experimental conditions used (see Material and Methods). Since the His-tagged proteins accumulated to higher levels than the MPB-tagged proteins (Figure 10), and the His-tagged proteins do not contain a large solubility tag that might interfere with down-stream analysis, the His-tagged AtDEK1-C2L and PpDEK1-C2L proteins were chosen for further work. To verify that the recombinant proteins produced were indeed C2L proteins, Western Blot analysis using custom made anti-DEK1-C2L primary antibody was performed on crude protein samples extracted from *E. coli* expressing putative AtDEK1-C2L and PpDEK1-C2L proteins (Figure 12). Protein bands corresponding to the expected size of the His-PpDEK1-C2L (Figure 12 A) and His- AtDEK1-C2L (Figure 12 A) are apparent in lane 2 and lane 4 respectively. Western blotting result clearly suggests that DEK1-C2L proteins were produced as intended. Since approximately the same amount of total proteins were loaded on the gel for similar samples (Figure 12 B) (as judged by the Commassie stain of a replicate gel), the results indicate that the anti-DEK1-C2L

antibody has much higher specificity and affinity towards the His-PpDEK1-C2L protein then the His-AtDEK1-C2l protein. This was an expected result since the epitope used for producing the anti-DEK1-C2L antibody was designed to be 100 % identical to the PpDEK1-C2L protein. The epitope similarity to the AtDEK1-C2L protein was 75 %.

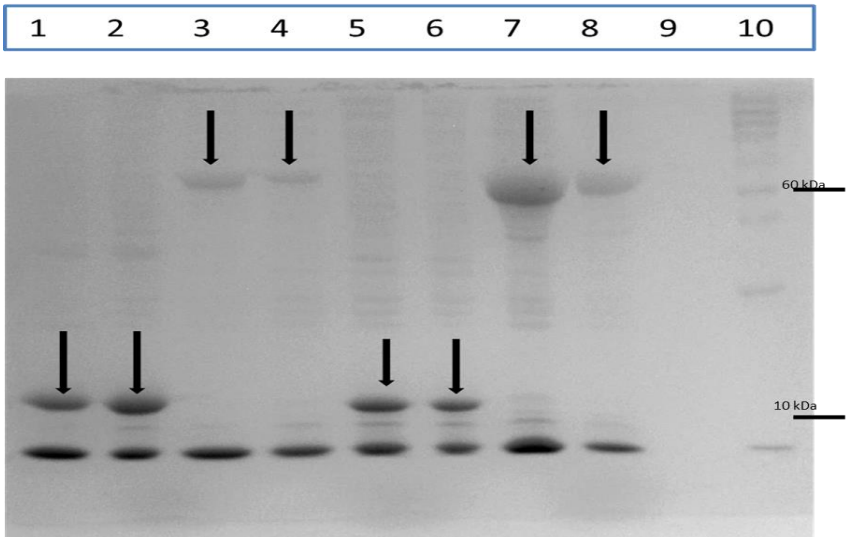


Figure 10: Insoluble protein fractions prepared from *E. coli* cultures harboring the various expression vectors described in Material and Method, separated by SDS PAGE and stained with comassie. Lane 1: His-PpDEK1-C2L IPTG-induced; lane 2: His-PpDEK1-C2L un-induced; lane 3: MPB-AtDEK1-C2L IPTG-induced; lane 4: MPB-AtDEK1-C2L un-induced; lane 5: His-AtDEK1-C2L IPTG-induced; lane 6: His-AtDEK1-C2L un-induced; lane 7: MPB-PpDEK1-C2L IPTG-induced; lane 8: MPB-PpDEK1-C2L un-induced; lane 9: Empty; lane 10 : Ladder.

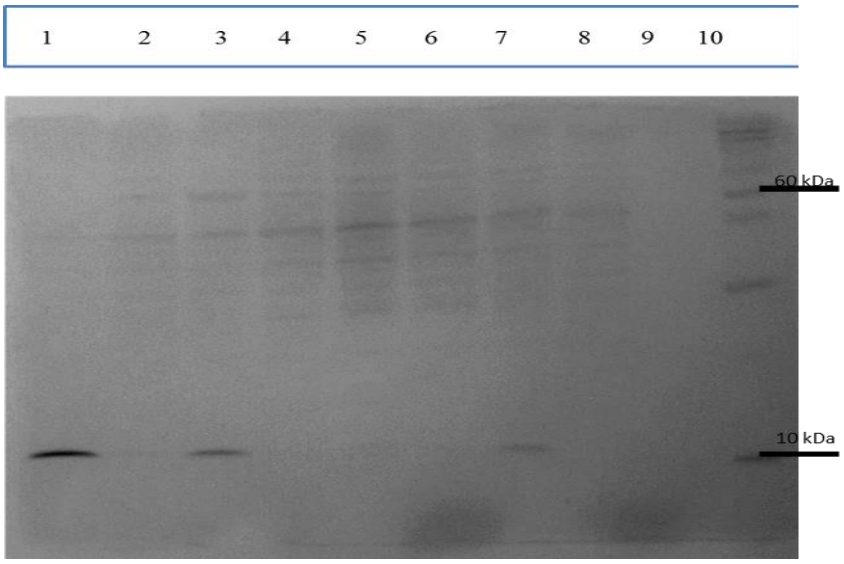


Figure 11: Insoluble protein fractions prepared from *E. coli* cultures harboring the various expression vectors described in Material and Method, separated by SDS PAGE and stained with comassie. Lane 1: His-PpDEK1-C2L IPTG-induced; lane 2: His-PpDEK1-C2L un-induced; lane 3: MPB-AtDEK1-C2L IPTG-induced; lane 4: MPB-AtDEK1-C2L un-induced; lane 5: His-AtDEK1-C2L IPTG-induced; lane 6: His-AtDEK1-C2L un-induced; lane 7: MPB-PpDEK1-C2L IPTG-induced; lane 8: MPB-PpDEK1-C2L un-induced; lane 9: Empty; lane 10 : Ladder.

