Functional Dissection of a Novel Rice C2-domain Protein

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Statement

All experimental works reported in this thesis were performed by the author, unless stated the otherwise.

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Abstract of thesis entitled:

Functional dissection of a novel rice C2-domain protein

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C2 domain in animal was a well-studied lipid-binding domain, yet scarce reports were published to investigate C2 domain in plants. Small C2 domain protein which contained only a single C2 domain was a group of plant-specific protein having great involvement in stress response. Despite the recent reports on their functions, the structure-function relationship in this group of protein was not yet established.

OsGAP1, which refers to GTPase-activating protein in rice, is a plant small C2 domain protein acting as both positive regulator of plant defense response and abiotic stress tolerance. Its interacting partner OsYchF1 belongs to the YchF subfamily of the Obg family in the TRAFAC class of P-loop GTPases and was the first plant YchF homologues reported by our group. Opposite to the role of OsGAP1, OsYchF1 was proved to play a negative role in both plant defense response and abiotic stress tolerance. OsGAP1 can regulate its GTPase activity, the unique ATPase activity and subcellular localization. However, detailed molecular mechanism of this protein pair has not been revealed.

To understand the structure-function relationship of OsYchF1- OsGAP1 pair, our group collaborates with Prof. KB Wong's group to resolve their

structures by X-ray crystallography. By structural and amino acid sequence comparison, OsGAP1 belongs to topology II C2 protein but also contains some signature features of topology I C2 protein, such as aspartate residues of the calcium binding pocket. It also contains two extra beta-strands which can distinguish it from other C2 domains.

In this project, site-directed mutagenesis based on the resolved crystal structure of OsGAP1 was performed aiming to dissect the structure-function relationship of OsGAP1. We have identified phospholipid and OsYchF1 interacting surface on OsGAP1 which was the first report of molecular dissection of a plant small C2 domain protein. Different interacting surface of OsGAP1 towards phospholipids and OsYchF1 allowed OsGAP1 to bridge the indirect interaction between phospholipids and OsYchF1. Lipid selectivity of OsGAP1 was also solved which gave us insight on the involvement of OsGAP1 in lipid signaling. Strong interaction of cardiolipin and phosphatidylinositol 4-phosphate may imply a sensing function of OsGAP1 in response to different stress conditions. Phospholipid binding ability of OsGAP1 was not required for the activation of salt stress tolerance in plant. All of the results can fit into the working model of OsGAP1-OsYchF1 pair proposed in the previous reports.

摘要

C2 結構域是最被廣泛研究的脂質結合結構域，但是植物的 C2 結構域就鮮有被研究。最近，一組只在植物出現的小 C2 結構域蛋白被發現結構中不尋常地只含有一個單一的 C2 結構域，而且和植物的脅迫反應有密切的關係。儘管它擁有如此特別的結構，最新報告只專注在它的功能，而並未找出這組蛋白質的結構與功能之間的關係。

同樣屬於小 C2 結構域蛋白的 OsGAP1 可以同時提高植物的防衛反應和抗逆反應。 和 OsGAP1 有相互作用的 OsYchF1 屬於一類未被廣泛研究的保守 G 蛋白。它的功能和 OsGAP1 恰恰相反，是會抑制植物的防衛反應和抗逆反應。跟據我們之前所做的實驗，我們推論 OsGAP1 可以透過激活 OsYchF1 的 GTPase 酶活度和 ATPase 酶活度，加上改變它的亞細胞定位去操控它對脅迫反應的影響，但是詳細的分子機制並未確定。

爲了了解 OsGAP1 的結構與功能之間的關係，我們的團隊聯同黃錦波教授的團隊以射綫衍射晶體分析法解構了 OsGAP1 的結構。跟據結構和氨基酸的序列比较，我們發現了 OsGAP1 除了擁有和保守 C2 結構域一樣的結構和一些特點之外，還有一些和其他 C2 結構域不同的特別地方。

在這個碩士研究報告，我根據 OsGAP1 的結構去設計基因定點突變的位置，從而解構 OsGAP1 的結構與功能之間的關係。這是第一個在分子層面解構了小 C2 結構域蛋白的報告。我們找到了 OsGAP1 與磷脂或 OsYchF1 相互作用的蛋白質分子表面。由於磷脂或 OsYchF1 的相互作用分子表面並于相同，所以 OsGAP1 可以同時與這兩種物質相互作用並因此有能力改變 OsYchF1 的亞細胞定位。近年的報告指出磷脂在細胞轉導中扮演重要的角色，所以我同時分析了 OsGAP1 對磷脂的選擇性。我發現了 OsGAP1 對心

肌磷脂和磷脂酰肌醇-4-單磷酸有強度的結合。這個發現指出 OsGAP1 有可能在脅迫反應中的磷脂細胞轉導中扮演傳感的角色。我們亦同時發現植物抗逆反應並不需要 OsGAP1 與磷脂結合。以上所有的實驗結果都與之前報告的結果吻合，亦可作爲我們之前假設的 OsGAP1-OsYchF1 分子機制模式的認證。

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General Abbreviations

%	percentage
µl	microLitre
µmol	micromole
µmol	micromolar
3`	3 prime end
3-D	3-dimentional
5`	5 prime end
Å	ångström
℃	degree Celsius
FDPB	finite-difference Poisson-Boltzmann
HPLC	High-performance liquid chromatography
hr	hour
kDa	kilodalton
L	Litre
m	meter
mg	milligram
min	minute
ml	millilitre

mm	millimeter
mM	milliMolar
MMDB	Molecular Modeling Database
NCBI	National Center for Biotechnology Information
nm	nanometer
rpm	Revolution per minute
s	second
UV	Ultraviolet
v:v	volume to volume
VAST	Vector Alignment Search Tool
X	times

Abbreviations of Scientific terms and Chemicals

ADP	Adenosine diphosphate
Agr	Arginine
AIDA-C2	Axin interaction dorsal-associated protein -C2
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
B9-C2	B9 domain -C2
CD	Circular dichroism
Col-0	Columbia zero
cPLA2	cytosolic phospholipase A2
DOCK-C2	Dock180/Dock1 proteins -C2
ECL	enhanced chemi-luminescence
G protein	Guanine nucleotide-binding protein
GDP	Guanosine diphosphate
GPS	Glycerophosphoserine
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
Ile	ileucine

IPTG	Isopropyl β-D-1-thiogalactopyranoside
MBP	Maltose binding protein
MS medium	Murashige and Skoog medium
NaCl	sodium chloride
NT-C2	N-terminal C2
OBG family	spo0B-associated GTP binding protein family
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCR	Polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI3K-C2	phosphatidylinositol-3 kinase -C2
PKC	protein kinase C
PLC	phospholipase C
P-loop	phosphate-binding loop
PS	phosphatidylserine
PtdIns(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate
PtdIns(4,5)P2	phosphatidylinositol-4,5-bisphosphate
PTEN-C2	Phosphatase and tensin homolog

SDS	Sodium dodecyl sulfate
SNAP-25	synaptosomal-associated protein of 25 kDa
Syt	synaptotagmin
TRAFAC class	Translation factor class
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
Tyr	Tyrosine
WT	wild type
H_3BO_3	Boric acid
$CuSO_4$	Copper (II) sulphate
LB broth	Luria Bertani Broth
NaOAC	sodium acetate
$MnCl_2$	Manganese chloride
$CaCl_2$	Calcium chloride
Na_2MoO_4	Sodium molybdate
MES	2-(N-morpholino)ethanesulfonic acid

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Chapter 1 Introduction and Project Objectives

Membrane structure forms a complicated dynamic system in eukaryotic cell which is essential for the control of many important cellular processes especially the signaling and trafficking event. The wide variety of phospholipid species in the membrane offers an architecture for different protein players to perform their function under different spatial and temporal conditions. The C2 domain is among the first group of lipid binding domain being discovered [1] and this allowed time for people to have extensive research on C2 domain of animal. However, the importance of the C2 domain has been overlooked in plant. The discovery of a novel subgroup called plant small C2 domain protein has recently drawn the attention due to its unique structure and diverse functions [2-7]. This group of proteins is plant specific and contains a single C2 domain alone. There are limited reports about their functions and detailed analysis on the relationship between its structure and function remains an important missing piece of knowledge before we can fully understand its molecular mechanism and put it into application.

Rice OsGAP1 belongs to plant small C2 domain protein which was discovered by our laboratory in a search of signaling component related to plant defense response [8]. It is induced upon pathogen inoculation and soon found to be an interacting partner of OsYchF1 [9]. YchF protein is conserved among bacteria and eukaryote, yet its function is not known. OsYchF1 is the first YchF protein reported in plant acting as a negative regulator for both biotic [9] and abiotic stress [10] while OsGAP1 plays an opposite functional role. Co-localization of OsGAP1 and OsYchF1 from cytoplasm to plasma

membrane has been observed in a double –labeling immunogold electron microscopy experiment, which suggested that the signaling of plant defense response is related to the plasma membrane interaction [9]. With the crystal structure of OsGAP1 being resolved (unpublished data, HML), dissecting its structure for analyzing its different functions becomes possible and a critical step towards a complete picture of its molecular mechanism.

The goal of this study is to investigate the function of different regions in OsGAP1 by dissecting it using site-directed mutagenesis. This finding can shed light on the structure-function relationship of small C2 domain protein in plants which is still remained unclear and give insight to the functional control of plant YchF protein. It might also be applied through genetic engineering to increase plant diseases resistance and abiotic stress tolerance and hence crop yield in the world.

The objectives of this project are:

(1) To identify specific binding sites of phospholipids and OsYchF1 on OsGAP1 by site-directed mutagenesis;
(2) To study the functional significance of phospholipid binding ability or OsYchF1 interaction of OsGAP1 on plant defense response and abiotic stress tolerance

Chapter 2 Background of Research

2.1 Previous studies by other research groups

2.1.1 Structure of the C2 domain

The C2 domain is one of the most prevalent eukaryotic lipid-binding domains. It is the first reported lipid-binding domain as one of the conventional protein kinase C conserved domain in late 1970s [1]. In spite of the low conservation in amino acid sequence C2 domain[11], structure was highly conserved among the known C2 domain superfamily. Its general structure was resolved as two beta-sheets: each consists of four antiparallel beta-strands and then folded into a beta-sandwich architecture [12-17]. Flexible loops were found between the beta-strands which their exposing residues formed possible sites for interaction with others. The conformation and residue sequence of the loops are of great variety among different C2 domains [11].

The members of C2 domain can be classified into two topologies based on their beta-strand arrangement [11]. They differed from each other by circular permutation of the terminal strand [18] (Figure 1). For most of the topology I C2 domains such as that in protein kinase Calpha [19] and C2A of synaptotagmin [20, 21], a calcium-binding pocket is formed by negative aspartate residues in conserved position. Hence former researches suggested most C2 domain functioned as a calcium sensor and its lipid binding ability was calcium-dependent, but it was later proved that those were not general for all of the C2 domains [22]. With

some exceptions, the lipid binding ability of topology II C2 domain proteins such as protein kinase C (PKC) epsilon [17] and protein kinase C (PKC) eta [23] are calcium-independent. This is due to their loss of the essential residues composing calcium-binding pocket in spite of the fact that they can still bind to lipid.

Although previous studies mostly focused on the PKC-C2 family, a recent study revealed there are a total of seven families within the C2 domain superfamily [24]. In contrary to the former belief that C2 domain must be a calcium sensor, topology I or calcium-binding-pocket-containing C2 domains were only present in PKC-C2 family among all families of C2 domain superfamily shown by sequence alignment, which proved topology II was more typical and the involvement of calcium in the lipid binding event was not conversed among the whole superfamily. This also suggested that the common ancestor of C2 domain is of topology II and without calcium binding ability [24].

2.1.2 Interaction target of C2 domain

C2 domain of animal proteins usually presents with other domains to form a functional protein. Most of them lacks enzymatic activities, but can interact with different substances to assist the functions of other domains. The most studied interactions are its interaction with calcium and phospholipids. Protein and some special ligands are also the target of some C2 domains. In the following session I will discuss the interaction of C2 domains to these

substances one by one and their inter-dependence on one another.

2.1.2.1 Calcium

Calcium regulation has long been studied in the conventional PKC-C2 domain family. Negatively charged aspartate residues on the loops between betastrand 2 and 3 and that between 6 and 7 of topology I C2 domain forms a calcium binding site which can bind to 2 or 3 calcium ions[19, 25]. This has made the C2 domain first known as a calcium binding domain but not a lipid binding domain. Positive cooperativity has been observed among the calcium ions associated with the C2 domain of cytosolic phospholipase A2 (cPLA2) in the equilibrium binding and stopped-flow kinetic experiments [26]. Hill coefficients of C2A of synaptotagmin I (Syt I) [21], C2A of Doc2â [27] and C2A of synaptotagmin IV (Syt IV) [28] also supports the existence of the cooperativity of calcium binding.

2.1.2.1.1 Role of calcium binding on phospholipid-binding

With more evidences to show the lipid binding ability of C2, how the binding of calcium to C2 domain influences the lipid binding drew the spotlight and can be concluded in mainly three aspects.

First, the binding of calcium can change the conformation either within the C2 domain or in the entire protein. Although the conformational change induced by calcium is not very significant for most of the C2 domains, some influential or even dramatic structural changes have been

reported. This model was strongly supported by an analysis on two proteins which both binds to two calcium ions but works by different mechanisms: cytosolic phospholipase A2 (cPLA2) and protein kinase C (PKC) alpha [29]. By surface plasmon resonance and monolayer studies, calcium ions in position 2 (CA2) of cPLA was shown to be the major player of membrane binding through the induction of intradomain conformational change and thus exposing the hydrophobic residues on the loop for membrane penetration. Calcium ions in position 1 (CA1), on the other hand, did not play an important role in membrane binding, but assists the activation of the protein [30]. In contrary, both CA1 and CA2 of PKC alpha exerted their effect interdomainly and induced the residues on other domain such as the two adjacent C1 domains to have membrane penetration. This interdomain conformational change may also help to recruit residues on other domains to have more specific interaction with PS. This model was supported by studies showing that the binding specificity to PS was much higher in intact PKC alpha than in the C2 domain fragment only [29]. Another important finding based on the comparison between the crystal structure of annexin V with and without calcium ions also gave us insight on the local conformational change. Trp185 was found interacting with lipid bilayer. “Closed form” was observed when the calcium binding site was empty so that the side chain of Trp185 was buried inside the structure. When a calcium occupied that binding site, “open form” was resulted so that Trp185 became exposed to the protein surface which may be ready for phospholipid interaction [31].

In more typical cases rather than conformational changes, calcium

exerts its impact on C2 domain by acting as a cation bridge to coordinate the negatively charged residues and anionic phospholipids directly. Crystal structure of calcium-annexin V complex with phospholipid head group [31] and co-crystal structure of PKC alpha -C2, calcium ion and phosphatidylserine (PS) resolved by X-ray [32] have provided solid evidences of this model. As shown in the PKC alpha-C2 structure, the head group of PS was co-ordinated directly and specifically towards CA1 and other residues in the calcium binding pocket. Docking position of the C2 domain to the membrane was also consistent with this model.

Finally, binding of calcium ion also changes the electrostatic status of the C2 domain which implies the function of C2 domain as an electrostatic switch. This offers either the non-specific lipid binding ability or the lipid specificity for the C2 domains of different structures. Binding of calcium greatly increases the electrostatic potential on the interaction surface on the C2 domain towards lipid [33]. This flavors the binding of C2 domain to negatively charged acidic phospholipids, yet the specificity towards any specific acidic phospholipids is small. This non-specific lipid binding ability induced by calcium was the dominant reason for the lipid binding the C2A of Syt I [34] and C2 of PKC beta [16].

Calculations of the electrostatic potential on the C2 domain surface by the accurate finite-difference Poisson-Boltzmann (FDPB) method [33] demonstrated that positive charge of calcium ions can also reduce the desolvation penalty for penetration of the hydrophobic residues of C2 domain into the phospholipid domain by neutralizing the negative charge

of the acidic residues. This could bring the C2 domain to a closer proximity to the membrane so that its residues can better penetrate into the membrane bilayer and exert their effect on the lipid selectivity. Hence calcium-dependent lipid binding C2 domain working in this model such as cPLA-C2, can bind to zwitterionic phospholipids rather than to only negatively charged phospholipids.

By changing the electrostatic potential, calcium binding can also directly change the lipid specificity of the C2 domain. C2B domain of Syt I which is of topology I binds inositol polyphosphate and phospholipids independent of the presence of calcium ions [35]. However Ca2+ ions switches the specificity of C2B binding from phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) at low calcium concentration to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) at high calcium concentration [36]. Another interesting observation related to the C2B domain of Syt I is that the orientation and mode of lipid binding changes in different calcium concentration. In low calcium concentration, polybasic region formed from lysine residues of C2B interacted with the lipid bilayer in a solely electrostatic way. This may prevent the interaction of C2B domain to other ligands as the interacting surface is greater in this way. When the calcium concentration is increased, the calcium binding pocket penetrated into the membrane by rotating the domain, which may allow the polybasic region to be exposed to other lipids [37]. This observation may be related to the change in lipid specificity but need more evidences to show their correlation.

2.1.2.1.2 Role of calcium binding on protein interaction

C2 domain-containing protein may have interaction with protein through other adjacent domains, or directly through C2 domain which would be discussed below. The interaction can be regulated by calcium binding in two levels.