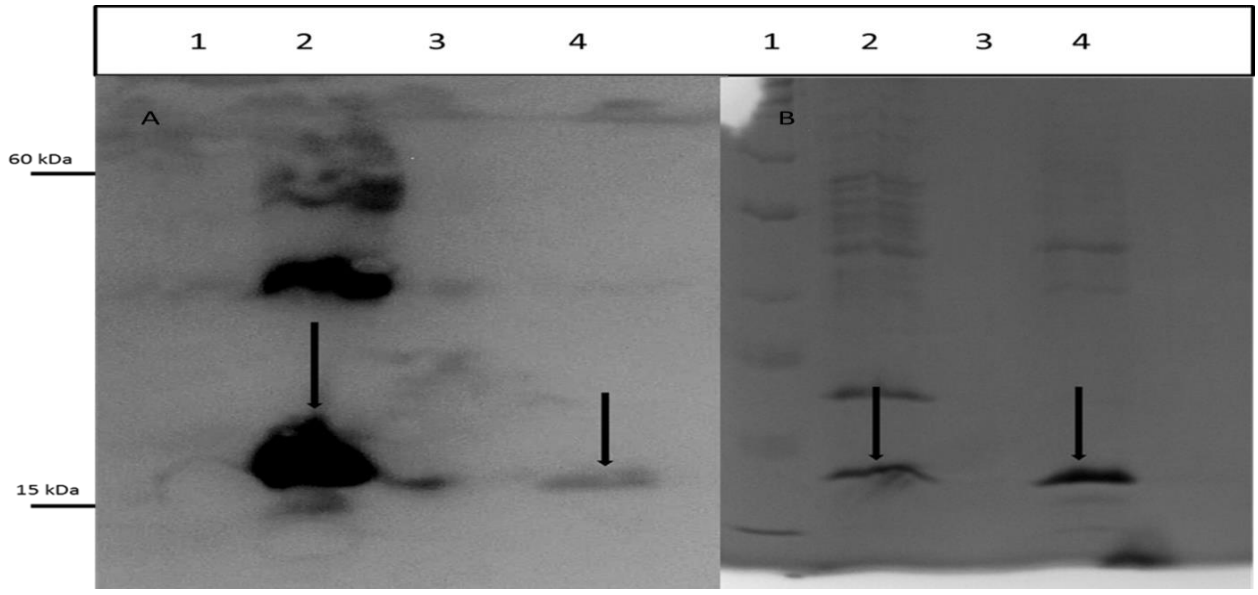


Figure 12: Western Blot (A) and SD-PAGE (B) of insoluble protein fraction from *E. coli* cultures harboring the 6X-His-PpDEK1-C2L plasmid and 6X-His-AtDEK1-C2L plasmid. Immunostaining was performed using anti-DEK1-C2L as the primary antibody on membrane obtained from western blotting. Lane 1: Ladder; Lane 2: His-PpDEK1-C2L IPTG-induced; Lane 3: Empty well; Lane 4: His-AtDEK1-C2L IPTG-induced.

4-2. Expression and purification of recombinant His-tagged *P. patens* DEK1-C2L protein

Both of the His-tagged DEK1-C2L proteins were proven to express well as inclusion bodies in *E. coli* (Figure 10). Since DEK1 function is currently investigated using the *P. patens* as a model organism in our lab, the recombinant His-tagged PpDEK1-C2L protein was chosen for further analysis. First, large scale protein production (from 100 mL culture volume) was performed to provide enough recombinant protein for down-stream processing and analysis. Before purification, proteins from the insoluble fraction prepared from the 100 mL culture volume were analyzed by Western analysis to confirm the production and presence of the His-tagged PpDEK1-C2L protein (Figure 13). Then, recombinant His-tagged PpDEK1-C2L proteins were purified from total insoluble proteins using HisPur^{TM} Cobalt Spin Columns. Before applying to the column, the insoluble protein samples were first dissolved in 8 M urea. Seven fractions were obtain from the His-column, including one flow through (FT) fraction, three wash (W1-3) fractions, and three elute (E1-3) fractions. All protein fractions obtained from the Spin Columns were analyzed by SDS-PAGE, western blotting and Immunostaining technique using the anti-DEK1-C2L antibody. Approximately the same amount of total proteins was loaded on the gel for both SDS-PAGE and western blotting analysis. Protein bands corresponding to the expected size of the His-PpDEK1-C2L on the gel resulted from SDS-PAGE (Figure 14B) indicates that His tagged PpDEK1-C2L proteins obtained from the columns as elute (E1-3) fractions in lane 7, lane 8 and lane 9 are purified from the rest of the insoluble protein in a high degree (as judged by the Commassie stain of the gel). The western blotting and Immunostaining result (Figure 14A) clearly shows that the eluted protein in elute (E1-3) fractions is His tagged PpDEK1-C2L.

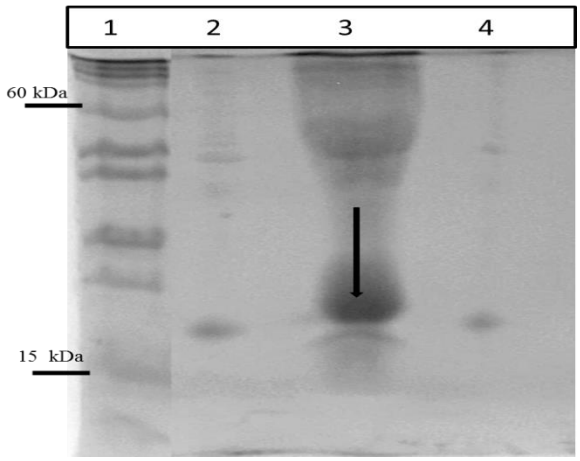


Figure 13: Insoluble protein fractions prepared from *E. coli* cultures harboring the His-tagged PpDEK1-C2L, analyzed by western blotting and Immunostaining technique using the anti-DEK1 C2L antibody. Lane 1: Ladder; lane 2: Empty; lane 3: His-PpDEK1-C2L IPTG-induced; lane 4: Empty

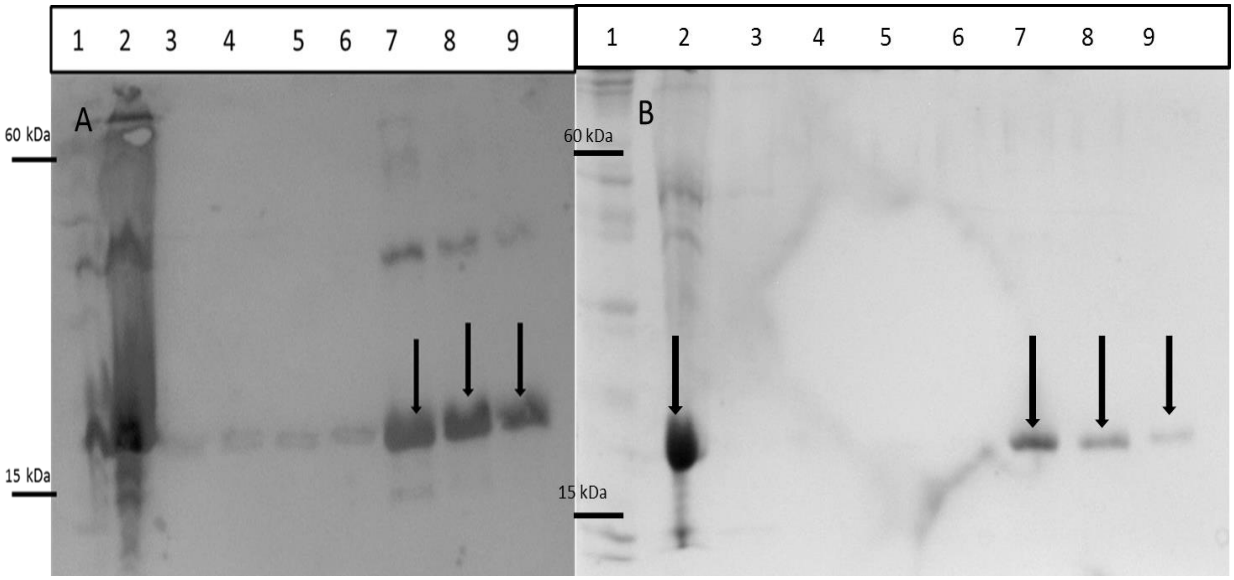


Figure 14: Insoluble protein fraction from *E. coli* cultures harboring the 6X-His-PpDEK1-C2L plasmids, purified using HisPur^{TM} Cobalt Spin Columns. All protein fractions obtained from the Spin Columns were analyzed by SDS-PAGE (Panel B), western blotting and Immunostaining technique (Panel A). Lane 1: Ladder; lane 2: insoluble protein fraction from *E. coli* cultures harboring the 6X-His-PpDEK1-C2L plasmids; lane 3: Flow Through; lane 4: Wash-1; Line 5: Wash-2; Line 6: Wash-3; Line 7: Elute-1; Line 8: Elute-2; Line 9: Elute-3; Line 10: Empty. Commassie stain of the SDS-PAGE gel indicates the degree of purity of His tagged PpDEK1-C2L proteins obtained from the columns as is shown in lane 7, lane 8 and lane 9 on the panel B. Analyzing the eluted proteins by immunostaining technique using the anti-DEK1 C2L antibody as primary anti-body verify that obtained proteins in elute fractions in lane 7, lane 8, and lane 9 in panel A is His-PpDEK1-C2L .

4-4. Re-folding and concentration of recombinant His-PpDEK1-C2L protein

Denatured eluted proteins from fraction E1-3 was subjected to refolding procedure by dialysis against an aqueous solvent. After complete dialysis as describe in Material and Method, the sample was centrifuged to pellet any insoluble material that might had formed and the sample from the soluble (supernatant) fraction was analyzed by western Blotting and confirmed the presence of the PpDEK1-C2L protein in the soluble fraction (data not shown). The refolded His-tagged PpDEK1-C2L protein was finally concentrated using Vivaspin 4 ml concentration column to a final concentration of approximately 500 µg/mL. The concentrated recombinant protein sample was analyzed and verified by western blotting and immunostaining techniques (Figure 15).

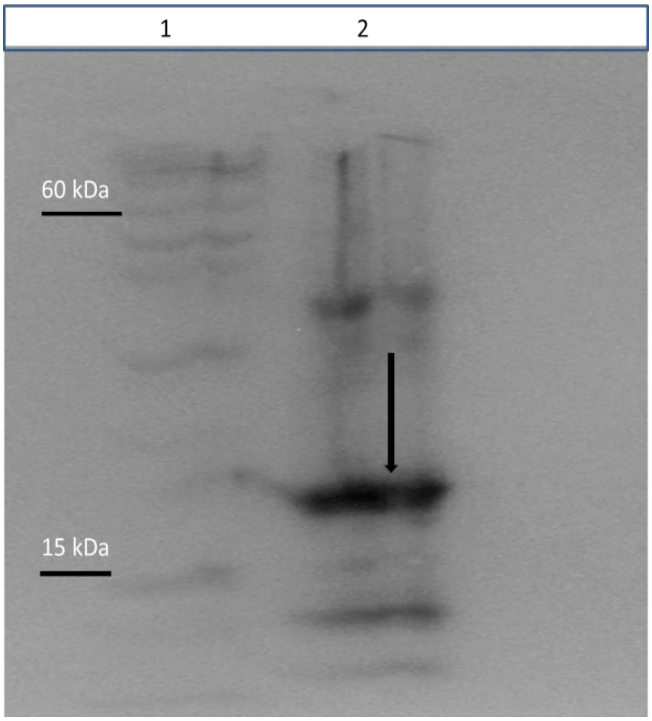


Figure 15: Soluble refolded His-tagged PpDEK1-C2L fractions resulted from dialysis concentrated using Vivaspin 4 ml concentration column then, analyzed by western blotting and immunostaining techniques. Lane 1: Ladder; lane 2: His-PpDEK1-C2L