Firstly the interaction can be calcium dependent. A classical model was the interaction between C2A domain of Syt I and plasma membrane protein syntaxin [38]. The surface charges of the calcium binding sites in Syt I changes dramatically after the calcium binding from a relatively neutral charge to highly positive. This allowed the ring of basic residues around the calcium binding sites to have less electrostatic repulsive force and directly bind to the syntaxin. Exocytosis followed by the release of neurotransmitter was resulted through the calcium-triggered synaptotagmin- syntaxin interaction. The interaction can be diminished with point mutation in the calcium binding site [39].

Besides, the binding of calcium can affect the function of the protein. Two calcium ions were incorporated into the structure of PKC alpha. While both of them were responsible for the interdomain conformational change to assist the membrane penetration of the adjacent C1 domain, conformation induced by CA2 can also activate the catalytic activity of the PKC [30]. Contrary to the mechanism in PKC alpha, only one of the calcium ions in cPLA mediates hydrophobic membrane binding cPLA and another oriented the membrane bilayer in proper geometory to activate

the interfacial enzyme activity [25].

2.1.2.2 Phospholipids

Besides the calcium-dependent lipid binding model suggested above, there are lipid interactions on C2 domain which are calcium-independent. When compared to the calcium-dependent phospholipid binding, calcium-independent binding is often more specific [11]. As shown above by the different lipid binding mode of Syt I, phospholipid binding of C2 domain is not limited to the calcium binding site of C2B domain but also the polybasic strand consisted of lysine residues [37]. Additional binding sites for anionic phospholipid were also revealed in the lysine-rich cluster of PKC alpha [40].

In fact, many of the C2 domains do not bind to calcium but still have the ability to bind phospholipid. One of the classic examples is PKC epsilon, which is a member of novel PKC [17]. PKC epsilon lacked the calcium binding pocket on the loops, but the bulky amino acid residues of Trp23, Ile89 and Tyr91 on the loops can provide the hydrophobic force for the membrane penetration. Arg26, Arg32 and Arg50 on the beta groove can also electrically interact with the phospholipids.

2.1.2.2.1 Phospholipid specificity

Phospholipid specificity of C2 domain was mainly offered by the side chain of the residues on the loops or on the beta-groove.

As the positive charges on the calcium ions were relatively very large, interaction directly involved calcium ion was always electrostatic and non-specific towards anionic phospholipids [21]. Side chain of the residues on the loops can interact with the lipids and provide and offer specificity. Calcium may then change the local conformation of the loops so that the side chains may have a proper orientation towards the membrane to carry out their functions [29]. Cationic residues on the calcium binding loops have a higher preference for anionic lipids [32, 41], while aromatic and aliphatic residues, especially tryptophan, mighty favor the binding to neutral phosphatidylcholine (PC) [14]. For the calcium-independent C2 domain, though their loops did not bind to calcium, some residues on them can still bind to phospholipids selectively [17, 42, 43].

Cationic beta-groove of both calcium dependent and independent C2 domain is a hotspot for the lipid binding and also has residues which are responsible for the lipid specificity. As its name suggested, the groove is formed by cationic residues which can have better interaction with anionic phospholipids[11]. Their specific interaction with inositol phosphates at different phosphorylation states was well studied [13, 19, 37, 44].

2.1.2.2.2 Role of phospholipid binding on calcium binding

Focuses have long been put on the effect of calcium binding on

phospholipid binding. However some evidences suggested that how the calcium ions interact with the C2 domain can also be a phospholipid-dependent process [45].

As shown in the crystal structure of rat annexin V-glycerophosphoserine (GPS) complex and data of solution measurement, binding of PS to annexin V can increase the calcium affinity [31]. In a crystal without phospholipid, though annexin V can still bind to two calcium ions, only one of the calcium ions was fully coordinated and the other was coordinated to only three ligands. In contrary, both of the calcium ions had the same high occupancy and fully coordinated as a configuration of pentagonal bipyramid when annexin V was at the present with PS.

The disassociation constant of the C2A domain of Syt I for calcium decreased dramatically for calcium-binding site II and site III respectively from 60μM and 400μM in the absence of phospholipid [46] to 3-4μM in the presence of acidic phospholipid [21]. This phenomenon can be briefly explained by an observation that a cluster of water was coordinated to the calcium ions in the calcium binding site so that phospholipids had to displace the water molecules in order to interact with the C2 domain. Upon phospholipid binding, the protein surface became less exposed to water and the calcium ions can be better coordinated in the domain to accomplish the higher affinity [44]. By interacting specifically with PtdIns(4,5)P2, calcium affinity of C2B domain of Syt I also has a significantly increase [47]. Before the lipid binding, the calcium binding

affinity of both C2 domains in the Syt I is too low to respond to the change in calcium concentration in cell. Phospholipid binding can provide calcium sensitivity for the protein to accomplish their functional role as a calcium sensor for the neurotransmitter releasing event.

These reports have suggested that the relationship of lipid binding and calcium binding may be interdependent and should not be considered as a single-way process.

2.1.2.2.3 Role of phospholipid binding on protein interaction

Phospholipid is also an important signaling compound in different cellular event. Conventional PKC isoforms can be activated by the binding to PS and calcium [16, 32], while the novel PKC isoforms is calcium independent and activated by negatively charged phospholipids or diacylglycerol [43]. These examples supported that importance of phospholipids in triggering protein functions.

2.1.2.3 Protein

Members of C2 domain superfamily were similar to each other in terms of their tertiary structure and pattern of secondary structures. Their amino acid sequences are highly variable that provide a basis for C2 domain to interact with a wide range of ligands including proteins [11]. Protein-protein interaction is not conserved among C2 domain but contributes to the important functions of some C2 domain-containing

protein, such as G protein signaling and vesicular trafficking which were briefly discussed below.

C2 domain containing phospholipase C (PLC) beta1 and beta2 are regulated downstream of G protein signaling [48]. Activated G alpha subunits of heterotrimeric G proteins interacted specifically to their C2 domains. C2 domain provided an anchor surface for the G alpha subunits which can then activate the enzymatic activities of the phospholipase.

Protein interaction is more common in C2 domain which is related to vesicular trafficking. Direct interaction of C2B domain of Rabphilin and the synaptosomal-associated protein of 25 kDa (SNAP-25) implies the functions of C2 domain in regulating membrane docking in exocytosis shown *in vitro* by immunoprecipitation [49] and *in vivo* by fluorescence resonance energy transfer [50]. C2A domain of a synaptic vesicular protein, Syt I, control the release of neurotransmitter by interacting with a plasma-membrane-localized protein required for vesicle fusion or docking in a calcium-dependent manner [34, 51].

In conclusion the binding of C2 domain to calcium and phospholipids are interdependent and act as a sensing event to the change in environment. On the other hand, the protein interaction of C2 domain is often dependent on the above two binding which protein is the effector of the signal received.

2.1.3 Function of C2 domain

Since the first discovery of C2 domain in protein kinase C, it was found in many different proteins related to signal transduction or membrane trafficking [24, 52-54] Three structurally distinct families were well recognized: PI3K-C2, PTEN-C2 and PKC-C2, followed by four newly identified families: AIDA-C2, NT-C2, DOCK-C2 and B9-C2 discovered by a more extensive analysis of this superfamily based on searches on sequence-profile, prediction on structure and analysis on phylogenetic and phyletic pattern[24]. More distinct functions were discovered in these families and this can give us an overview on the diverse functions of C2 domain.

Based on the phylogenetic study, descendants of ancient C2 domains were originally recruited in PTEN-C2 and PI3K-C2 families for the generation of phospholipid metabolites that could act as intracellular chemical messengers [24]. PTEN-C2 family is present in phosphatase and tensin homolog protein that functions in phosphoinositide phosphatase pathway. Their C2 domain binds to PtdIns (3,4,5)P3 in the membrane, brings it to the active site of phosphatase and then dephosphorylates it to PtdIns (4,5)P2 [55, 56]. This signal can prevent the cell from uncontrolled division by triggering programmed cell death (apoptosis) whenever necessary [57, 58]. On the other hand, C2 domains in phosphatidylinositide 3-kinases are prototype of PI3K-C2 domain. Phosphatidylinositol-3 kinases involved in a tumor-development-relating signaling pathway that crosstalks with phosphoinositide phosphatase

pathway [59, 60]. Three classes of phosphatidylinositide 3-kinases generate different inositol phosphates that act as messengers and regulate cellular processes including differentiation, proliferation and intracellular trafficking [61].

Recruitment of C2 domain in generation of lipid messengers has expanded during the evolution of other lipid-processing enzymes such as phospholipases in PKC-C2 family [41]. This type of C2 domains also involved in receiving and responding to the lipid signal by being present in peptidases and protein kinases with other catalytic or adaptor domains [53]. PKC-C2 family is the most-studied among different families and comprehensive researches have been carried out on its interaction with lipid to give insight on that of other C2 domains. It is the only family that contains members that bind to calcium and hence has calcium-related functions [45] such as regulation of membrane localization during signaling pathway or vesicular trafficking [52, 62]. Some very typical examples include isoforms of PKC and cPLA2.

Function of the three C2 families is related to the cytoskeleton. A family of C2 domain which always located at the N-terminal of protein (NT-C2) can link microfilament or actin to membrane. Its functions also extend to cellular trafficking such as organelle migration [63] and endocytosis [64] due to its nature of anchoring membranous substances to the cytoskeleton.

AIDA-C2 familiy is found in the C terminal of axin-interacting and

dorsal-associated protein of zebrafish which interacts with microtubule and act as a scaffold protein for the players in the Wnt signaling cascade [65]. Different conserved domains which have myosin interaction were usually present together with AIDA-C2 domain which transportation of this cytoskeletal protein to the membrane can occur directly rather than depended on microfilament movement. Hence, AIDA-C2 domain may contribute a cytoskeletal functions.

Besides the actin-myosin dynamics, C2 domain also interacts with microtubules. In the cilia there are three types of C2 domain-containing proteins that have interaction with lipid and co-localized to the centrosome or basal body [66]. The C2 domains in these proteins can be identified as a distinct family: B9-C2 domain. Serious diseases can be resulted from the mutation of these proteins since the normal formation of cilia was hindered [66-68].The architecture of proteins with B9-C2 domain was rather simple that almost all members are consisted of C2 domain as the only domain in the protein.

Another family of C2 domains, DOCK-C2 domain, is related to the G-protein function. Its presence was confirmed by crystallographic structural data [69] in one of the two conserved regions of an atypical group of guanine nucleotide exchange factors which regulates small GTPases that performed functions on phagocytosis and cell-migration [70, 71].

To summarize, C2 domain exhibited a diversified type of cellular

functions including mostly the regulation of intracellular lipid signaling networks and vesicular trafficking, followed by control of cellular cytoskeleton and activation of G protein.

2.1.4 Plant small C2 domain protein

Most of the C2 domains existed with other domains in a protein to give particular function. Recent research has shown the presence of unusual small C2-domain proteins consisting of a single C2-domain solely in plant. This distinct group of protein was understudied despite the thorough understanding of structure and function of other members of C2 domain in animal.

Among the limited studies about small C2-domain protein, most of them have focused on their functions in plant. For example, pollen specific OsPBP1 was responsible for the germination and fertility of the pollen grains [4]. Many members of this group of proteins were found involved in plant stress and defense response. Arabidopsis AtBAP1 is a negative regulator of cell death and defense response towards bacterial and oomycete pathogens [72] and its family member AtBAP2 was found to have control on programmed cell death [3]. Some other members work at opposite way. When rice OsSMCP1 is overexpressed, resistance towards both abiotic and biotic stress is enhanced [7]. On the other hand, OsERG1 was suggested to be related to defense response as its expression is found induced in a fungal elicitor treated rice cDNA library [2]. Translocalization of OsERG1 to the plasma membrane under the

fungal elicitor treatment was also observed which suggested a possible relationship between the function and the localization of OsERG1. Phosphorylation was observed in OsERG1 protein and the degree of phosphorylation decreased when Ser41 was mutated to alanine [73]. The plasma membrane localization of OsERG1 was also abolished by this mutation, but the effect of changing localization on physiological function of OsERG1 has not yet been studied. Another small C2 domain protein identified in canola was found interacting with the elicitor of necrotrophic fungus [74]. Despite the induced expression under fungal inoculation, this canola small C2 domain protein also showed a dynamic subcellular localization in different physiological calcium concentration which implied its possible role in plant defense response.

In conclusion, the current study does not give us a complete picture on the detailed relationship between structure and function of plant small C2 domain protein. My project concentrated on finding out this important missing piece of the puzzle by dissecting a small C2 domain protein, OsGAP1 using site-directed mutagenesis approach, based on the previous knowledge of its structure, subcellular localization and function attained in our laboratory.

2.2 Previous Studies by Prof. Hon-Ming Lam's laboratory

2.2.1 Biochemical characterization of OsGAP1-OsYchF1 protein pair

The gene *OsGAP1* was identified as a candidate in our laboratory

during a search for disease-related gene by subtraction library in rice *Oryza sativa* [8]. Its interacting partner was later found as the first YchF protein identified in plant and named OsYchF1. YchF1 protein was classified as an unconventional P-loop GTPase belonged to the Obg family of TRAFAC class [9]. It is a distinct and conserved group among bacteria and eukaryota domain, yet its importance and function have been overlooked until several recent reports on worm human YchF.

In rice the interaction of OsGAP1 and OsYchF1 has been confirmed by co-immunoprecipitation, bimolecular fluorescence complementation, yeast-two-hybrid [8] and double labeling immunogold electron microscopy[9]. Different from other G protein, OsYchF1 can bind to both GTP and ATP and perform hydrolytic activities on these substrates (unpublished data, HML). Both of the activities were activated by OsGAP1 but the extent of activation was relatively low when compared to other GTPase-activating proteins. OsYchF1 was distinct from other G protein by having a non-canonical amino acid sequence NMSE in the G4 motif of the G domain which controls the nucleotide binding specificity while the canonical sequence was NXSD. Site-specific mutation of OsYchF1 back to the canonical sequence can remove the ATP binding and hydrolytic activity of OsYchF1 but retained its GTPase activity (unpublished data, HML).

By searching the conserved domain database, OsGAP1 is regarded as a small protein which only consisted of a single C2 domain. Most C2 domain is a soluble protein that interacts reversibly with phospholipids as

mentioned in many literatures. Phospholipid dot blot experiment has confirmed its interaction with phospholipids [9]. Though OsYchF1 has no known domain with phospholipid interacting ability and it did not interact with phospholipids directly, indirect interaction between OsYchF1 and phospholipids can occur under the presence of OsGAP1.

2.2.2 Physiological characterization of OsGAP1-OsYchF1 protein pair

Being the one of the two conserved groups of G protein understudied, YchF proteins have surprising scare information on its functions. It has been reported to be involved in growth, translation and metal utilization in different bacteria [75-77] and protein turnover in yeast and parasite *Trypanosoma cruzi* [78, 79]. YchF homolog in human is related to reactive oxygen species response and overexpressed in cancer of different occasions which implies a regulatory role in tumor development [80, 81].

The first YchF identified in plant OsYchF1 and its interacting partner OsGAP1 were related to mechanism to combat both biotic and abiotic stress. Ectopic expression of OsGAP1 in *A. thaliana* improved the plant defense response and that of OsYchF1 has opposite effect in pathogen inoculation test [9]. While the expression of OsGAP1 was induced by pathogen inoculation, that of OsYchF1 was unchanged. This implied that the negative role of OsYchF1 in biotic stress may not be regulated at transcriptional level but may be more possible to be

regulated by the activation of OsGAP1. The subcellular localization of OsGAP1 and OsYchF1 was also related to the plant disease defense mechanism. As shown by the double labeling immunogold election microscopy experiment, both of the proteins in wild type rice localized in the cytoplasm under normal condition [9]. Under wounding condition, the expression of OsGAP1 increased and relocalized with OsYchF1 to the plasma membrane. As implied by the phospholipid dot blot experiment, OsGAP1 which contains a phospholipid-interacting C2 domain is more likely the active player in the relocalization event.

Antagonistic function of OsGAP1 and OsYchF1 can also be seen in the plant tolerance towards abiotic stress[10]. Similar to the effect on plant defense response, ectopic expression of OsGAP1 in *A. thaliana* enhances the abiotic stress tolerance in terms of higher chlorophyll content, higher anti-oxidation enzyme activity level and lower lipid peroxidation under salt treatment comparing to wild type. OsYchF1 again gave an opposite response under the same treatment. However, relocalization of OsGAP1 and OsYchF1 did not happen under salt treatment. Both of the proteins were localized in the cytoplasm under mock treatment or salt treatment which suggested localization to plasma membrane is not involved in the mechanism of abiotic stress tolerance.

As mentioned above, G4-mutated OsYchF1 lost its ATPase activity (unpublished data, HML). At the same time it lost its negative regulator role in biotic stress but not in abiotic stress. This strongly supported the

signaling relationship between ATPase and plant defense response and that between GTPase and abiotic stress tolerance.

In short conclusion, OsGAP1 plays a positive regulator role in both biotic and abiotic stress while OsYchF1, on the other hand, plays a negative regulator role. Biotic stress affected the subcellular localization of the protein pair but abiotic stress did not. ATPase activity of OsYchF1 was involved in the defense mechanism against biotic stress while GTPase activity was involved in the tolerance mechanism against abiotic stress.

2.2.3 Current working model of the OsGAP1-OsYchF1 protein pair

A working model of OsGAP1-OsYchF1 protein pair has been proposed based on the previous research. OsYchF1 was present as an ATP-bound form and a negative regulator towards biotic stress. Involvement of G protein in signaling pathway is common by switching between their GTP-bound and GDP-bound form and GDP-bound form was always identified as an inactive form. This can ensure the plant defense response was not activated until necessary to avoid energy wastage. Under pathogen invasion, the expression of OsGAP1 was induced and activated the hydrolytic power of OsYchF1 on ATP. OsYchF1 was also relocalized by OsGAP1 towards the plasma membrane and may initiate some signaling event there. In ADP-bound form, the negative regulator role of OsYchF1 was inactivated and hence the plant defense response was enhanced to combat against

pathogen.

On the other hand, GTP-bound form of OsYchF1 suppressed the abiotic stress tolerance which can be inactivated by enhancing the conversion from GTP to GDP by OsGAP1. This differed from the biotic stress model from not relocalizing to the plasma membrane.

Chapter 3 Experimental Design

3.1 Analysis on the structure of OsGAP1 protein

Through a collaborative effort between our laboratory and Prof. KB Wong's laboratory, the structure of OsGAP1 has been recently resolved using X-ray crystallography to a resolution of 1.63Å (unpublished data, HML). The basic structure of OsGAP1 was an independently folded beta-sandwich which is typical among all known C2 domain, which is consistent with the search result from the Conserved Domain Database. Further structural analysis on OsGAP1 was done by performing structural alignment using Vector Alignment Search Tool in Molecular Modeling Database (VAST-MMDB) developed by the NCBI Structure Group [82]. This tool allowed the comparison of proteins mainly based on their molecular geometry but not on their sequences to discover proteins with similar structure. PKC-C2 family was found to be closest to OsGAP1 among the seven structural distinct C2-domain families reported. Plant homologues of OsGAP1 also possessed the conserved aromatic residues and lysine residues that form the cationic beta-groove. These are the common residues conserved among most members in the PKC-C2 family regardless of topology [17, 19] and may be responsible for the specific binding of phospholipids.

Topology II members of PKC-C2 family was identified as the top candidates which had the highest structural homology to OsGAP1 (Figure 2). Despite the type II topology of OsGAP1, some amino acid residues which are highly conserved in most members of type I topology members can also be

found conserved in the plant homologues of OsGAP1, i.e. aspartate residues forming the calcium binding pocket which may confer calcium binding and phospholipid binding ability.

Except the possession of type II topology and the conserved residues in members of type I topology, OsGAP1 and its homologues had unique features which allowed them to form a distinct subfamily from other PKC-C2 family members (Figure 2). First, OsGAP1 is a small protein which only consisted of a single C2 domain which was exceptional among other members of PKC-C2 family. Second, instead of having eight beta-strands, OsGAP1 and its homologues had two more beta-strands inserted between the strand 7 and 8 of typical topology II C2 domain. These two special features were conserved among the known members of small C2 domain proteins in plants according to the sequence alignment based on secondary structure predication.

3.2 Rational

Based on the current working model suggested that OsYchF1 could shuttle between cytoplasm and plasma membrane under wounding condition through its interaction with OsGAP1, some specific sites on OsGAP1 are responsible for its interaction with phospholipids and OsYchF1 and these interactions have significance on the physiological function of OsYchF1-OsGAP1 pair. To identify specific interaction sites of phospholipids and OsYchF1 on OsGAP1, site-directed mutagenesis is used to dissect OsGAP1. Deletion mutagenesis was not chosen as OsGAP1 folds into only one single C2 domain. Deletion of any residues may destroy the overall

structure to maintain the different interaction sites. Hence, to identify specific binding sites on OsGAP1, mutations have to be done on OsGAP1 to disturb the interaction without destroying the overall protein structure.

3.3 Site-directed mutagenesis design

By comparing to other plant homologues of OsGAP1, conserved amino acid residues on the 3-D protein structure were identified. Five clusters of residues were chosen as the targeted mutation sites (Figure 3, 4). The main criteria for choosing these residues were that they must be surface residues and be conserved among other plant OsGAP1 homologues. Other criteria such as polarity or hydrophobicity were also taken into consideration. As most binding require the side chain on the residues for interactions, all of the residues in clusters indicated (Figure 3, 4) were mutated to alanine to remove the effect of side chain on interaction.

Two nonpolar aliphatic residues on betastrand 1 and two uncharged polar residues on betastrand 4 constitutes cluster 1. Two aspartate residues of the calcium binding pockets were mutated in cluster 2. Not all the calcium binding pocket residues were mutated since the changes in charges may be too large which may affect the protein in other ways rather than the side chain effect only. Positively charged lysine and arginine on betastand 3 which formed the cationic beta-groove were mutated in cluster 3. Lastly, four charged residues on the loop between betastrand 7 and 7a and on the loop between betastrand 7b and 8 were mutated as cluster 4 and cluster 5 respectively (Figure 3, 4) (Table 1).

Chapter 4 Methods and materials

4.1 Construction of five designed mutated OsGAP1 clones by site-directed mutagenesis

Five mutated constructs were produced by overlapping PCR [83]. During the first round of PCR, fragment with N-terminal and C-terminal of OsGAP1 with overlapping mutated regions were generated respectively with the overlapping forward and backward primers designed to mutate the regions (Table 2). The two fragments were then mixed together and polymerized to generate full length mutated construct. The PCR products were then inserted into *EcoRI* and *SalI* site of pMAL-c2 expression vector for the generation of in frame MBP fusion protein [84]. DNA sequencing were done to confirm the success of mutagenesis and cloning.

4.2 Expression and purification of in-frame MBP fusion proteins of OsGAP1 and its mutants and in-frame GST fusion protein of OsYchF1

After the pMAL-C2 constructs were transformed into the BL21 *E. coli* strain, protein expression was performed by IPTG overnight induction in LB medium with 100 mg/L Ampicillin at 20℃. The proteins were then purified by SpinClean™ MBP Excellose® Spin kit (Mbiotech 23020), following the procedures described in the user manual. Successful expression and purification of MBP fusion protein were confirmed with clear bands of correct sizes shown in SDS-PAGE (Figure 5). In-frame GST fusion protein

of OsYchF1 was obtained by expressing the construct in the pGEX-4T-1 expression vector as mentioned in the previous report [9] and purified by MagneGST™ Protein Purification System (Promega V8603), following the procedures described in the user manual.

4.3 Circular dichroism measurement of native and mutated OsGAP1 protein

Circular dichroism measurements were performed with a JASCO J-815 spectrometer using 1 mm rectangular quartz cuvett. The spectra of all samples with protein concentration of 0.2mg/ml were collected in dark at 25℃.

4.4 Phospholipid dot blot

For initial screening on phospholipid binding ability of the OsGAP1 mutants, 1 ul of phospholipid mixture for HPLC from soybean (Supelco P3817-1VL) was dotted on supported nitrocellulose membrane (Bio-Rad 162-0095). The membrane was then blocked with 3% skim milk blocking solution and incubated with 0.2 mg/ml MBP fusion proteins of different mutated or native protein respectively in blocking solution and washed as described [9]. The incubation and subsequent washing steps were performed in TBS buffer supplemented with 0.1% v:v Tween 20. MBP fusion proteins were detected by mouse monoclonal anti-MBP antibody (Sigma M6295) and then sheep anti-mouse HRP-linked antibody (GE Healthcare NA931).

Chemiluminescent signal can then be detected with X-ray film (Fujifilm 47410 08399) after adding of ECL substrate (GE Healthcare 89168-782). Biological repeat was done to confirm the result.

4.5 Lipid specificity test

Commercial membrane dotted with fifteen different lipid components of cell membrane (Echelon P-6002) was first blocked 3% skim milk blocking solution and then incubated with 0.2 mg/ml MBP fusion protein of native OsGAP1 or its mutants respectively in blocking solution for two hours. Washing and detection procedures were the same as those in the phospholipid dot blot described above[9]. Biological repeat was done to confirm the result.

4.6 Co-immunoprecipitation

E. coli cell lysates with expression of MBP-OsGAP1 native and mutated proteins were added respectively to GST-OsYchF1 pre-incubated with MagneGST™ Glutathione Particles of the MagneGST™ Protein Purification System (Promega V8603). The mixture was washed and eluted according to the user manual. Presence of mutated MBP-OsGAP1 was detected by mouse monoclonal anti-MBP antibody (Sigma M6295) and then goat anti-mouse AP-linked antibody (Sigma A0162). Color detection was done after adding of SIGMA FAST™ BCIP/NBT substrate (Sigma B5655). Western blotting against GST was repeated to ensure even GST-OsYchF1 protein loading on different lanes. Biological repeat

was done to confirm the result.

4.7 Preparation of transgenic tobacco BY2 cells

The coding regions of the mutant constructs were sub-cloned into binary vector V7 respectively under the control of the cauliflower mosaic virus 35S promoter. For transgenic tobacco BY2 cells, the V7 constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 which were then used to transform the tobacco BY2 cells following the previous protocol [85]. The transformed BY2 cells were selected on MS agar plate with 50 mg/L Kanamycin and 500 mg/L Cephalomycin and subcultured biweekly for 5 times to ensure stable transformation before generation of cell suspension for later experiment. Ectopic expression of the mutated proteins was confirmed by western blotting. Cell suspensions derived from BY2 cells callus were grown in 250-mL Erlenmeyer flasks containing 50 mL of MS medium in the dark at 25℃ on a rotary shaker at 100 rpm and subcultured weekly. Cell suspensions used for all experiments were 3-day-old.

4.8 Growth condition of *A. thaliana*

Floragard universal potting soil from Floragard Vertriebs GmbH (Gerhard-Stalling, Germany) was used for the growth of *A. thaliana*. They were then cultivated in a growth chamber with temperature of around 22°C, relative humidity of 70–80%, light intensity of 80–120 μmol m^{-2} s^{-1} on a 16 hr light: 8 hr dark cycle.

4.9 Preparation of transgenic *A. thaliana*

To constituently express OsGAP1 mutants constructs in *A. thaliana*, the respective V7 constructs was transformed into wild type Col-0 by Agrobacterium-mediated vacuum infiltration method [86] via *Agrobacterium tumefaciens* strain GV3101 (pMP90) [87]. T1 were screened on MS agar plate supplemented with 50 mg/L Kanamycin. Phenotypic ratio of Kanamycin resistance of T2 generation was statistically analyzed with chi-square test. A ratio of 3:1 indicated a single locus insertion event. Homozygous lines screened from T3 generation were used in the later experiment. Real-time PCR were done to confirm the expression of the transgenes.

4.10 Cell viability staining with trypan blue after salt treatment of tobacco BY-2 cell

Three-day-old tobacco BY-2 cell suspensions ectopically expressing mutated or native OsGAP1 were washed with MS solution before treatment. MS solution supplemented with 150mM NaCl or MS solution only was added to the cell suspension as salt treatment or mock treatment respectively. The cell suspensions were then incubated in the dark at 25℃ on a rotary shaker at 100 rpm for 20 hrs. After the treatment, the cells were allowed to settle and stained in new 2ml micro-centrifuge tubes with 1:1(v:v) 0.4% Trypan blue solution (Sigma T8154) for 10 min . They were then observed under a light microscope at a magnification of

200X and the viability were recorded by counting around 100 cells per sample. At least five microscopic fields were taken for each sample. Wild type and BY2 cells transformed with empty vector were included as control. Biological repeat was done to confirm the result.

4.11 Statistical analysis

Statistical Package for Social Sciences (ver. 15.0) was used to analyze the quantitative data. The mean difference was analyzed using one-way analysis of variance following the Games-Howell or Tukey's post-hoc test.

Chapter 5 Results

5.1 All of the mutated proteins were successfully expressed in full length and folded properly

MBP fusion protein of native OsGAP1 and its mutants were successfully expressed and purified as confirmed by SDS-PAGE (Figure 5). The sizes of the MBP fusion proteins were about 55 kDa as expected which showed the successful expression of full length fusion protein.

Circular dichroism (CD) spectroscopy made use of the fact that most macromolecules had molecular asymmetry such that the mirror image of its structure was not superimposable on its mirror image [88]. This chiral property allowed different absorbance to be detected between protein samples in circularly polarized light of different wavelength. Different structural conformation can give different CD spectra. It was widely used in protein engineering to show successful protein folding of the modified protein [89, 90]. The circular dichroism spectra shape of OsGAP1 mutants in far UV region were similar to that of the native protein with a negative value between 203-245 nm (Figure 6) which indicated secondary structure (i.e. beta strands) of the mutants were properly folded as the native protein [91]. Besides, the near UV spectra of the mutants can be well superimposed to that of the native protein (Figure 7) which reflected the micro-environment between the side chains of aromatic residues on all the proteins was similar [91]. This implied the native OsGAP1 as well as its

mutants folded properly in their tertiary structure.

Taken together the results of the far UV and near UV circular dichroism spectra, OsGAP1 mutants folded properly in terms of both secondary and tertiary structure. No significant distortion of structure was seen in the mutants as compared to the native OsGAP1. This allowed a better analysis that any loss in function was mainly due to the removal of the side chain of the mutated residues but not the destruction of the native structure.

5.2 OsYchF1 interacting ability of OsGAP1 was lost when cluster 1 or 3 was mutated

One of the distinctive features of OsGAP1 was its interaction with OsYchF1 which may properly confer functions. To identify the regions of OsGAP1 that interacted with OsYchF1, co-immunoprecipitation was performed. Among the mutants of OsGAP1, cluster 1 and 3 mutant lost interaction with OsYchF1 while the others still retained the OsYchF1 interaction (Figure 8).

5.3 Lipid binding ability was lost in OsGAP1 with mutations at cluster 2, 4 and 5

OsGAP1 contained a C2 domain that interacted with phospholipids in a calcium-independent manner [9]. Hence phospholipid binding ability is one of the biochemical properties that have to be tested in the OsGAP1 mutants. Initial screening of the mutants was done by incubating a

phospholipid mixture dotted membrane with MBP fusion protein of different OsGAP1 mutants respectively. Result showed OsGAP1 protein with mutation site at cluster 2, 4 or 5 lost their interaction with phospholipids but that at cluster 1 or 3 still retained their interaction though the interaction of phospholipids to OsGAP1 mutated at cluster 3 was reduced (Figure 9).

Mutation at clusters that required for phospholipid binding (i.e. cluster 2, 4 and 5) did not affect the OsYchF1 binding (Table 3). OsGAP1 was able to interact with phospholipids and OsYchF1 by different residues at the same time. This result was compatible with the previous report that suggested OsGAP1 can bridge the interaction between OsYchF1 and phospholipids [9].

5.4 OsGAP1 mainly interacted with cardiolipin, anionic phosphatidylinositol 4-phosphate and phosphatidylserine

The lipid specificity of OsGAP1 has not been investigated in the previous reports. Primary screening of phospholipid species binding to OsGAP1 was performed by incubating MBP fusion protein of OsGAP1 with commercial membrane pre-dotted with fifteen cell-membrane-constituting phospholipids (Figure 10, 11). In this assay, loose lipid selectivity of OsGAP1 was observed. OsGAP1 recombinant protein was found to have strong interaction selectively with phosphatidylserine, cardiolipin and phosphatidylinositol 4-phosphate (PtdIns(4)P). Weaker interaction can also be detected in the dots with phosphatidylethanolamine,

phosphatidylglycerol, phosphatidylinositol phosphates, phosphatidic acid and cholesterol in decreasing intensity.

5.5 Mutation of the cationic residues batch in cluster 3 demolished the interaction between OsGAP1 and phosphatidylinositol phosphates

To find out the role of different residues on lipid selectivity, lipid specificity test with commercially available membrane was repeated in the OsGAP1 mutants which still retained phospholipid binding ability in the phospholipid dot blot assay. Mutants of cluster 1 had a similar phospholipid binding pattern as the native OsGAP1 while mutation at cluster 3 (with three lysine and one arginine converted to alanine) abolished the interaction with phosphatidylinositol phosphates (Figure 10, 11). This result implied that the residues in cluster 3 were required for the specific binding of OsGAP1 to phosphatidylinositol phosphates.

5.6 Only mutant in cluster 2 of OsGAP1 retained the positive regulator role of salt stress in cell-level

Cell death is one of the direct consequences from salt stress. To assess the change of functional role in OsGAP1 after site-directed mutagenesis, salt treatment was performed in the respective transgenic tobacco BY2 cells. Rate of cell death was then counted by method of trypan blue staining. Cell lethality in the transgenic tobacco BY2 cell lines which ectopically expressed OsGAP1 clusters 2 mutants or native OsGAP1 were

similar (Figure 12, 13). Other lines expressing OsGAP1 mutants lost tolerance towards salt stress and had comparable cell lethality to the wild type or empty vector line.

Chapter 6 Discussion

6.1 OsYchF1 interaction site on OsGAP1 was identified

Interaction of OsGAP1 to a universally conserved G protein OsYchF1 was one of the distinctive and important features of OsGAP1 which provided clues for its molecular working mechanism. Protein-protein interaction of plant small C2 domain protein was rarely documented except our first report of OsGAP1-OsYchF1 [8] and the recent report of interaction between a fungal elicitor Sspg1d from necrotrophic *Sclerotinia sclerotiorum* and the small C2 domain protein IPG-1 in canola during infection [74]. Though the interaction between OsGAP1 and OsYchF1 was affirmed with several experimental approaches [8-10], the specific interaction site has not been determined. Co-immunoprecipitaion of OsYchF1 with mutants of OsGAP1 showed that mutation at cluster 1 (L5A, L8A, T58A, S60A) and cluster 3 (K37A, K39A, K41A, R43A) of OsGAP1 can abondon its binding to OsYchF1 (Figure 8) suggesting that these sites are essential for the interaction between OsGAP1 and OsYchF1.