4-5. Analysis of the liposome binding ability of recombinant PpDEK1-C2L protein

The liposome binding ability of the recombinant His-tagged *P. patents* DEK1-C2L protein was analyzed using size exclusion chromatoghraphy spin columns (see Materials and Methods). In this method binding of proteins to liposomes will increase the size of the resulting complex, decreasing the retention time in the column thereby improving the elution of the protein complex. Exactly same amount of protein was applied into each column also the amount of control protein sample not applied to the column (Figure 16, lane 2). Elutes were run on 12 % SDS gel. First, the experiment was performed with bovine serum albumin (BSA) as a control since BSA is not able to bind to liposomes (Tompa et al., 2001) (Figure 16 C). Investigation of differences in intensity between protein bands corresponding to expected size of BSA in lane 3 (BSA), lane 4 (BSA along with liposomes), lane 5 (BSA along with liposomes and Ca^{2+}) and lane 6 (BSA along with liposomes and EGTA) reveals that presence of liposomes or Ca^{2+} does not affect elution of BSA from the column. According to the SDS-PAGE results, the intensity of protein band corresponding to the expected size of His tagged *P. patents* DEK1-C2L protein increases in presence of liposome (Figure 16 A). So that, the protein band in lane 4, corresponding to the eluted His tagged PpDEK1-C2L in presence of liposomes, seems stronger than the protein band in the lane 3, corresponding to eluted His tagged PpDEK1-C2L protein in the absence of liposomes. Also protein bands in lane 5, corresponding to the eluted His tagged PpDEK1-C2L in presence of liposomes and Ca^{2+}, is stronger than the protein band in lane 4, indicating that the presence of Ca^{2+} along with liposomes increases elution of His tagged *P. patents* DEK1-C2L proteins from the size exclusion chromatography spin columns. Also His tagged *P. patents* DEK1-C2L proteins eluted from the size exclusion chromatography spin columns were investigated by western blotting and immunostaining techniques using the anti-DEK1 C2L antibody as the primary anti-body (Figure 16 B). The immunostaining result confirms the SDS-PAGE result (Figure 14, panel B).

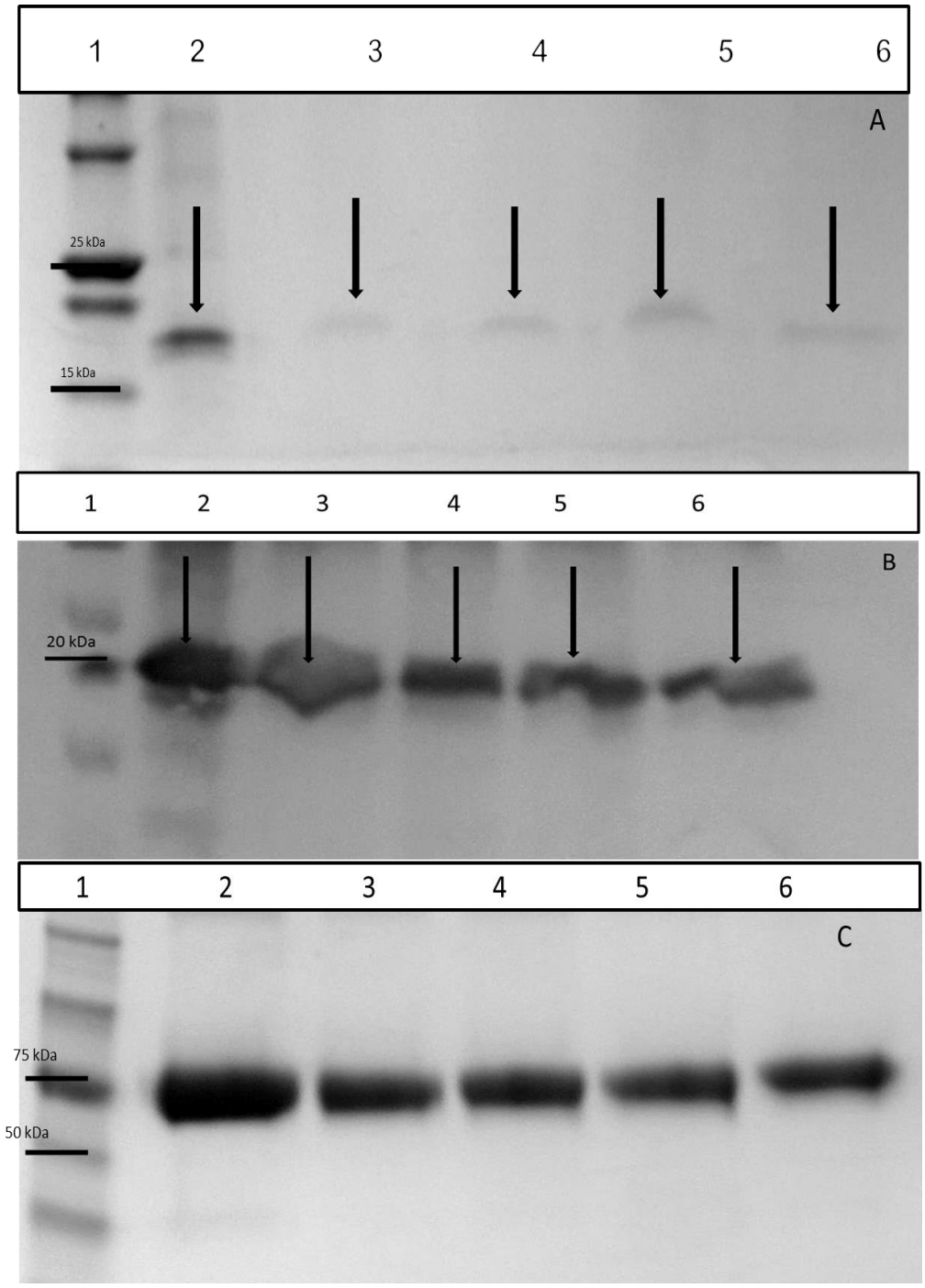


Figure 16: Soluble refolded His tagged *P.patents*-DEK1-C2L fractions were applied to the spin columns with a wet bed volume of 170 µl with or without liposome and EDTA or Ca^{2+}. Collected elutes were run on 12 % SDS gel and analyzed by western blotting and immunostaining techniques using the anti-DEK1 C2L antibody as primary anti-body, also the experiment was performed with bovine serum albumin (BSA) as a control. **A**; SDS-PAGE results of elutions from size exclusion chromatography spin column for His tagged *P.patents*-DEK1-C2L. Lane 1: Ladder; lane 2: refolded recombinant *P.patents*-DEK1-C2L (0.3 mg/ml) un-applied into the spin column; lane 3: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L; lane 4: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L along with 50 mg/ml liposomes; lane 5: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L along with 50 mg/ml liposomes and 5mM Ca^{2+}; lane 6: elution of 0.3 mg/ml

refolded recombinant *P.patents*-DEK1-C2L along with 50 mg/ml liposomes and 5 mM EGTA. **B**; immunostaining results of elutions from size exclusion chromatography spin column for His tagged *P.patents*-DEK1-C2L. Lane 1: Ladder; lane 2: refolded recombinant *P.patents*-DEK1-C2L (0.3 mg/ml) applied in the spin column; lane 3: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L; lane 4: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L along with 50 mg/ml liposomes; lane 5: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L along with 50 mg/ml liposomes and 5 mM Ca^{2+}; lane 6: elution of 0.3 mg/ml refolded recombinant *P.patents*-DEK1-C2L along with 50 mg/ml. **C**; SDS-PAGE results of elutions from size-exclusion chromatography spin columns for BSA. Lane 1: Ladder; lane 2: bovine serum albumin (0.1 mg/ml) applied in the spin column; lane 3: elution of 0.1 mg/ml bovine serum albumin; lane 4: elution of 0.1 mg/ml bovine serum albumin along with 50 mg/ml liposomes; lane 5: elution of 0.1 mg/ml bovine serum albumin along with 50 mg/ml liposomes and 5 mM Ca^{2+}; lane 6: elution of 0.1 mg/ml bovine serum albumin along with 50 mg/ml liposomes and 5mM EGTA