6.2 OsGAP1 may be a cardiolipin-sensing protein functioned in plant defense response

By a search in Conserved Domain Database, OsGAP1 was identified to be a small protein contained only a C2 domain. Most C2 domain can interact with phospholipids. Compatible with the search

result, experimental data has shown OsGAP1 was able to interact with lipid in a calcium-independent manner [9]. Phospholipids were also greatly involved in signal transduction in addition to their presumed structural function. Different types of phospholipids with diverse chemical properties which allowed them to function as different cellular signals under different conditions in organism. Hence, knowing the type of phospholipids which OsGAP1 interacted with can provide a better understanding of the niche of OsGAP1 in signaling pathway.

To test for the lipid specificity of OsGAP1, its MBP fusion protein was incubated with a commercially available membrane pre-dotted with fifteen phospholipids commonly found in biological membrane followed by detection (Figure 10). OsGAP1 was found interacting strongly with cardiolipin, PtdIns(4)P and PS, followed by phosphatidylethanolamine (PE) , phosphatidylglycerol (PG) and PtdIns(4,5)P2. Weak association can also be observed with cholesterol (Figure 11). Both negatively charged (i.e. PS, polyphosphatidylinositides and PG) and zwitterionic phospholipids (i.e. cardiolipin and PE) [92] were found interacting with OsGAP1 which may imply multiple phospholipid interacting sites on the protein.

One of the attention-drawing results was the interaction between cardiolipin and OsGAP1. In eukaryote, cardiolipin was a phospholipid present exclusively in the inner mitochondrial membrane [93] and it was also the only phospholipid synthesized in the mitochondria which meant that this phospholipid cannot be found in the cytosol and plasma

membrane [94, 95]. This result seemed contradictive with the previous finding that OsGAP1 was localized in cytosol and relocalized to the plasma membrane under wounding condition [9]. However, one important fact should be noted was that the cell membrane of bacteria also contained cardiolipin [94]. Phospholipids in the bacterial membrane can trigger the defense mechanism of plant. For example, phospholipid components of the cell membrane of *Phytophthora infestans* was reported as a signaling compound to trigger defense response in potato [96]. In the case of cardiolipin, when the pathogen interacts with the plant during invasion, membrane lipid fragments which may contain cardiolipin can possibly be released from fungi and bacteria. Cardiolipin can then be a signal to activate the plant defense response by binding to cardiolipin-sensing OsGAP1. Some reports have proved that cardiolipin can activate the binding ability of C2 domain to other phospholipids [97]. This may also be the reason why OsGAP1 re-localized to the plasma membrane under wounding condition [9].

6.3 Canonical and novel lipid binding surfaces of C2 domain were found in OsGAP1

There are plenty of reports on the interaction of C2 domain with phospholipids in animal but no previous report was found for the specific interaction region of plant C2 domain to phospholipids. We previously showed that OsGAP1 interacts with the plasma membrane and the extract of total phospholipids from plant [9]. In this project, the candidate residue clusters on OsGAP1 possible for interaction was mutated to alanine by

site-directed mutagenesis so that the phospholipid interaction site can be located.

Using the phospholipid dot blot experiment, it was found that mutations in cluster 2, 4 or 5 abolished the lipid binding ability of OsGAP1. Cluster 2 (D23A, D28A), cluster 4(R117A, N119A, E123A, E124A) and cluster 5 (R141A, R143A, E146A, E149A) formed a ridge on the convex surface of OsGAP1 which was critical for the binding of phospholipid (Figure 4). Cluster 2 consisted of two aspartate residues which were conserved in the calcium binding loop in some C2 domains [16, 19, 25, 27, 28]. Moreover, several pieces of previous studies demonstrated the involvement of the calcium binding loop to phospholipid binding in both calcium-dependent [16, 19, 25, 27, 28] and independent manner [17, 22, 37]. This observation is also supported our results. The new discovery on the involvement of residues in cluster 4 and 5 of OsGAP1 in phospholipid binding provides new clues to understand this phospholipid interaction of other C2 domains. Residues in these clusters were conserved [4] or chemically similar [2, 6] to other plant small C2 domain proteins suggesting that lipid binding site of cluster 4 and 5 in OsGAP1 may be common in plant- specific small C2 domain proteins but non-canonical in other well-studied C2 domains. On the other hand, the other side of OsGAP1 which consisted of cluster 1 and 3 was not critical for phospholipid binding, since the mutation in cluster 1 or 3 did not demolish the phospholipid interaction (Figure 9).

6.4 Lipid binding surface and OsYchF1 binding surface in OsGAP1 mostly did not overlap each other

Cluster 2, 4 and 5 of OsGAP1 formed a surface critical for the binding to phospholipid while cluster 1 and 3 which was on the opposite site of the protein formed another surface critical for the interaction with OsYchF1 (Figure 4) (Table 3). OsGAP1 was able to interact simultaneously with phospholipids and OsYchF1 using different residues. This result supports our proposed model explaining the working mechanism of OsGAP1-OsYchF1 protein pair [9]. Double labeling immunogold electron microscopy showed that OsYchF1 and OsGAP1 re-localized from cytosol to plasma membrane under wounding situation [9]. It was suggested that this re-localization event was mediated by OsGAP1 since OsGAP1 can bridge the indirect interaction between the OsYchF1 and phospholipid as shown by phospholipid dot blot experiments [9]. To fulfill its function, OsGAP1 should bind to OsYchF1 and phospholipid at the same time. Characterization of different mutants of OsGAP1 showed that the main lipid binding surface and OsYchF1 binding surface are located on the opposite sites of OsGAP1 and hence simultaneous binding of OsGAP1 to the two ligands is possible.

6.5 Cationic beta groove (cluster 3) of OsGAP1 may regulate OsYchF1 interaction in a polyphosphoinositides-sensing manner

Mutation in cluster 3 of OsGAP1 lost its binding to polyphosphoinositides and OsYchF1 (Figure 8, 11). This indicated that cationic residues in cluster 3 (K37A, K39A, K41A, R43A) had dual binding ability towards OsYchF1 and polyphosphoinositides. The

position of cluster 3 in OsGAP1 was conserved to that of the cationic beta-groove in other C2 domains. Our data was consistent to previous reports on the specific interaction between polyphosphoinositides and cationic beta-groove in other C2 domains [13, 19, 37, 44] and also the protein-protein interaction between C2 domain and other proteins [98].

Questions raised as mutation in cluster 2, 4 or 5 also abolished the polyphosphoinositides binding (Figure 9). This can be possibly explained by the cooperativity of phospholipid binding [99].The binding of lipid ligands to C2 domain can be depended on each other. Binding to some phospholipids can even activate the protein binding to other phospholipids [97]. Cluster 2, 4 and 5 may bind to phospholipids such as cardiolipin which was critical for the binding of other phospholipids such as polyphosphoinositides. Hence, destruction of phospholipid binding site at cluster 2, 4 or 5 can also destroy the polyphosphoinositides-binding ability at cluster 3. In contrary, polyphosphoinositides-binding did not affect the binding ability of OsGAP1 to other phospholipids so that mutation at cluster 3 only abolished the binding to polyphosphoinositides.

Cluster 3 was formed by three lysine and one arginine residues which also composed the cationic beta groove conserved in C2 domains (Figure 2). Previous research has shown this site can bind to polyphosphoinositides [13, 19, 37, 44] and contribute to one of the lipid binding modes of C2 domain [37]. The two lipid binding modes can switch to each other under different situation. For example C2B of Syt I

can bind to the membrane either by the flexible loops which allowed the protein interacting site to expose in the cytosol, or by the cationic beta groove which immersed the protein interacting site into the membrane and hence blocked the protein interaction [37]. Similar scenario may be seen in OsGAP1, binding to polyphosphoinositides can be a chemical signal which switched OsGAP1 from OsYchF1-binding-available state to OsYchF1-binding-unavailable state under certain physiological conditions.

6.6 Enhanced salt tolerance was retained in OsGAP1 mutated in cluster 2 which lost binding to phospholipids but still interacted with OsYchF1

In the previous report on the involvement of OsYchF1 and OsGAP1 in salinity tolerance, double labeling immunogold electron microscopy showed that OsYchF1 and OsGAP1 did not re-localize to the plasma membrane [10] as they did under wounding condition [9]. This implied that interaction with the plasma membrane did not contribute to the enhanced abiotic tolerance caused by expressing OsGAP1. In the salinity tolerance test of using transgenic tobacco BY2 cells ectopically expressing mutated or native OsGAP1, only transgenic lines expressing OsGAP1 with cluster 2 mutated still retained the enhanced salt tolerance similar to that of native OsGAP1(Figure 12,13). OsGAP1 with cluster 2 mutated can interact with OsYchF1 but not with phospholipids (Table3). This was consistent with the previous finding that salinity tolerance was not dependent on interaction with the plasma membrane.

It also indicated that OsGAP1 and OsYchF1 needed to interact to perform their functions in salt tolerance since expressing OsGAP1 mutants which did not interact with OsYchF1 (i.e. cluster 1 and 3) could not enhance salinity tolerance of the BY2 cells. For OsGAP1 with cluster 4 or 5 mutated, despite that these mutated OsGAP1 could still interact with OsYchF1, tobacco BY2 cells ectopically expressing these mutated OsGAP1 did not exhibit enhanced salt tolerance. The mutation in cluster 4 or 5 may have demolished the OsYchF1 activation ability of OsGAP1 which will have to be confirmed by NTPase activity assay.

The above observation points to two possible molecular working mechanisms of OsGAP1-OsYchF1. In one model, interaction with plasma membrane was simply not required for salinity tolerance meaning that OsGAP1 and OsYchF1 did not need to interact with any components in the plasma membrane to regulate the salinity tolerance. In another model, both OsGAP1 and OsYchF1 are needed to function in the cytosol to activate the salt tolerance mechanism. In the later case, if OsYchF1 and OsGAP1 did not localize in the cytosol, the salt tolerance might be affected.

Chapter 7 Conclusion and future prospects

In this project, site-directed mutagenesis based on the resolved crystal structure of a rice small C2 domain protein, OsGAP1, was performed aiming to dissect the structure-function relationship of OsGAP1. We have identified phospholipid and OsYchF1 interacting surface on OsGAP1 which was the first report of molecular dissection of a plant small C2 domain protein. Different interacting surface of OsGAP1 towards phospholipids and OsYchF1 allowed OsGAP1 to bridge the indirect interaction between phospholipids and OsYchF1. Lipid selectivity of OsGAP1 was also solved which gave us insight on the involvement of OsGAP1 in lipid signaling. Physiological function of OsGAP1 was also dissected such that phospholipid binding ability of OsGAP1 was not required for the activation of salt stress tolerance in plant. All of the results can fit into the working model of OsGAP1-OsYchF1 pair proposed in the previous reports.

To provide concrete evidence to the molecular working mechanisms of OsGAP1-OsYchF1 suggested above and proposed in the previous reports, several experiments have to be done. First, GTPase and ATPase activity of OsYchF1 in the presence of different OsGAP1 mutants should be assayed to determine if the mutations affect the activation ability of OsGAP1 on OsYchF1.

To confirm the lipid specificity of OsGAP1 and its mutants, vesicle binding

studies have to be done. This experiment can also be used to determine whether cardiolipin can activate the binding of OsGAP1 to phosphatidylserine or phosphatidylinositol 4-phosphate.

Abiotic tolerance test and pathogen inoculation test of transgenic *A. thaliana* ectopically expressing mutated or native OsGAP1 can also be performed to determine whether the loss of binding to either phospholipids or OsYchF1 or OsGAP1 will affect the salt stress tolerance or disease resistance in plant.

Table 1) Table to show the residues in different clusters mutated to alanine

Cluster 1	L5A	L8A	T58A	S60A
Cluster 2	D23A	D28A		
Cluster 3	K37A	K39A	K41A	R43A
Cluster 4	R117A	N119A	E123A	E124A
Cluster 5	R141A	R143A	E146A	E149A

Table 2) Table to show primers used in the site-directed mutagenesis of OsGAP1

Description		Sequences (5' to 3')
Universal primers used as outer boundary primers to amplify coding region of C2-modified OsGAP1 for cloning into the vector*	Forward primer	CCGAATTCATGTTGGGGCATCTGGTTG
	Reverse primer	AACTCGAGAATAGGCAGAGTACAGAGTTC
Outer primer to introduce part 1 of cluster 1 mutation**	Forward primer	CCGAATTCATGTTGGGGCATGCGGTTGGGGCGGTGAAG
Overlapping primer pairs to introduce part 2 of cluster 1 mutation	Forward primer	GATGAACTCGCCCTCGCGATCGAAGAT
	Reverse primer	ATCTTCGATCGCGAGGGCGAGTTCATC
Overlapping primer pair to introduce cluster 2 mutation	Forward primer	GTCCGCGCCCTCCGCTCCAGCGCCCCCTAC
	Reverse primer	GTAGGGGGCGCTGGAGCGGAGGGCGCGGAC
Overlapping primer pair to introduce cluster 3 mutation	Forward primer	ATGGGCGCACAGGCATTGGCAACAGCAGTCATA
	Reverse primer	TATGACTGCTGTTGCCAATGCCTGTGCGCCCAT
Overlapping primer pair to introduce cluster 4 mutation	Forward primer	AATGCACAGGCCTGCCTAGCTGCAGCGAGC
	Reverse primer	GCTCGCTGCAGCTAGGCAGGCCTGTGCATT
Overlapping primer pair to introduce cluster 5 mutation	Forward primer	GTTCTCGCACTAGCGGATGTGGCATGCGGGGCAATT
	Reverse primer	AATTGCCCCGCATGCCACATCCGCTAGTGCGAGAAC

**EcoRI* and *XhoI* site were added to the end of the outer boundary primer pair for the later cloning of fragment into vector.

** No overlapping primers are required for the mutation of cluster 1 (part 1) as the mutation region lies on the forward primer of the outer boundary primer pair. Forward primer for this mutation is designed to replace the outer universal forward primer and used with the overlapping reverse primer of cluster 1 (part 2) mutation to produce N-terminal OsGAP1 fragment with cluster 1 mutated.

Table 3) Table to summarize the biochemical characterization of different mutants of OsGAP1

	Clu1	Clu2	Clu3	Clu4	Clu5	native	MBP
Phospholipid binding	++	-	+	-	-	++	-
OsYchF1 binding	-	++	-	++	++	++	-

- **:** **No interaction**

+ + **:** **Strong interaction**

+ **:** **Reduced interaction**

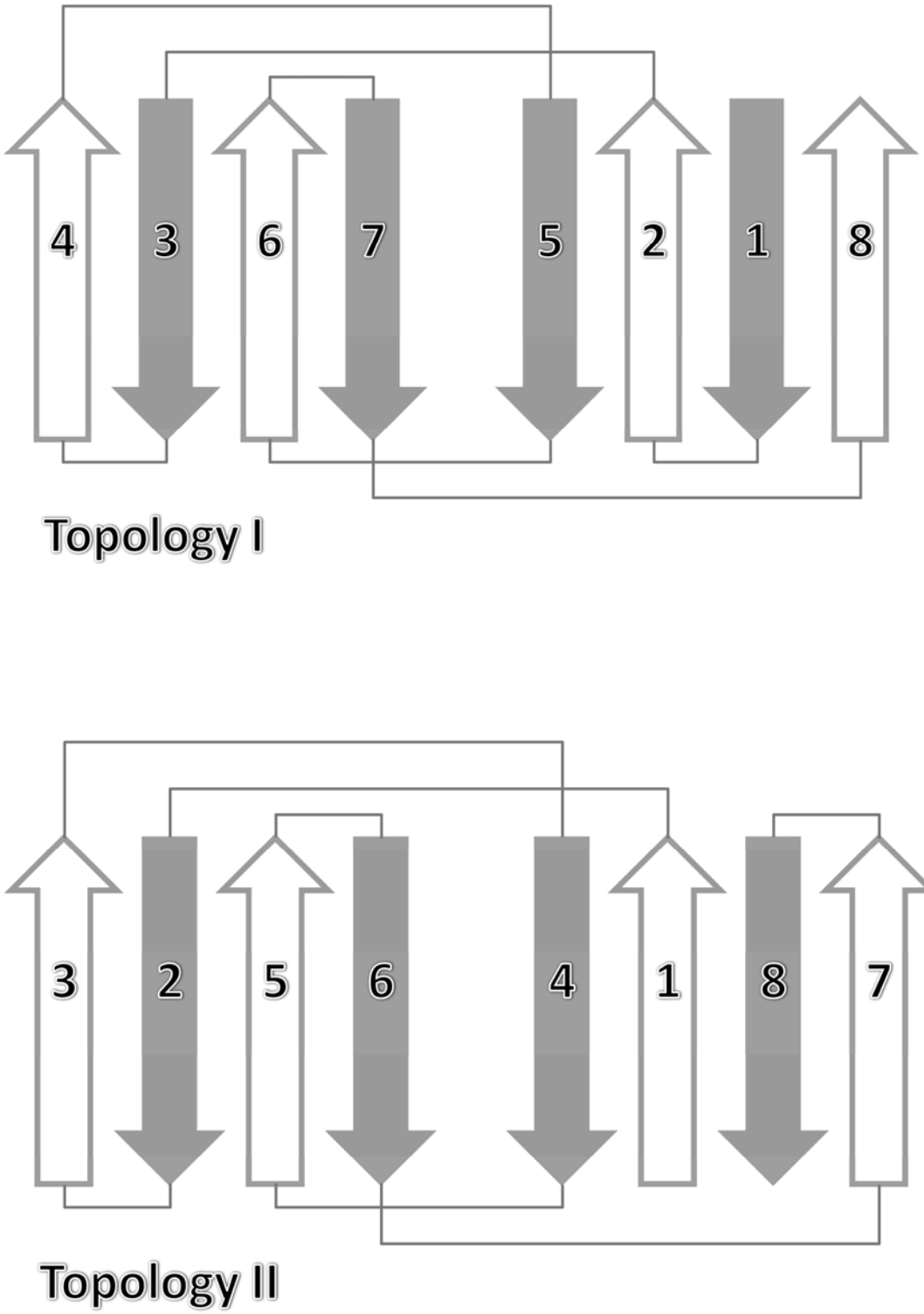


Figure 1) Schematic diagram to represent the secondary structure pattern of two typical C2 domain topologies modified from Figure 3A of [18].