5. DISCUSSION AND CONCLUSION

5.1 Expression and purification of recombinant DEK1-C2L proteins

In order to study the biochemical properties and characteristics of proteins, the protein is needed in larger amounts in its native and biological active state. Isolation of the protein from its native source may be advantages since the protein thus can be isolated in its chemically modified active and soluble form. However, isolation of proteins from its native source is often very time-consuming and expensive and may also results in a low protein yield, not sufficient for down-stream biochemical analysis. Therefore, production of the protein of interest in heterologous systems is often advantageous. In this study the *P. patens* and *A. thaliana* DEK1-C2L proteins were expressed in *E. coli* as C-terminal fusion partners with either an N-terminal His or MBP tag. Both His and MBP tags can be used as affinity tags for purification of the resulting recombinant proteins. In addition, the MBP tag, but not the His tag, is commonly used to increase the solubility of the produced proteins. The exact mechanisms of how MBP may increase solubility are, however, not known. In this study, both the His- and MBP-tagged DEK1-C2L proteins were expressed mainly as inclusion bodies (Figure 10 & 11). Protein bands in expected size of the His tagged DEK1-C2L are much stronger than protein bands in expected size of the MBP tagged DEK1-C2L. It can because of the difference between the molecular mass of MBP and His. The molecular mass of MBP is approximately 42.5 kDa that is much larger than approximate molecular mass of DEK1-C2L domain (17.5 kDa), while His tag adds just 1 kDa to the molecular mass of fused recombinant protein. The larger size of MBP tagged DEK1-C2L likely decrease the amount of yield. Another issue about recombinant protein expression, as it is obvious in Figure 10, is that difference between the intensity of protein bands in expected size of induced samples by IPTG (lane 1, lane 5 for His tagged DEK1-C2L and lane 3, lane 7 for MBP tagged DEK1-C2L) and protein band corresponding to the expected size of un-induced samples is not significant. Since the gene of interest was cloned into pETM-41 and pET-302 vectors that contain a T7 promoter the BL21 (DE3) strain of *E. coli* was used to express recombinant DEK1-C2L protein. This strain has a RNA polymerase gene from the T7 bacteriophage that is extremely promoter-specific and transcribes only DNA downstream of a T7 promoter. T7 RNA polymerase in BL21 *E. coli* (DE3) is under the control of the Lac repressor. Lac repressor is a protein that is produced in *E. coli* under native conditions. Its normal role is to adjust the level of lactose metabolic proteins in the response to the concentration of lactose in cytoplasm of *E. coli*. The Lac repressor interacts with the operator that regulates T7 RNA

polymerase encoding gene and prevents RNA polymerase from interacting with the promoter. Presence of lactose leads to formation of lactose-Lac repressor complex that breaks the interaction of the Lac repressor with the operator allowing transcription from the T7 promoter. However, the presence of lactose also triggers the expression of enzymes that metabolize lactose and convert it to a molecule which is not able to bind to the Lac repressor (McAllister, 1993). To avoid this problem IPTG (isoproplythio- galactoside) is used as a non-metabolizable mimic of lactose to induce the expression of recombinant proteins. The Lac repressor is not tightly regulated and a basal level of expression is expected (Gilbert & Müller-Hill, 1966). It can explain recombinant protein expression in un-induced samples (Figure 10). But why induced samples (Figure 10) do not give significantly higher expression than un-induced samples? It is unknown. As it is clear in Figure 10 and Figure 11, despite of using MBP tag as a fusion partner to increase the solubility of expressed recombinant DEK1-C2L desired protein mainly expressed as insoluble aggregates. It was tried to decrease the induction temperature from 37 °C to 30 °C and increase the induction time up to 24 hours to achieve more soluble yield (data not shown). In general, decrease in temperature cause decrease in protein yield (Michael, Daniel, Elaine, & Peter, 1996). Considering all these information, along with undesired conditions were faced related to the high molecular mass of MBP tag and inducing with IPTG (mentioned above) it turned to a challenge to produced enough amount of soluble recombinant protein for downstream processing using expression vectors with MBP tag. Also there was another problem with continuing experiment using MBP tagged DEK1-C2L proteins. Considering the relatively large size of MBP tag it has the possibility to interact with phospholipids in the phospholipid binding assay and cause false positive results. This possibility would raise the urge to either remove the MBP tag from the recombinant C2L protein or express the MBP tag separately and use it as a control in the phospholipid binding assay. To avoid time consuming additional steps to produce enough amounts of recombinant protein and downstream processes, His tagged DEK-1 was chosen to continue the experiment. Also worth mentioning, in this experiment recombinant *A. thaliana* and *P. patens* DEK1-C2L recombinant proteins were both initially expressed but purification and analysis steps were performed using only the *P. patens* DEK1-C2L. This decision was taken base on the fact that epitope used for antibody production was specifically designed against the P. patens DEK1-C2L protein (Figure 12). His tagged *P. patens* DEK1-C2L proteins were expressed mainly in the form of IBs. To recover active proteins from IBs it was necessary to solubilize and refold the *P. patens* DEK1-C2L proteins. Proteins in the form of IBs contain host-cell-derived contaminants such as lipids, nucleic acids, lipopolysaccharides,

and membrane proteins (Thatcher, 1990). These impurities may interfere with the refolding of desired proteins, so prior to the refolding step, purification was performed to minimize interference by mentioned impurities (Tsumoto, Ejima, Kumagai, & Arakawac, 2003). Since expressed recombinant protein had 6x Histidine tag, cobalt based affinity chromatography spin columns were chosen as an effective method used under chaotropic conditions, that is suitable to isolate protein of interest from inclusion bodies. IBs can only be solubilized using strong denaturant agents, so insoluble His tagged DEK1-C2L fractions were dissolved in Equilibration/Wash buffer containing 8 M urea, to achieve monomolecular dispersion and minimize non-native intra- or inter-chain interactions in the structure of recombinant protein to make histidine residues available for immobilized cobalt ions. Despite the wide spread use of urea in biochemical studies, the molecular mechanism of its ability to denature proteins is not fully understood. It is suggested that urea cause denaturation directly by binding to the protein, or indirectly, by altering the solvent environment (Pace, 1986).The cobalt ions bind specifically to the imidazole ring present in the histidine residues, and the strength of bonds between cobalt ions and histidine residues increase significantly when more histidine residues are available in the sample applied in to spin column. In the next step, these the bonds between cobalt ions and histidine residues tagged to recombinant protein were weakened using Imidazole. Imidazole dissolved in the Elution buffer competes directly with the imidazole rings present in the His-tag and binds to immobilized cobalt ions in the resin of column and dissolved recombinant protein elutes. Also affinity chromatography spin columns with immobilized nickel and copper ions are available which can bind His tagged protein as well. Nickel ions has higher capacity to bind histidine residues than cobalt ions (Bolanos-Garcia & Davies, 2006), but using Nickel based columns to purify histidine tagged recombinant DEK1-C2L proteins caused more contamination of the His-tagged proteins with other proteins (Hendriks, 2013). This contamination may be a result of Nickel ions tend to bind non-specifically (Bolanos-Garcia & Davies, 2006). Using of copper ions in chromatography column result in the strongest but less specific bonds in compare with cobalt and nickel based columns (Bolanos-Garcia & Davies, 2006). Elutes from affinity spin columns were investigated by SDS-PAGE to assess the level of recombinant protein purity. Protein bands in Figure 14 panel B suggests that recombinant proteins in elution fractions has acceptable level of purity for refolding step. In this step recombinant protein which was unfolded by dissolving in 8 M urea refolded in native state by decreasing the concentration of denaturant against an aqueous buffer (25 mM Tris-HCl) using dialysis method. Unlike the recombinant μ-calpain-C2L protein, in which refolding is possible only under high ionic

strength (above 1 m NaCl) (and even under high ionic conditions this domain tends to aggregates) (Tompa et al., 2001), DEK1-C2L can be refold using conventional dialysis method and stay refolded without high ionic condition (see Material and Method). To avoid re-aggregation during the dialysis process, the denaturant buffer was exchanged with aqueous buffer gradually. This consideration increased the time of dialysis process up to several days so whole process was performed in 4 °C to protect recombinant protein from changes in their biological functions. Purified and solubilized recombinant proteins were investigated by SDS-PAGE (results are not shown), and protein bands corresponding to expected size of His tagged PpDEK1-C2L were analyzed and verified by immunoistaning technique using anti-DEK1-C2L as the primary antibody (Figure 15).

5.2 Analysis of the phospholipid binding ability of *P. patens* DEK1-C2L domain

The purified and solubilized *P. patens* DEK1-C2L protein produced in the first part of this work was finally investigated for its ability to bind to liposomes, which are composite structures made of phospholipids, using size exclusion chromatography technique, in presence and absence of Ca^{2+} (Figure 17 panel A and B). For this purpose liposomes were prepared from phosphoinositides sodium salt, obtained from crud bovine brain extract, by hydrating dried phosphoinositides sodium salt films to form multilamellar vesicles by agitation from hydrated lipid sheets driven by the hydrophobic effect (Lasic, 1998), then vesicles were sonicated to reduce the size of the particles. Crude bovine extract contains di- and triphosphoinositide, phosphatidylinosidite and phosphatidylinositol and phosphatidilserine (Sigma P6023). The lipid composition of the cell membrane varies with cell type, developmental stage, and environment in plants, but the phospholipids mentioned previously exist commonly in eukaryotic cell membranes (Furt, Simon-Plas, & Mongrand, 2010). It is possible to stain liposome vesicles using hydrophilic dyes such as Sudan Black B (Briz, Serrano, Macias, & J.J., 200) and analyze the liposomes using light microscopy to verify the formation and quantity of liposomes. The principle of the method used in this work was that binding of DEK1-C2L proteins to the phospholipids increases the size of resulted component, which results in improvement in elution. Analysis of elutes with SDS-PAGE and western techniques suggests that the DEK1-C2L domain has the ability to bind phospholipid *in vitro* and that this ability is enhanced in the presence of Ca^{2+}. This conclusion is drawn from the difference in intensity (amount) of the protein bands of desired protein in lane 3 (elution of His tagged PpDEK-C2L protein in absence of phospholipid) and lane 4 (elution of His tagged

PpDEK1-C2l protein in presence of phospholipid) in Figure 16 A. Also, investigating differences between intensity of protein bands in lane 4 (elution of DEK1-C2L in presence of phospholipids) and lane 5 (elution of DEK1-C2L in presence of phospholipids and Ca^{2+}) in Figure 16 A suggests that Ca^{2+} enhance the ability of DEK1-C2L to bind phospholipids. This finding may shed light on the role of the C2L domain in a functional DEK1 enzyme in land plants.