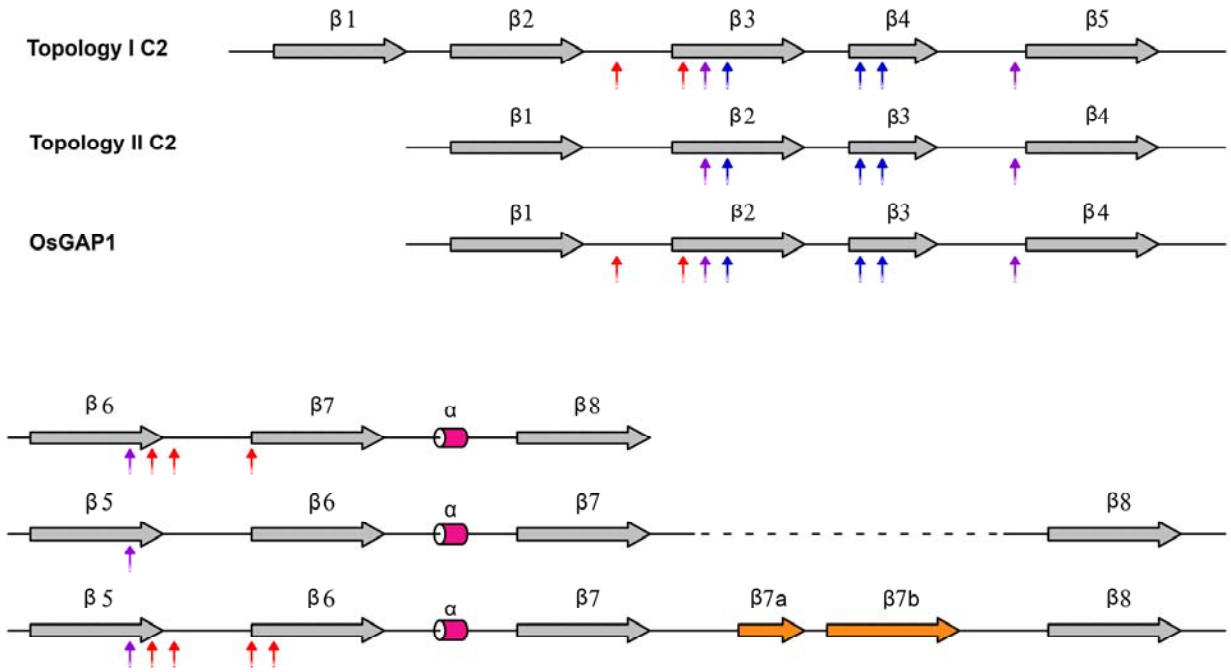


Figure 2) Schematic diagram to show the comparison of secondary structure pattern between OsGAP1 and C2 domain with topology I, II. By structural alignment, OsGAP1 is closest to the PKC-family and retains the common features of family members indicated by colored arrows (Lysine patch: blue, aromatic residues: purple). It belongs to topology II but also has the signature residues conserved in topology I C2 domain indicated by red arrows (Aspartate residues composing calcium binding pocket). However, OsGAP1 has 2 extra beta strands (7a and 7b shown in orange) and extra aspartate residues which is unique to other PKC-C2 domains.

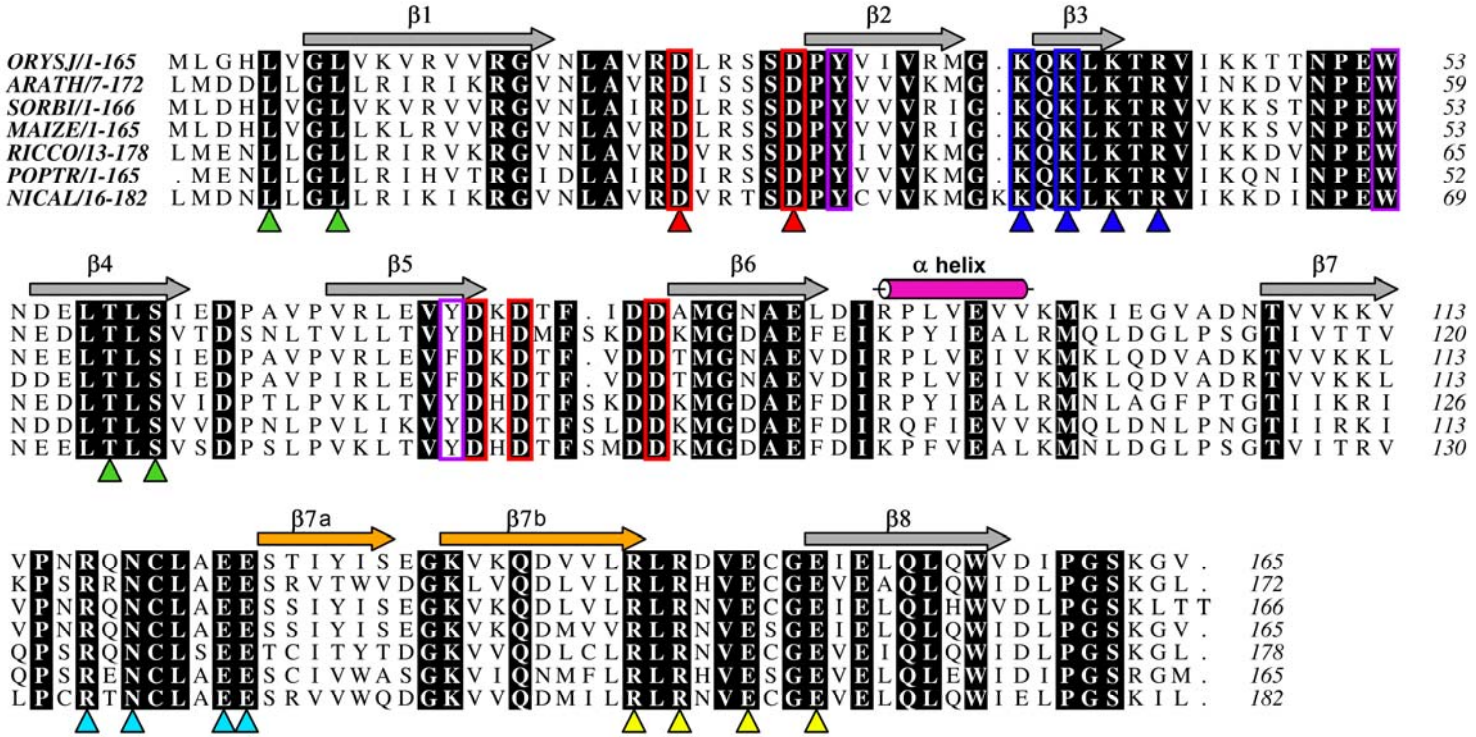


Figure 3) Sequence alignment of plant homologues of OsGAP1. Identical residues among homologues are highlighted in black. Clusters designed for mutation are labeled in colored arrows respectively (green: cluster 1; red: cluster 2; blue: cluster 3; cyan: cluster 4; yellow: cluster 5), while conversed residues with PKC-C2 domain are boxed in color (Red: aspartate residues composing calcium binding pocket, blue: lysine patch, purple: aromatic residues). Five clusters of residues were chosen as the targeted mutation sites. The main criteria for choosing these residues were that they must be surface residues and be conserved among other plant GAP1 homologues.

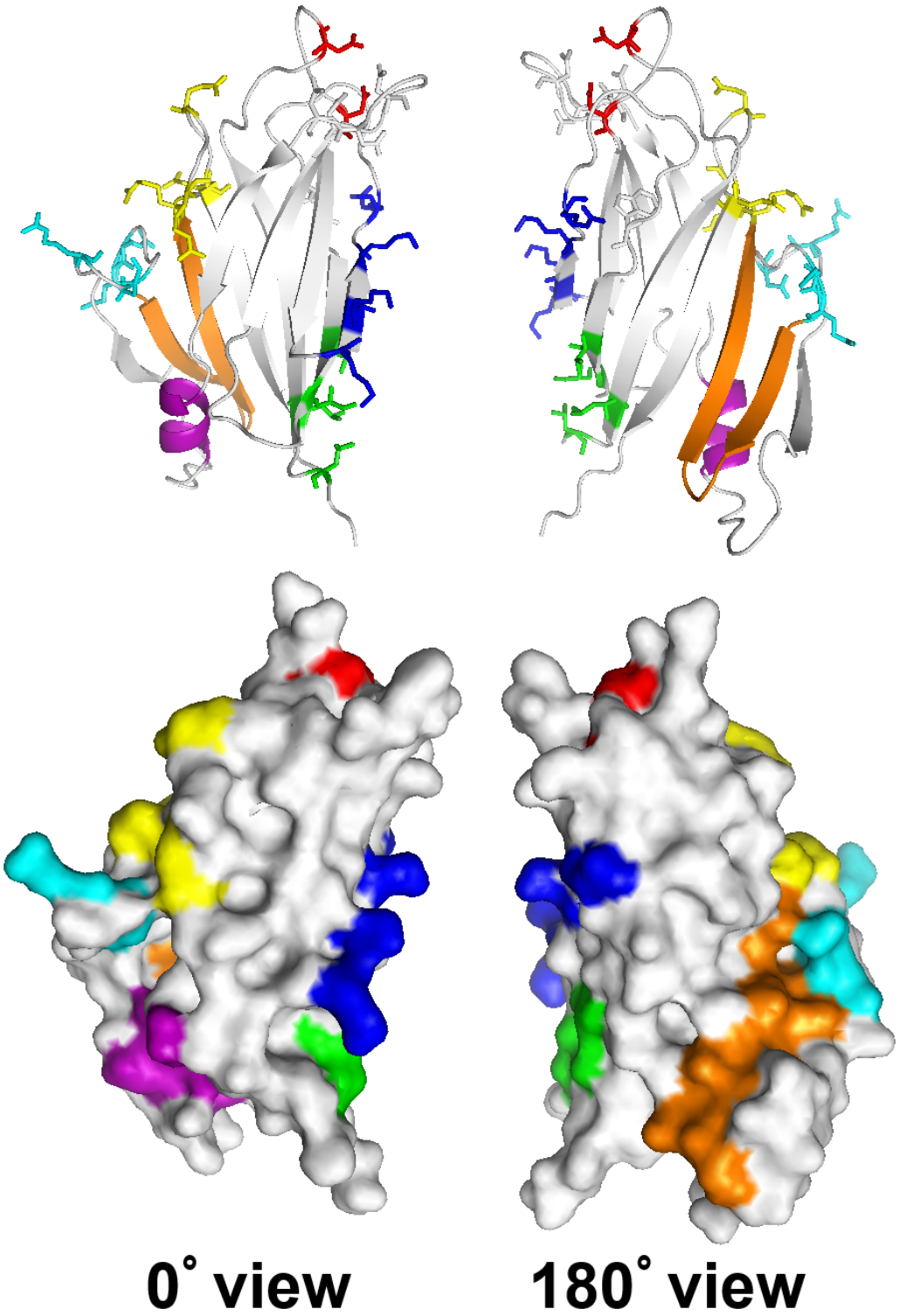


Figure 4) Diagram to show the location of designed mutation clusters on OsGAP1 with color indicated in (Figure 3) as shown as ribbon (above) and surface (below). Crystal of OsGAP1 was resolved to the resolution of 1.63Å.

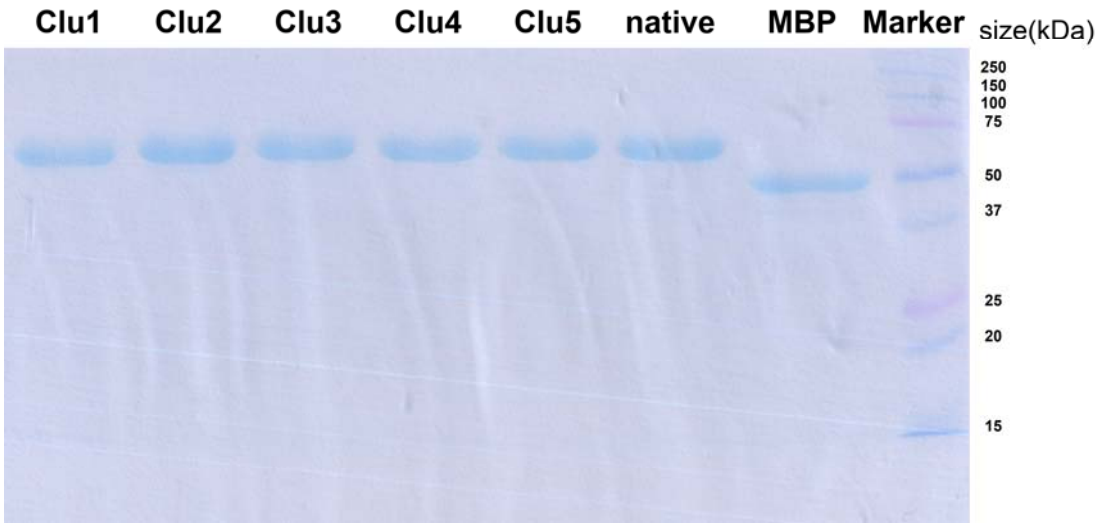


Figure 5) SDS-PAGE protein gel electrophoresis result showing successful expression and purification of MBP-OsGAP1 protein. Elution fraction of MBP-OsGAP1-C2'-(from left) cluster 1 to 5, native MBP-OsGAP1 and MBP only respectively were collected from protein purification process by SpinClean™ MBP Excellose® Spin kit (23020; Mbiotech). Precision Plus Protein Dual Color Standards (M) (Bio-Rad 161-0374) was used for protein size comparison. Clear single bands of around 55 kDa were observed which showed the successful purification of MBP fusion protein.

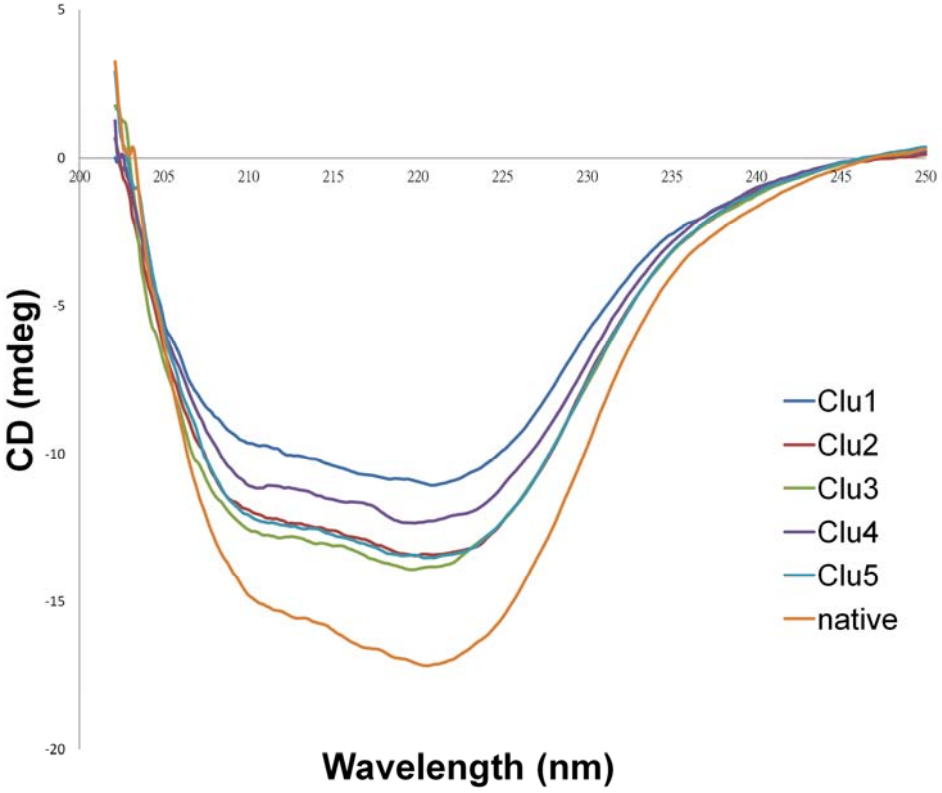


Figure 6) Circular dichroism spectra of native OsGAP1 and its mutants (Clu 1-5) in far UV region.