Activation of animal calpain is associated with binding to cell membranes (Shao et al., 2006). Animal calpains (m-calpain and μ-calpain) are strongly involved in the regulation of cell motility which is critical for wound healing (Leloup et al., 2010). Recent studies indicate that m-calpain activity is regulated by phosphorylation during wound healing process (Leloup et al., 2010). Also, it has been proposed that the C2L domain of animal calpain is the main phospholipid binding site in presence of Ca^{2+} (Tompa et al., 2001). Based on experiments performed in 2001 by Tompa et al. it was suggested that the C2L domain of animal calpain orchestrate enzyme activity via binding to phospholipids and Ca^{2+}. This finding is supported by the results of previews studies that indicates inactivity of calpain enzyme with deleted C2L domain (Vilei et al., 1997). Three dimensional modeling of DEK1-C2L domain shows high similarity with animal the C2L domain structure. Also amino acid sequence alignment between animal calpains and DEK1 reveals high conservation between animal C2L and DEK1-C2LDEK1 enzyme (Wang et al., 2003). Because of high similarity in spatial structure and amino acid sequence, similarity in function of animal and DEK1 C2L domain is expected. As previous mentioned, animal calpain activity is associated with attaching to cell membranes, same property is suggested for DEK1 (Johnson et al., 2008). Also, DEK1 C2L domain is indispensable for an active DEK1 enzyme *in vitro* (Wang et al., 2003). Considering the results of this experiment it can be proposed that DEK1-C2L domain regulates enzyme activity through binding to phospholipids in cell membranes. Increasing of phospholipid binding ability of DEK1-C2L in the presence of Ca^{2+} ,suggested by the results obtained in this work, is corroborated by the results of an experiment carried out at 2003 by Wang, et al. In that experiment recombinant DEK1-CysPc-C2L protein was incubated with purified casein. Results of the assay demonstrated that casein was degraded by the enzyme significantly faster in the present of Ca^{2+}. Increasing of phospholipid binding ability in presence of Ca^{2+} in animal calpain C2L domain is understandable due to negatively charged amino acid in domain structure and ionic interactions with other domains of enzyme (Tompa et al., 2001). However, DEK1-C2L lack the acidic residues and it is unlikely to attach Ca^{2+}

(Wang et al., 2003). It needs father investigations to clear how Ca^{2+} increases DEK1-C2L phospholipid binding capacity.

5-3. Further Studies

Through this experiment recombinant *P.patent*-DEK1-C2L was expressed in BL21 (DE3) *E. coli*, then purified and refolded. In next step concentrated refolded protein investigated from phospholipid binding aspect in presence and absence of Ca^{2+}. The results of this study are not fully conclusive but indicative. It is suggested to use the Fat Western method. Fat Western is used to screen for protein-lipid interactions. In this case phospholipid binding capacity of DEK1-C2L domain can be tested. In this method hydrophobic membranes that have been spotted with different biologically important lipids found in eukaryotic cell membranes are used to investigate how protein of interest interacts with the phospholipides that are fixed on membrane in presence and absence of Ca^{2+}. Technically, fat westerns are based on immunoblotting techniques in which lipid-protein binding precedes the antibody detection steps.

6. Appendix

6-1. Primer overview

Coding direction, plant name and inserted restriction site are shown in the primer's name.

For use in pET302 vector:

1 SP_AtC2L_PmlI: CACGTGCGTGAGATGCGCTACTCTG
ASP_AtC2L_BamHI: GGATCCTTACAAAGCTTCAAGAACAATG

2 SP_PpC2L_pMlI: CACGTGCCTGAGATGAAGTATTCAG
ASP_PpC2L_BamHI: GGATCCTTAAAGCGGTTCCAAGATGATAG

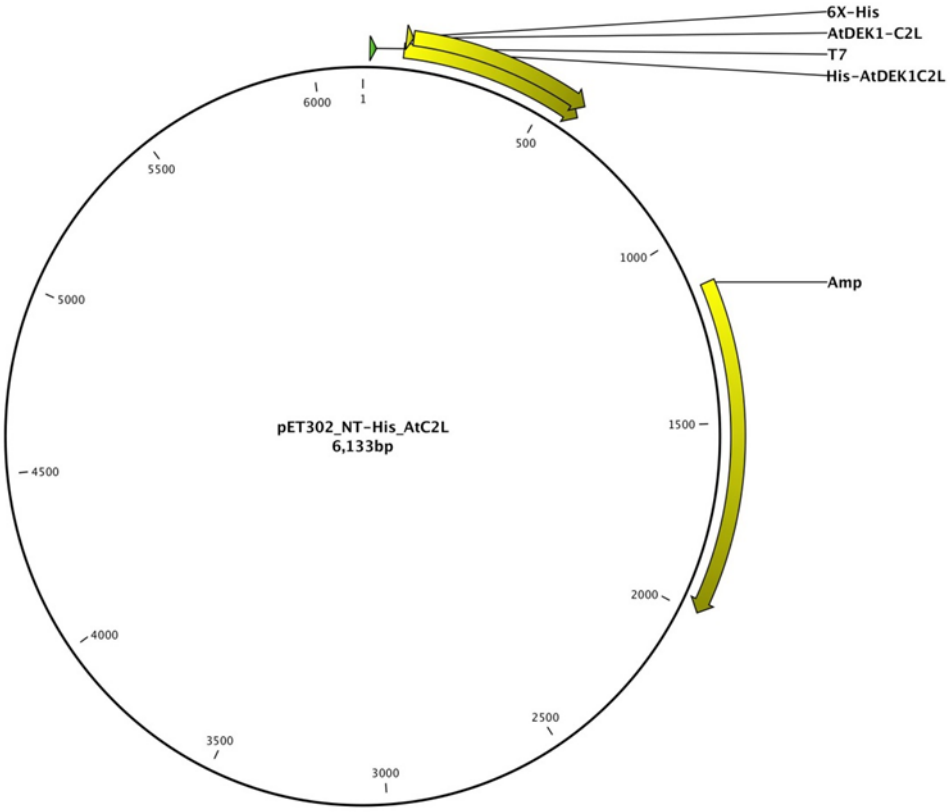
For use in pETM-41 vector:

3 SP_AtC2L_NCoI: ATTGCCATGGCCCGTGAGATGCGCTACTCTG
ASP_AtC2L_NotI: TGAAGCGGCCGCTTACAAAGCTTCAAGAACAATG

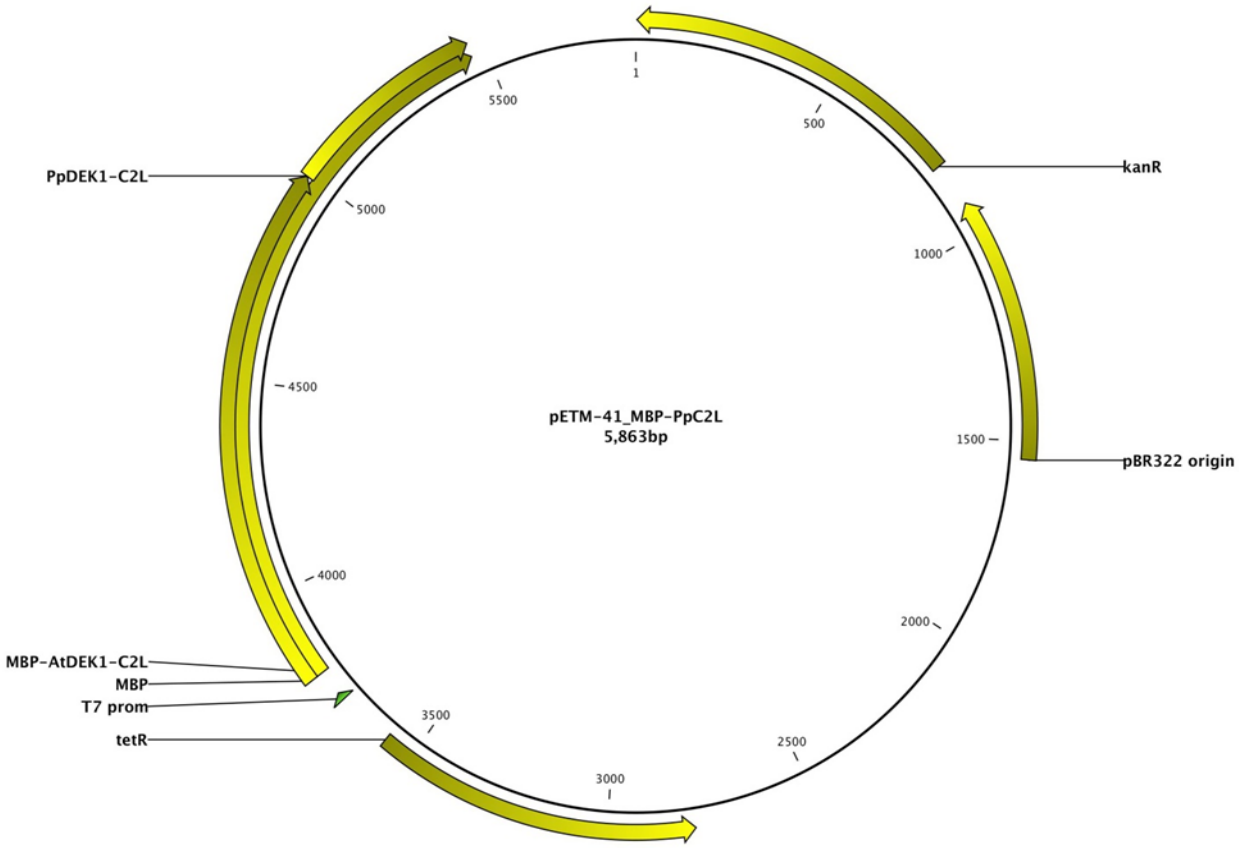
4 SP_PpC2L_NcoI: ATTGCCATGGCACCTGAGATGAAGTATTCAG
ASP_ PpC2L_NotI: TGAAGCGGCCGCTTAAAGCGGTTCCAAGATGAT

6-2. Vectors Maps

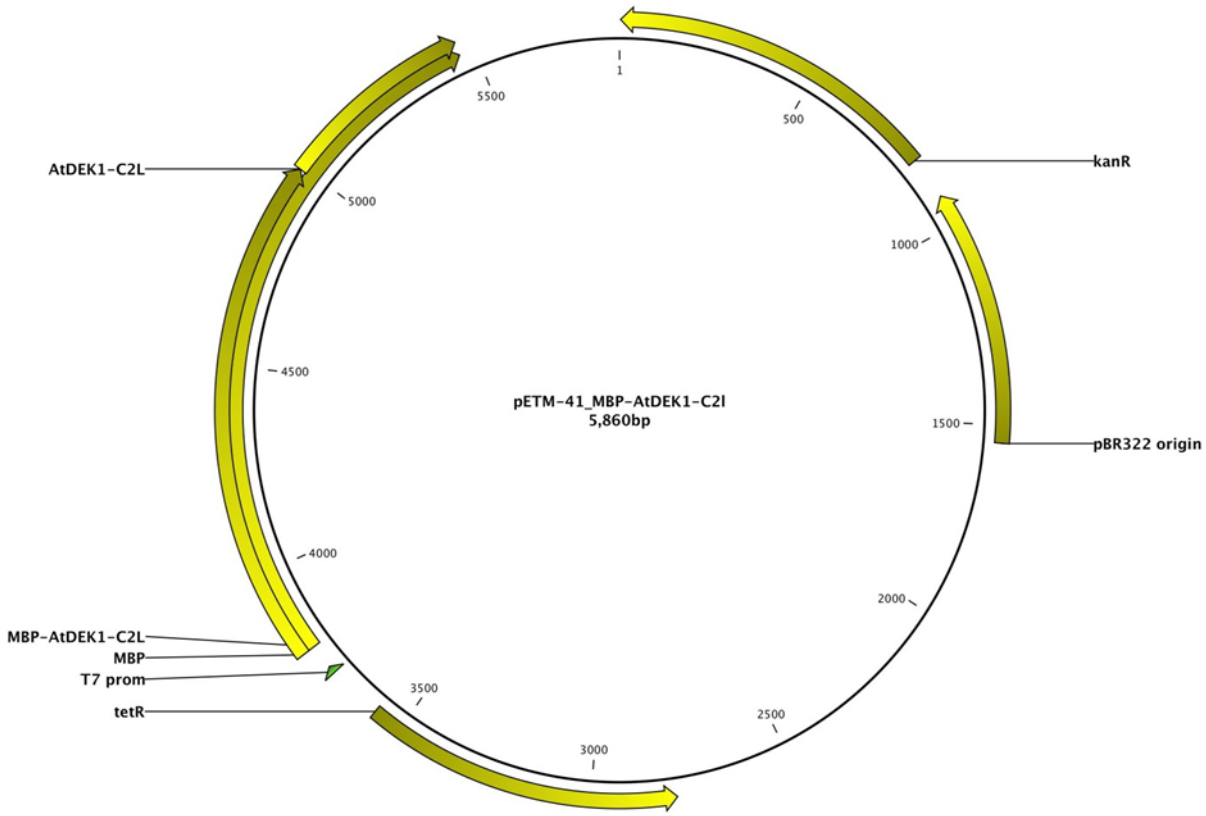
1. pET302_NT-His_AtC2L plasmid



2. pETM-41_MBP-PpC2L plasmid



3. pETM-41_MBP-AtC2L plasmid



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