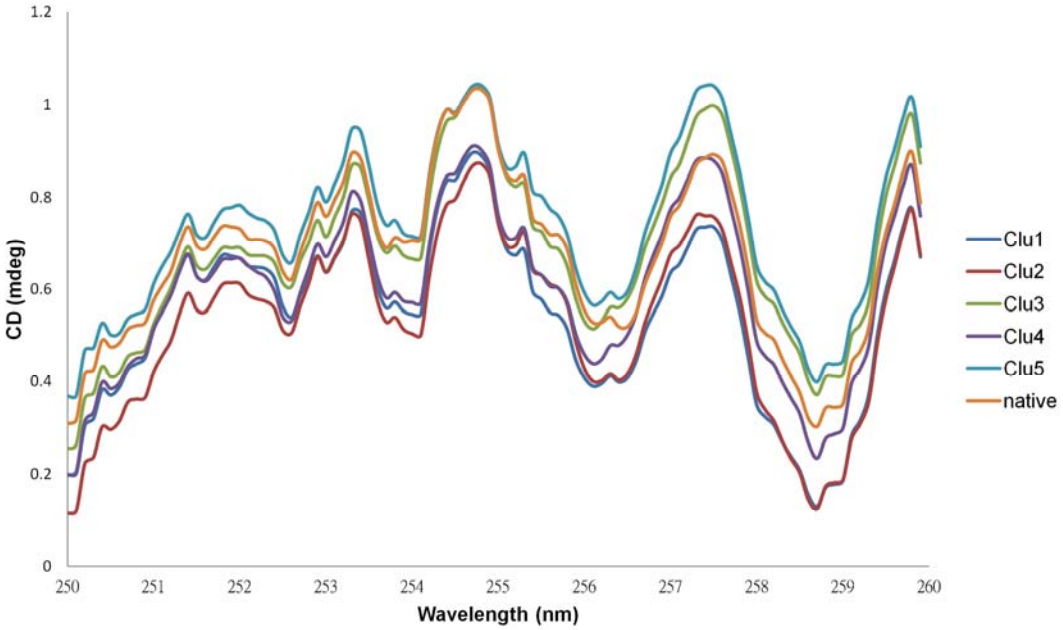


Figure 7) Circular dichroism spectra of native OsGAP1 and its mutants (Clu 1-5) in near UV region.

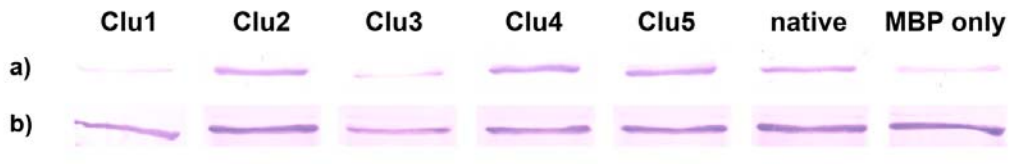


Figure 8) Co-immunoprecipitation to show interaction between OsYchF1 and MBP fusion protein of OsGAP1 mutants (Clu1-5) respectively. (a) Co-immunoprecipitation by mixing GST-OsYchF1 with MBP fusion protein of OsGAP1 mutants which were then pull down by MagneGST™ Glutathione Particles from the MagneGST™ Protein Purification System (Promega V8603) and detected by anti-MBP antibody. MBT fusion protein of native OsGAP1 and MBP protein was used as positive and negative control respectively. (b) Western blotting against OsYchF1 was performed to show the even loading of GST-OsYchF1.

Figure 9) Phospholipid dot blot assay with MBP fusion protein of OsGAP1 mutants (Clu1-5). Native OsGAP1 and MBP protein was used as positive and negative control respectively.

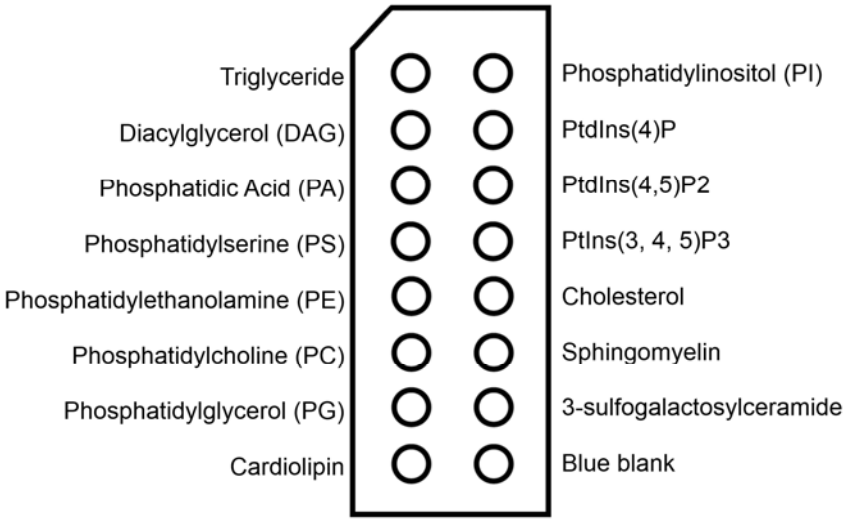


Figure 10) Position of fifteen lipid species pre-dotted on membrane lipid strips (Echelon Biosciences, P-6002). This membrane was used to detect lipid specificity of phospholipid binding protein.

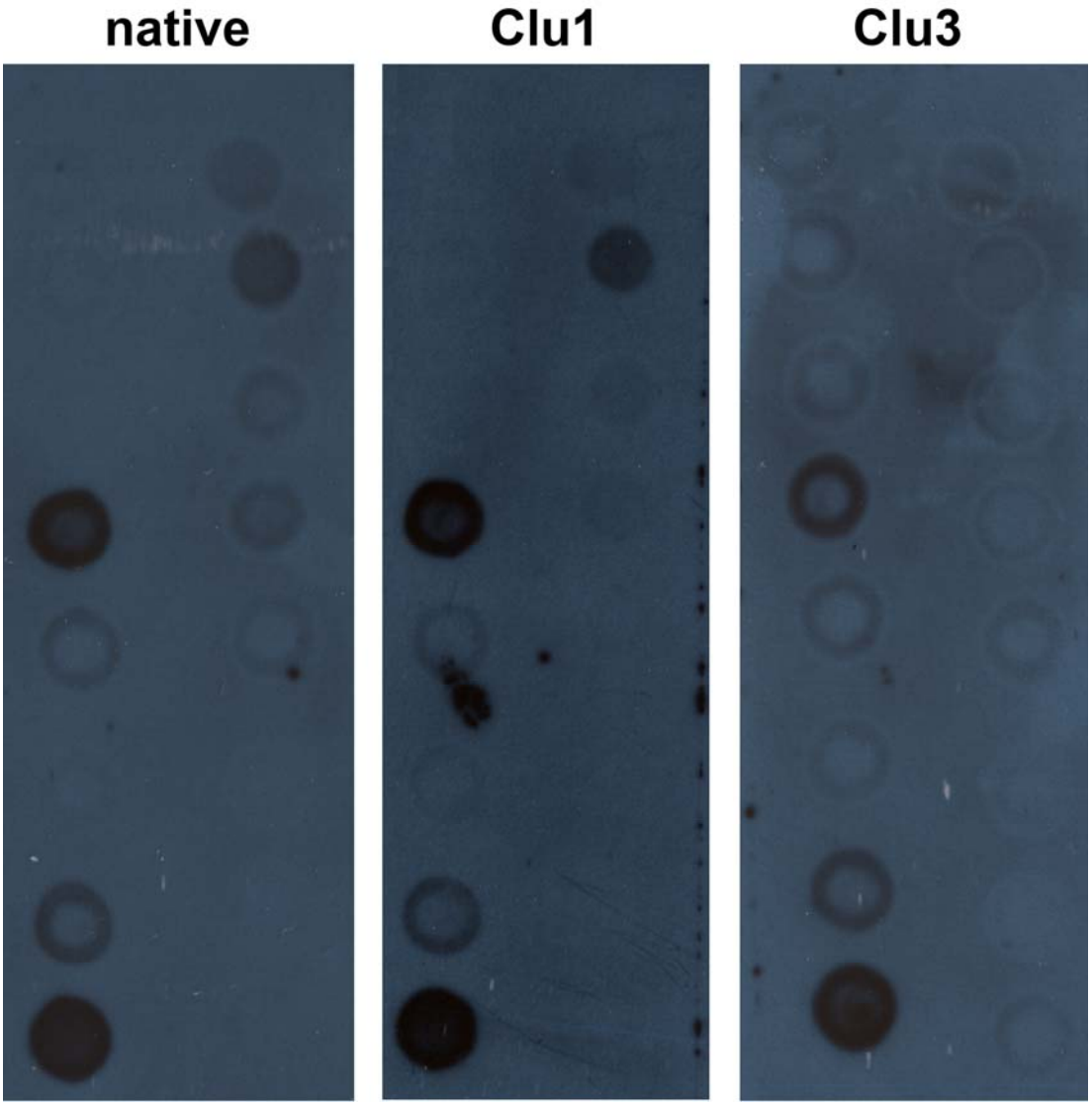


Figure 11) Lipid specificity test of (from left) native OsGAP1 and OsGAP1 mutated in cluster 1 and 3 respectively. Commercial membrane dotted with fifteen different lipid components of cell membrane (Echelon P-6002) (Figure 10) was used.

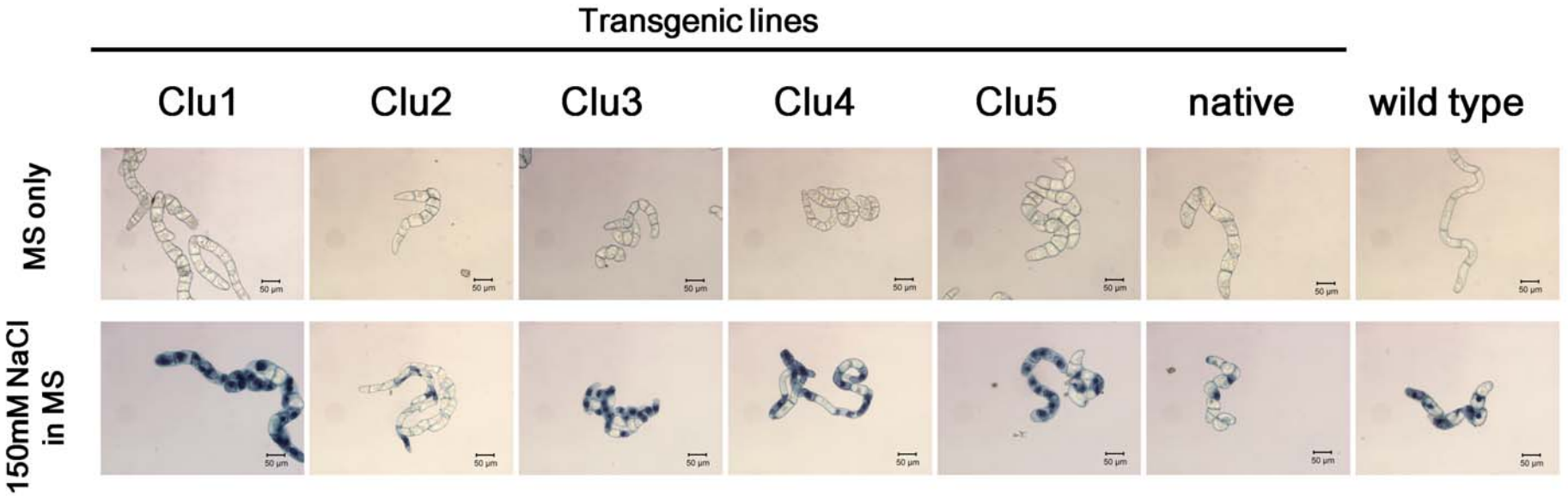


Figure 12) Cell viability test under salt stress of transgenic tobacco BY-2 cell ectopically expressing mutated or native OsGAP1. (from left) Ectopic expresser of OsGAP1 cluster 1-5 mutants (Clu1-5) and native OsGAP1 were treated overnight with MS solution supplemented with 150mM NaCl (below) and MS solution only (above). BY-2 cell was observed under light microscope after staining with 0.4% trypan blue in 1:1 (v:v) ratio for 10 mins. Black bars represent 50 μ m.

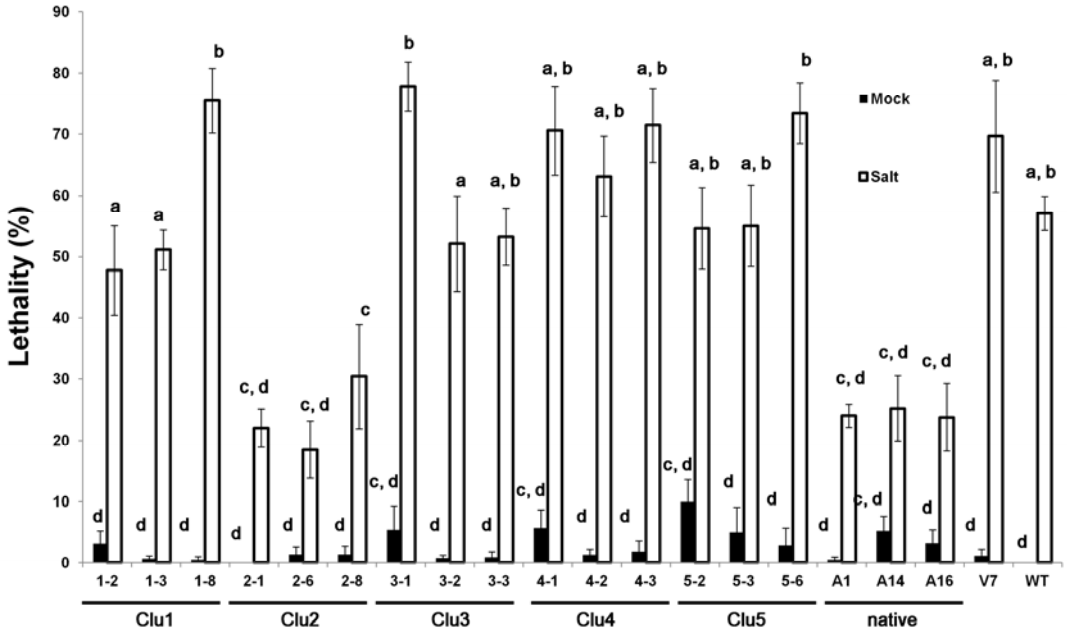


Figure 13) Statistical analysis of results in cell viability test under salt stress of transgenic tobacco BY-2 cell ectopically expressing mutated or native OsGAP1. Three independent cell lines were included for all transgenic cell lines in order to compare with wild type and the cell line transformed with empty vector. At least five microscopic fields were taken and around 100 cells in totally for each line were counted for the statistical analysis of the cell lethality. One-way ANOVA with the Games-Howell posthoc test were done to identify the statistically distinct groups at $p<0.05$ as indicated by the letters on the top on top of the bars.

References

1. Takai Y, K.A., Iwasa Y, Kawahara Y, Mori T, Nishizuka Y, *Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids.* J Biol Chem., 1979. **254**(10): p. 3692-5.
2. Kim CY, K.Y., Jin JB, Moon BC, Kang CH, Kim ST, Park BO, Lee SY, Kim ML, Hwang I, Kang KY, Bahk JD, Lee SY, Cho MJ., *Rice C2-domain proteins are induced and translocated to the plasma membrane in response to a fungal elicitor.* Biochemistry, 2003. **42**(40): p. 11625-33.
3. Yang H, Y.S., Li Y, Hua J., *The Arabidopsis BAP1 and BAP2 genes are general inhibitors of programmed cell death.* Plant Physiol, 2007. **145**(1): p. 135-46.
4. Yang WQ, L.Y., Li MN, Xu WY, Xue YB., *A Novel C2-Domain Phospholipid-Binding Protein, OsPBP1, Is Required for Pollen Fertility in Rice.* Mol Plant., 2008. **1**(5): p. 770-85.
5. de Silva K, L.B., Brown C, Sederoff HW, Khodakovskaya M., *Arabidopsis thaliana calcium-dependent lipid-binding protein (AtCLB): a novel repressor of abiotic stress response.* J Exp Bot, 2011. **62**(8): p. 2679-89.
6. Kang CH, M.B., Park HC, Koo SC, Jeon JM, Cheong YH, Chung WS, Lim CO, Kim JY, Yoon BD, Lee SY, Kim CY., *Rice OsERG3 encodes an unusual small C2-domain protein containing a Ca(2+)-binding module but lacking phospholipid-binding properties.* Biochim Biophys Acta, 2011. **1810**(12): p. 1317-22.
7. Yokotani N, I.T., Kondou Y, Maeda S, Iwabuchi M, Mori M, Hirochika H, Matsui M, Oda K., *Overexpression of a rice gene encoding a small C2 domain protein OsSMCP1 increases tolerance to abiotic and biotic stresses in transgenic Arabidopsis.* Plant Mol Biol., 2009. **71**(4-5): p. 391-402.
8. Cheung MY, Z.N., Tong SW, Li WY, Xue Y, Zhao KJ, Wang C, Zhang Q, Fu Y, Sun Z, Sun SS, Lam HM., *Constitutive expression of a rice GTPase-activating protein induces defense responses.* New Phytologist, 2008. **179**(2): p. 530-45.
9. Cheung MY, X.Y., Zhou L, Li MW, Sun SS, Lam HM., *An ancient P-loop GTPase in rice is regulated by a higher plant-specific regulatory protein.* J Biol Chem., 2010. **285**(48): p. 37359-69.
10. Cheung MY, L.M., Yung YL, Wen CQ, Lam HM., *The unconventional P-loop NTPase OsYchF1 and its regulator OsGAP1 play opposite roles in salinity stress tolerance.* Plant Cell Environ., 2013.
11. Cho W, S.R., *Membrane binding and subcellular targeting of C2 domains.*

Biochim Biophys Acta., 2006. **1761**(8): p. 838-49.

12. Sutton RB, D.B., Berghuis AM, Südhof TC, Sprang SR., *Structure of the first C2 domain of synaptotagmin I: a novel Ca2 phospholipid-binding fold.* Cell, 1995. **80**(6): p. 929-38.
13. Essen LO, P.O., Cheung R, Katan M, Williams RL., *Crystal structure of a mammalian phosphoinositide-specific phospholipase C delta.* Nature, 1996. **380**(6575): p. 595-602.
14. Perisic O, F.S., Lynch DE, Bycroft M, Williams RL., *Crystal structure of a calcium-phospholipid binding domain from cytosolic phospholipase A2.* J Biol Chem. , 1998. **273**(3): p. 1596-604.
15. Shao X, F.I., Südhof TC, Rizo J, *Solution structures of the Ca2+-free and Ca2+-bound C2A domain of synaptotagmin I: does Ca2+ induce a conformational change?* Biochemistry, 1998. **37**(46): p. 16106-15.
16. Sutton RB, S.S., *Structure of the protein kinase C beta phospholipid-binding C2 domain complexed with Ca2+.* Structure, 1998. **6**(11): p. 1395-405.
17. Ochoa WF, G.-G.J., Fita I, Corbalan-Garcia S, Verdaguer N, Gomez-Fernandez JC., *Structure of the C2 domain from novel protein kinase Cepsilon. A membrane binding model for Ca(2+)-independent C2 domains.* J Mol Biol., 2001. **311**(4): p. 837-49.
18. Zhang W, S.Y., Xiong G, Guo Y, Deng L, Li B, Yang J, Qi C., *Crystal structure of human Intersectin-2L C2 domain.* Biochem Biophys Res Commun, 2013. **431**(1): p. 76-80.
19. Guerrero-Valero M, F.-O.C., Querol-Audí J, Marin-Vicente C, Fita I, Gómez-Fernández JC, Verdaguer N, Corbalán-García S., *Structural and mechanistic insights into the association of PKC alpha-C2 domain to PtdIns(4,5)P2.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6603-7.
20. Stevens CF, S.J., *The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission.* Neuron, 2003. **39**(2): p. 299-308.
21. Davletov BA, S.T., *A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding.* J Biol Chem, 1993. **268**(35): p. 26386-90.
22. Sossin WS, S.J., *Ca(2+)-independent protein kinase Cs contain an amino-terminal domain similar to the C2 consensus sequence.* Trends Biochem Sci., 1993. **18**(6): p. 207-8.
23. Osada S, M.K., Saido TC, Suzuki K, Kuroki T, Ohno S, *A new member of the protein kinase C family, nPKC theta, predominantly expressed in*

skeletal muscle. Mol Cell Biol., 1992. **12**(9): p. 3930-8.

24. Zhang D, A.L., *Identification of novel families and classification of the C2 domain superfamily elucidate the origin and evolution of membrane targeting activities in eukaryotes.* Gene, 2010. **469**(1-2): p. 18-30.
25. Bittova L, S.M., Cho W., *A structure-function study of the C2 domain of cytosolic phospholipase A2. Identification of essential calcium ligands and hydrophobic membrane binding residues.* J Biol Chem, 1999. **274**(14): p. 9665-72.
26. Nalefski EA, S.M., Falke JJ., *Ca2+-Signaling Cycle of a Membrane-Docking C2 Domain.* Biochemistry, 1997. **36**(40): p. 12011-8.
27. Kojima T, F.M., Aruga J, Mikoshiba K., *Calcium-dependent phospholipid binding to the C2A domain of a ubiquitous form of double C2 protein (Doc2 beta).* J Biolchem., 1996. **120**(3): p. 671-6.
28. Fukuda M, K.T., Mikoshiba K., *Phospholipid composition dependence of Ca2+-dependent phospholipid binding to the C2A domain of synaptotagmin IV.* J Biol Chem., 1996. **271**(14): p. 8460-4.
29. Stahelin RV, C.W., *Roles of calcium ions in the membrane binding of C2 domains.* Biochem J., 2001. **359**(3): p. 679-85.
30. Medkova M, C.W., *Mutagenesis of the C2 domain of protein kinase C-alpha. Differential roles of Ca2+ ligands and membrane binding residues.* J Biol Chem, 1998. **273**(28): p. 17544-52.
31. Swairjo MA, C.N., Kaetzel MA, Dedman JR, Seaton BA., *Ca(2+)-bridging mechanism and phospholipid head group recognition in the membrane-binding protein annexin V.* Nat Struct Biol. , 1995. **2**(11): p. 968-74.
32. Verdaguer N, C.-G.S., Ochoa WF, Fita I, Gómez-Fernández JC., *Ca(2+) bridges the C2 membrane-binding domain of protein kinase Calpha directly to phosphatidylserine.* EMBO J., 1999. **18**(22): p. 6329-38.
33. Murray D, H.B., *Electrostatic Control of the Membrane Targeting of C2 Domains.* Mol Cell., 2002. **9**(1): p. 145-54.
34. Zhang X, R.J., Südhof TC., *Mechanism of phospholipid binding by the C2A-domain of synaptotagmin I.* Biochemistry., 1998. **37**(36): p. 12395-403.
35. Fukuda M, A.J., Niinobe M, Aimoto S, Mikoshiba K., *Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin II.* J Biol Chem, 1994. **269**(46): p. 29206-11.
36. Schiavo G, G.Q., Prestwich GD, Söllner TH, Rothman JE., *Calcium-dependent switching of the specificity of phosphoinositide*

binding to synaptotagmin. Proc. Natl. Acad. Sci. USA, 1996. **93**: p. 13327-32.

37. Kuo W, H.D., Ellena JF, Cafiso DS., *The calcium-dependent and calcium-independent membrane binding of synaptotagmin 1: two modes of C2B binding.* Journal of Molecular Biology, 2009. **387**(2): p. 284-94.
38. Shao X, L.C., Fernandez I, Zhang X, Südhof TC, Rizo J., *Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch.* Neuron, 1997. **18**(1): p. 133-42.
39. Li C, U.B., Zhang JZ, Anderson RG, Brose N, Südhof TC., *Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins.* Nature., 1995. **375**(6532): p. 594-9.
40. Ochoa WF, C.-G.S., Eritja R, Rodríguez-Alfaro JA, Gómez-Fernández JC, Fita I, Verdaguer N., *Additional Binding Sites for Anionic Phospholipids and Calcium Ions in the Crystal Structures of Complexes of the C2 Domain of Protein Kinase Cα.* Journal of Molecular Biology, 2002. **320**(2): p. 277-291.
41. Ananthanarayanan B, D.S., Rhee SG, Murray D, Cho W., *Membrane targeting of C2 domains of phospholipase C-delta isoforms.* J Biol Chem., 2002. **277**(5): p. 3568-75.
42. Corbalán-García S, J.G.-G.J., Sánchez-Carrillo MS, and Gómez-Fernández JC., *Structural characterization of the C2 domains of classical isozymes of protein kinase C and novel protein kinase Cε by using infrared spectroscopy.* Spectroscopy, 2003. **17**(2-3): p. 399-416.
43. Littler DR, W.J., She YM, Finerty PJ Jr, Newman EM, Dhe-Paganon S., *Structure of human protein kinase C eta (PKCeta) C2 domain and identification of phosphorylation sites.* Biochem Biophys Res Commun., 2006. **349**(41): p. 1182-9.
44. Essen LO, P.O., Lynch DE, Katan M, Williams RL., *A ternary metal binding site in the C2 domain of phosphoinositide-specific phospholipase C-delta1.* Biochemistry, 1997. **36**(10): p. 2753-62.
45. Bazzi MD, N.G., *Protein kinase C interaction with calcium: a phospholipid-dependent process.* Biochemistry, 1990. **29**(33): p. 7624-30.
46. Shao X, D.B., Sutton RB, Südhof TC, Rizo J., *Bipartite Ca2+-binding motif in C2 domains of synaptotagmin and protein kinase C.* Science., 1996. **273**(5272): p. 248-51.
47. Radhakrishnan A, S.A., Jahn R, Fasshauer D., *The Ca2+ affinity of*

synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate. J Biol Chem., 2009. **284**(38): p. 25749-60.

48. Wang T, P.S., Elliott JT, Dowal L, Gupta E, Rebecchi MJ, Scarlata S., *Selective interaction of the C2 domains of phospholipase C-beta1 and -beta2 with activated Galphaq subunits: an alternative function for C2-signaling modules.* Proc Natl Acad Sci USA., 1999. **96**: p. 7843-6.
49. Tsuboi T, F.M., *The C2B domain of rabphilin directly interacts with SNAP-25 and regulates the docking step of dense core vesicle exocytosis in PC12 cells.* J Biol Chem, 2005. **280**(47): p. 39253-9.
50. Lee JD, C.Y., Kao FJ, Kao LS, Lin CC, Lu AC, Shyu BC, Chiou SH, Yang DM., *Detection of the interaction between SNAP25 and rabphilin in neuroendocrine PC12 cells using the FLIM/FRET technique.* Microsc Res Tech, 2008. **71**(1): p. 26-34.
51. Thomas DM, E.L., *Functional Analysis of the C2A Domain of Synaptotagmin 1: Implications for Calcium-Regulated Secretion.* J. Neurosci., 1998. **18**(10): p. 3511-20.
52. Farah CA, S.W., *The role of C2 domains in PKC signaling.* Adv Exp Med Biol, 2012. **740**: p. 663-83.
53. Mellor, H.a.P., Peter J., *The extended protein kinase C superfamily.* 1998.
54. W., C., *Membrane targeting by C1 and C2 domains.* J Biol Chem, 2001. **276**(35): p. 32407-10.
55. Das S, D.J., Cho W., *Membrane-binding and activation mechanism of PTEN.* Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7491-6.
56. Lee JO, Y.H., Georgescu MM, Di Cristofano A, Maehama T, Shi Y, Dixon JE, Pandolfi P, Pavletich NP., *Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association.* Cell., 1999. **99**(3): p. 323-34.
57. Parsons, R., *Human cancer, PTEN and the PI-3 kinase pathway.* Seminars in Cell & Developmental Biology, 2004. **15**(2): p. 171-176.
58. Raftopoulou M, E.-M.S., Self A, Nicholls S, Hall A., *Regulation of cell migration by the C2 domain of the tumor suppressor PTEN.* Science., 2004. **303**(5661): p. 1179-81.
59. Vanhaesebroeck B, L.S., Panayotou G, Waterfield MD., *Phosphoinositide 3-kinases: a conserved family of signal transducers.* Trends Biochem Sci., 1997. **22**(7): p. 267-72.
60. Vanhaesebroeck B, S.L., Hawkins P., *PI3K signalling: the path to discovery and understanding.* Nat Rev Mol Cell Biol, 2012. **13**(3): p.

195-203.

61. Sarró E, T.O., Plana M, Meseguer A, Itarte E., *Phosphoinositide 3-kinase inhibitors protect mouse kidney cells from cyclosporine-induced cell death.* Kidney International, 2008. **73**(1): p. 77-85.
62. Alvi F, I.-B.J., Baldys A, Raymond JR, Hannun YA., *Regulation of membrane trafficking and endocytosis by protein kinase C: emerging role of the pericentrion, a novel protein kinase C-dependent subset of recycling endosomes.* Cell Mol Life Sci., 2007. **64**(3): p. 263-70.
63. DeBlasio SL, L.D., Hangarter RP., *A Plant-Specific Protein Essential for Blue-Light-Induced Chloroplast Movements.* Plant Physiol., 2005. **139**(1): p. 101-14.
64. Blume JJ, H.A., Behrendt D, Paulsson M, Plomann M., *EHD proteins are associated with tubular and vesicular compartments and interact with specific phospholipids.* Exp Cell Res. , 2007. **313**(2): p. 219-31.
65. Rui Y, X.Z., Xiong B, Cao Y, Lin S, Zhang M, Chan SC, Luo W, Han Y, Lu Z, Ye Z, Zhou HM, Han J, Meng A, Lin SC., *A beta-catenin-independent dorsalization pathway activated by Axin/JNK signaling and antagonized by aida.* Dev Cell, 2007. **13**(2): p. 268-82.
66. Bialas NJ, I.P., Li C, Robinson JF, Parker JD, Healey MP, Davis EE, Inglis CD, Toivonen T, Cottell DC, Blacque OE, Quarmby LM, Katsanis N, Leroux MR., *Functional interactions between the ciliopathy-associated Meckel syndrome 1 (MKS1) protein and two novel MKS1-related (MKSR) proteins.* J Cell Sci., 2009. **122**(5): p. 611-24.
67. Kyttälä M, T.J., Salonen R, Kopra O, Kohlschmidt N, Paavola-Sakki P, Peltonen L, Kestilä M., *MKS1, encoding a component of the flagellar apparatus basal body proteome, is mutated in Meckel syndrome.* Nat Genet,, 2006. **38**(2): p. 155-7.
68. Williams CL, W.M., Schafer JC, Michaud EJ, Yoder BK., *Functional redundancy of the B9 proteins and nephrocystins in Caenorhabditis elegans ciliogenesis.* Mol Biol Cell., 2008. **19**(5): p. 2154-68.
69. Premkumar L, B.A., Patel M, Jaroszewski L, Bankston LA, Stec B, Vuori K, Côté JF, Liddington RC., *Structural basis of membranetargeting by the Dock180 family of Rho-family guanine exchange factors (RhoGEFs).* J Biol Chem., 2010. **285**(17): p. 13211-22.
70. Côté JF, V.K., *Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity.* J Cell Sci., 2002. **115**(24): p. 4901-13.
71. Gumienny TL, B.E., Tosello-Trampont AC, Kinchen JM, Haney LB,

Nishiwaki K, Walk SF, Nemergut ME, Macara IG, Francis R, Schedl T, Qin Y, Van Aelst L, Hengartner MO, Ravichandran KS., *CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration.* Cell., 2001. **107**(1): p. 27-41.

72. Yang H, L.Y., Hua J., *The C2 domain protein BAP1 negatively regulates defense responses in Arabidopsis.* Plant J., 2006. **48**(2): p. 238-48.
73. Kang CH, M.B., Park HC, Koo SC, Chi YH, Cheong YH, Yoon BD, Lee SY, Kim CY., *Rice small C2-domain proteins are phosphorylated by calcium-dependent protein kinase.* Mol Cells, 2013.
74. Wang X, L.Q., Niu X, Chen H, Xu L, Qi C., *Characterization of a canola C2 domain gene that interacts with PG, an effector of the necrotrophic fungus Sclerotinia sclerotiorum.* J. Exp. Bot., 2009. **60**(9): p. 2613-20.
75. Chen YC, C.Y., *A conserved GTPase YchF of Vibrio vulnificus is involved in macrophage cytotoxicity, iron acquisition, and mouse virulence.* Int J Med Microbiol., 2011. **301**(6): p. 469-74.
76. Danese I, H.V., Delrue RM, Tibor A, Lestrate P, Stevaux O, Mertens P, Paquet JY, Godfroid J, De Bolle X, Letesson JJ., *The Ton system, an ABC transporter, and a universally conserved GTPase are involved in iron utilization by Brucella melitensis 16M.* Infect Immun., 2004. **72**(10): p. 5783-90.
77. Dassain M, L.A., Colosetti L, Carolé S, Bouché JP., *A new essential gene of the 'minimal genome' affecting cell division.* Biochimie., 1999. **81**(8-9): p. 889-95.
78. Gradia DF, R.K., Umaki AC, de Souza FS, Probst CM, Correa A, Holetz FB, Avila AR, Krieger MA, Goldenberg S, Fragoso SP., *Characterization of a novel Obg-like ATPase in the protozoan Trypanosoma cruzi.* Int J Parasitol., 2009. **39**(1): p. 49-58.
79. Godon C, L.G., Lee J, Buhler JM, Kieffer S, Perrot M, Boucherie H, Toledano MB, Labarre J., *The H2O2 stimulon in Saccharomyces cerevisiae.* J Biol Chem., 1998. **273**(35): p. 22480-9.
80. Koller-Eichhorn R, M.T., Gail R, Wittinghofer A, Kostrewa D, Kutay U, Kambach C., *Human OLA1 defines an ATPase subfamily in the Obg family of GTP-binding proteins.* J Biol Chem. , 2007. **282**(27): p. 19928-19937.
81. Zhang, J., et al., *OLA1, an Obg-like ATPase, suppresses antioxidant response via nontranscriptional mechanisms.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(36): p. 15356-15361.

82. Gibrat JF, M.T., Bryant SH., *Surprising similarities in structure comparison.* Curr Opin Struct Biol., 1996. **6**(3): p. 377-85.
83. Ho SN, H.H., Horton RM, Pullen JK, Pease LR., *Site-directed mutagenesis by overlap extension using the polymerase chain reaction.* Gene., 1989. **77**(1): p. 51-9.
84. Kapust RB, W.D., *Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused.* Protein Sci., 1999. **8**(8): p. 1668-74.
85. G, A., *High efficiency transformation of cultured tobacco cells.* Plant Physiol., 1985. **79**(2): p. 568-70.
86. Bechtold N, P.G., *In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration.* Methods Mol Biol. 1998;82:259-66., 1998. **82**: p. 259-66.
87. Koncz C, S.J., *The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector.* Mol. Gen. Genet., 1986. **204**(3): p. 383-96.
88. Corrêa DHA, R.C., *The use of circular dichroism spectroscopy to study protein folding, form and function.* Afr. J. Biochem. Res., 2009. **3**(5): p. 164-173.
89. Karnaukhova E, S.K., Crouch RK., *Circular dichroism and cross-linking studies of bacteriorhodopsin mutants.* Amino Acids, 2006. **30**(1): p. 17-23.
90. Kumar LV, R.T., Rao CM., *Structural and functional consequences of the mutation of a conserved arginine residue in alphaA and alphaB crystallins.* J Biol Chem., 1999. **274**(34): p. 24137-41.
91. Kelly SM, P.N., *The use of circular dichroism in the investigation of protein structure and function.* Curr Protein Pept Sci., 2000. **1**(4): p. 349-84.
92. Kleinschmidt JH, M.J., Thomas DD, Marsh D., *Interaction of bee venom melittin with zwitterionic and negatively charged phospholipid bilayers: a spin-label electron spin resonance study.* Biophys J., 1997. **72**(2): p. 767-78.
93. Houtkooper RH, V.F., *Cardiolipin, the heart of mitochondrial metabolism.* Cell. Mol. Life Sci., 2008. **65**(2493-506).
94. M., S., *Cardiolipin synthesis for the assembly of bacterial and mitochondrial membrane.* J Lipid Res., 2008. **49**(8): p. 1607-20.
95. Frentzen M, G.R., *Biosynthesis of Cardiolipin in Plant Mitochondria.* Plant Physiol., 1994. **106**(4): p. 1527-32.

96. Bostock RM, K.J., Laine RA., *Eicosapentaenoic and Arachidonic Acids from Phytophthora infestans Elicit Fungitoxic Sesquiterpenes in the Potato.* Science., 1981. **212**(4490): p. 67-69.
97. Ghomashchi F, N.G., Bollinger JG, Aloulou A, Lehr M, Leslie CC, Gelb MH., *Interfacial kinetic and binding properties of mammalian group IVB phospholipase A2 (cPLA2beta) and comparison with the other cPLA2 isoforms.* J Biol Chem, 2010. **285**(46): p. 36100-11.
98. Benes CH, W.N., Elia AE, Dharia T, Cantley LC, Soltoff SP., *The C2 domain of PKCdelta is a phosphotyrosine binding domain.* Cell., 2005. **121**(2): p. 271-80.
99. Mosior M, M.S., *Electrostatics and reduction of dimensionality produce apparent cooperativity when basic peptides bind to acidic lipids in membranes.* Biochim Biophys Acta., 1992. **1105**(1): p. 185-7.

Appendix I Chemicals

Ampicillin	USB 11259
Agarose	Invitrogen 16520-050
Bacto-Agar	Difco 214010
Bacto-peptone	BPDS 211677
Bromophenol blue	Sigma B0128
Calcium chloride	Merck 2380
Copper (II) sulphate	Sigma C1297
Ethanol (absolute)	Merck 100986
Ethidium bromide	Sigma E7637
Gelrite gellan gum	Sigma G1910
Gentamicin sulfate	Sigma G3632
Hydrochloric acid (36%)	Ajax 1364
Kanamycin, monosulfate	USB 17924
LB Broth, Ready-Made Powder	USB 75852
b-mercaptoethanol	Sigma M6250
Manganese chloride	Sigma M9272
MES	USB 18886
Methanol	Merck 6007
Murashige & Skoog salt mixture	Caisson C032
Phenol-chloroform-isoamylalcohol (25:24:1)	Amersco 883
Potassium dihydrogen orthophosphate	RdH 04248
PVDF membrane	Bio-Rad 1620184
Rifampicin	lL 3511858
SDS	Bio-Rad 161-0302
sodium acetate	Sigma S2889
Sodium chloride	RdH 31434
Sodium molybdate	RdH 31439
Streptomycin sulfate	Sigma S6501
Sucrose	USB 75825
Tris-HCl	Amresco 0826
Trypan blue solution (0.4%)	Sigma T8154
trytone	BDH 211705
yeast extract power	USB 23547

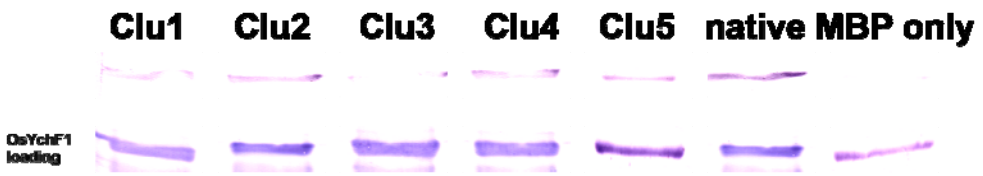
Appendix II Formulations of buffer, medium and solution

Buffer/ medium/ solution	Formulation
buffer for CD spectrometery	10mM maltose, 0.3M NaCl, 50mM NaOAc, pH 6.0
MS agar plate for Arabidopsis	4.3g/ L Murashige & Shoog salt mixture (GibcoBRL), 3% sucrose, 0.05% MES, 0.9% bacto-agar (pH 5.7) with vitamin B5
MS agar plate for BY-2 cells	4.3g/L Murashige & Skoog Basal salt mixture (Sigma), 3% sucrose, 1.8mM KH_2PO_4, 3% gelrite gellan gum (pH 5.8) with vitamin B5
MS medium for BY-2 cell suspension culture	4.3g/L Murashige & Skoog Basal salt mixture (GibcoBRL), 3% sucrose, 1.8mM KH_2PO_4 (pH 5.0) with vitamin B5
YEP medium for Agrobacterium	10g/L trytone, 10g/L yeast extract, 5g/L NaCl
LB agar plate for E. coli	20g/L LB Broth powder, 1% bacto-agar
TBS buffer	10 mM Tris, 150 mM NaCl, pH 8.0
vitamin B5 (1000X)	70mM boric acid, 14mM MnCl2, 5mM CuSO4, 0.2mM NaMoO4, 10mM NaCl, and 0.01mM CuCl2

Appendix III Equipments and facilities

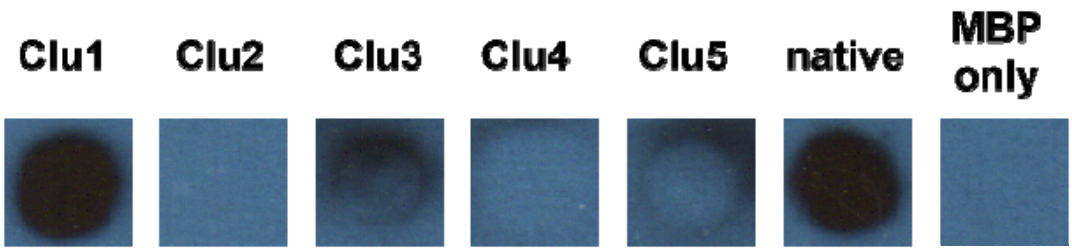
Biological Safety Cabinet	Baker SG600E 59419
Centrifuge J2-MI	Beckman T373 with JA-14 rotor
Growth chamber	Percival AR-32L 3859-05-971
Light microscope	Nikon Eclipse 80i
Orbital shaker	Lab line 4628-1
Programmable Thermal Controller	MJ Research PTC100 96VHB 200003879
Refrigerated Centrifuge 5810R	Eppendorf 03463
TELCO incubator	Cole-Parmer 39352-02
Mini-PROTEAN® Tetra Cell with Mini Trans-Blot®Module	BIO-RAD 165-8064
Circular dichroism Spectrometer	JASCO J-815

Appendix IV Result of biological repeat of co-immunoprecipitation to show interaction between OsYchF1 and MBP fusion protein of OsGAP1 mutants (Clu1-5) respectively



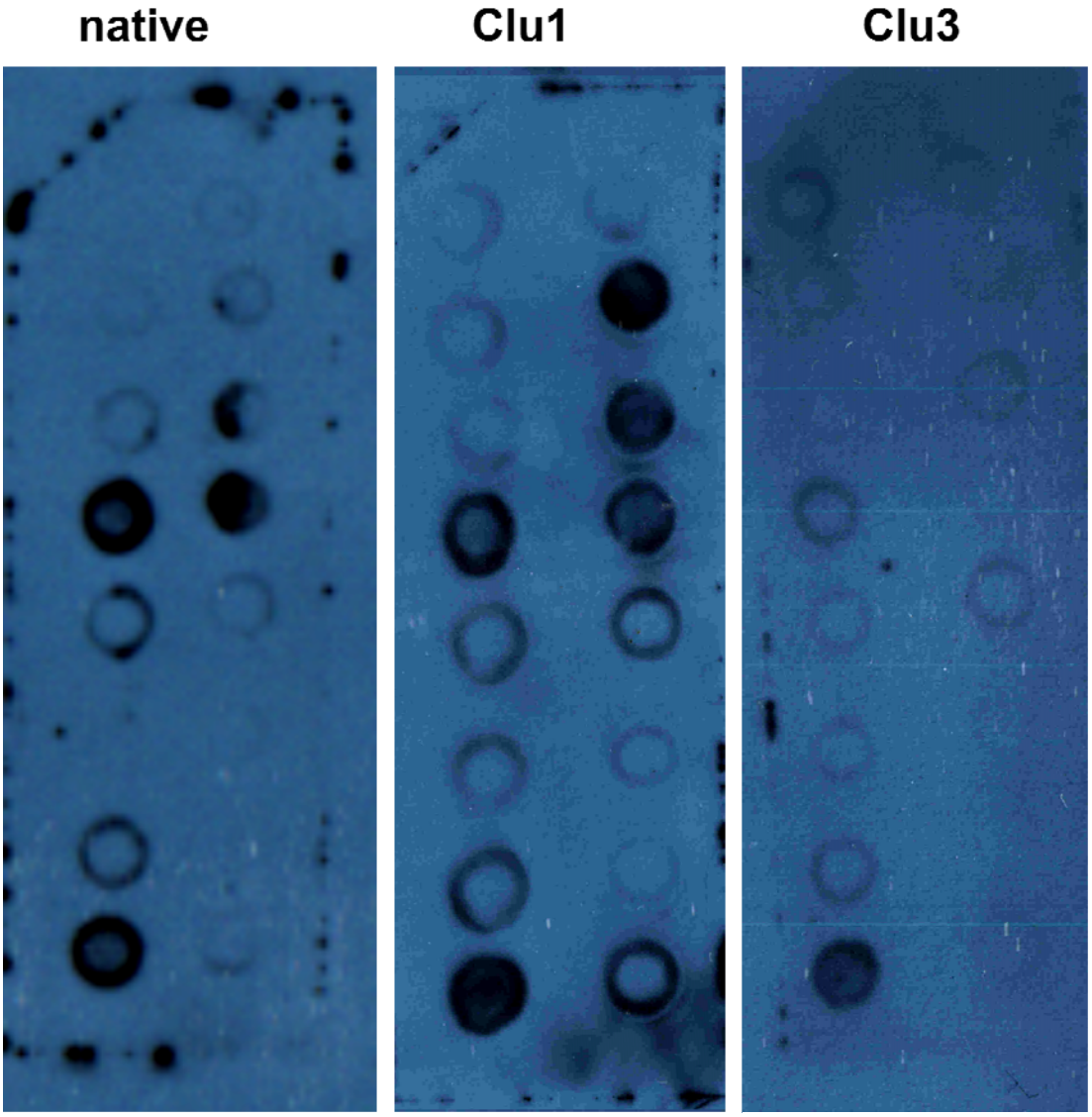
(above) Mutants at cluster 2, 4 and 5 still retained the strong interaction with OsYchF1 while the other two lost the interaction. Native OsGAP1 and MBP protein was used as positive and negative control respectively. (below) Western blotting against OsYchF1 was performed to show the even loading of OsYchF1.

Appendix V Result of biological repeat of phospholipid dot blot assay with MBP fusion protein of OsGAP1 mutants (Clu1-5).



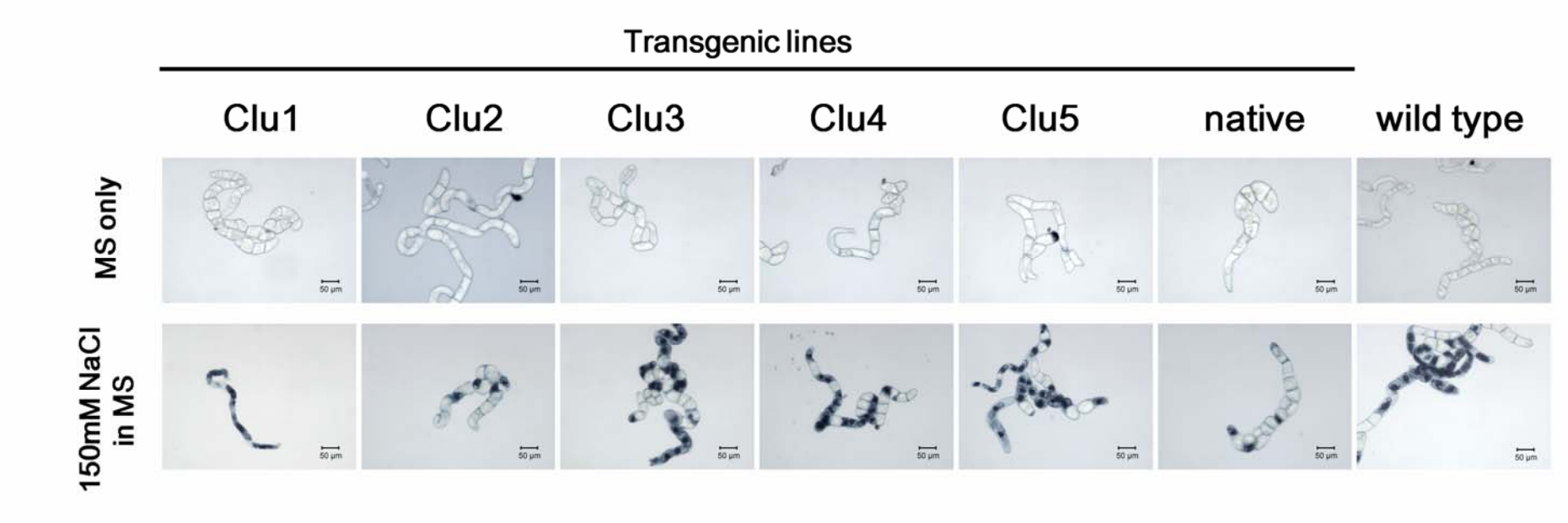
Native OsGAP1 and MBP protein was used as positive and negative control respectively. The lipid binding ability of OsGAP1 with mutation in cluster 1 was similar to that of the native protein. Mutant of cluster 3 still interacted to lipid but the extent of binding was reduced. Other mutants lost their binding to phospholipids.

Appendix VI Result of biological repeat of lipid specificity test of (from left) native OsGAP1 and OsGAP1 mutated in cluster 1 and 3 respectively.



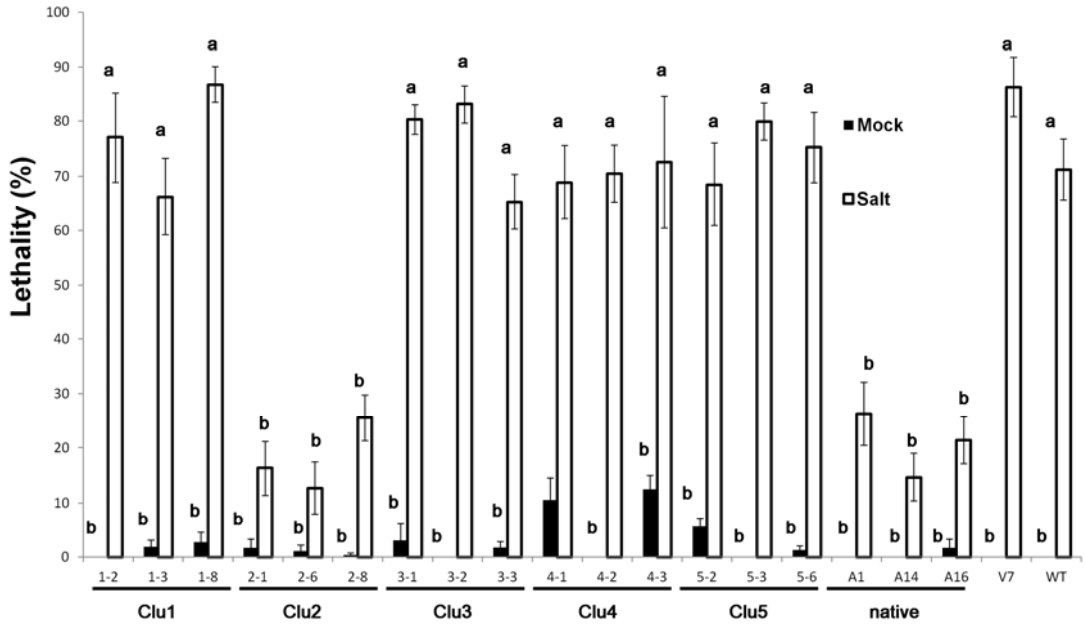
All of the proteins showed strong binding towards cardiolipin, phosphatidylserine and phosphatidylinositol 4-phosphate. Weaker association was also shown in phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol polyphosphates, phosphatidic acid and cholesterol.

Appendix VII Result of biological repeat of cell viability test under salt stress of transgenic tobacco BY-2 cell ectopically expressing mutated or native OsGAP1.



(from left) Ectopic expresser of OsGAP1 cluster 1-5 mutants (Clu1-5) and native OsGAP1 were treated overnight with MS solution supplemented with 150mM NaCl (below) and MS solution only (above). BY-2 cell was observed under light microscope after staining with 0.4% trypan blue in 1:1 (v:v) ratio for 10 mins.

Appendix VIII Result of biological repeat of statistical analysis of results in cell viability test under salt stress of transgenic tobacco BY-2 cell ectopically expressing mutated or native OsGAP1.



Three independent cell lines were included for all transgenic cell lines in order to compare with wild type and the cell line transformed with empty vector. At least five microscopic fields were taken and around 100 cells in totally for each line were counted for the statistical analysis of the cell lethality. One-way ANOVA with the Games-Howell posthoc test were done to identify the statistically distinct groups at $p<0.05$ as indicated by the letters on the top on top of the bars.