Crystal structure of an *Arabidopsis* BON1 providing insights into the copine protein family

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Short title: The crystal structure of BON1

Keywords

Copines, BON1, C2 domain, vWA domain, X-ray structure, Phospholipid, Membrane.

Significance Statement

BON1 is a calcium-dependent membrane-binding protein which plays an important role in the inhibition of programmed cell death and other cellular defense responses in plant. Here we report the first structure from the copine family and present the biochemical properties of BON1, which will help the rational design of compounds to treat plant infection and disease.

Summary

The *Arabidopsis thaliana* *BON1* gene product is a member of the evolutionary conserved eukaryotic calcium-dependent membrane-binding protein family. The copine protein is composed of two C2 domains (C2A and C2B) followed by a vWA domain. The BON1 protein is localized on the plasma membrane and is known to suppress the expression of immune receptor genes and to positively regulate stomatal closure. The first structure of this protein family has been determined to 2.5 Å resolution and shows the structural features of the three conserved domains C2A, C2B and vWA. The structure reveals the third Ca^{2+} -binding region in C2A domain is longer than classical C2 domains and a novel Ca^{2+} binding site in the vWA domain. The structure of BON1 bound to Mn^{2+} is also presented. The binding of the C2 domains to phospholipid (PSF) has been modelled and provides an insight into the lipid binding mechanism of the copine proteins. Furthermore, the selectivity of the separate C2A and C2B domains and intact BON1 to bind to different phospholipids has been investigated and we demonstrated that BON1 could mediate aggregation of liposomes in response to Ca^{2+} . These studies have formed the basis of further investigations into the important role that the copine proteins play *in vivo*.

Introduction

Copines are a group of ubiquitous eukaryotic calcium-dependent phospholipid-binding proteins, which were originally identified in *Paramecium tetraurelia* (Creutz *et al.*, 1998). The French name copine (friend) reflects the properties of these proteins that bind to lipid membranes as "companions" (Creutz *et al.*, 1998). Genome analysis indicates that eukaryotic organisms often have multiple genes for copine proteins that are highly conserved, suggesting that they play a critical and diverse biological role in cellular activities (Creutz *et al.*, 1998).

In plant copines have been demonstrated to play central roles in controlling cell growth and development. Silencing the *copine* gene increases the ability of wheat to resist powdery mildew (Zou *et al.*, 2018). The *copine* genes of *Oryza sativa*, *OsBON1* and *OsBON3*, act as negative regulators by inhibiting broad-spectrum disease resistance (Yin *et al.*, 2018). The *Arabidopsis thaliana* genome contains three *copine* genes, *BON1*, *BON2* and *BON3*. The BON1 protein acts alongside BON2 and BON3 in inhibiting programmed cell death and other cellular defense responses (Yang *et al.*, 2006b, Lee and McNellis, 2009). BON1 is a negative regulator of the receptor NLR gene *SNCI* (Suppressor of npr1-1, constitutive 1) and its loss-of-function mutant has an enhanced disease resistance and consequently a temperature dependent growth defect (Hua *et al.*, 2001, Jambunathan *et al.*, 2001). In addition, BON1 can promote abscisic acid- and pathogen-triggered stomatal closure, and this function is independent of its role in *SNCI* regulation (Clement *et al.*, 2011, Gou *et al.*, 2015). Using yeast two-hybrid screen, it was shown that both the full length BON1 and the vWA domain of BON1 interact with two homologous calcium-binding proteins BAP1 (BON association protein 1) and BAP2, and these two proteins also play a role in the regulation of immunity (Hua *et al.*, 2001, Yang *et al.*, 2006a). Moreover, yeast two-hybrid screen have demonstrated that BON1 can interact with BIR1 (BAK1-interacting receptor like kinase 1), which contribute to a negative regulation of defense responses (Wang *et al.*, 2011).

In mammals, copines localized in different tissues, including brain, lung, heart,

intestine, and liver (Tomsig and Creutz, 2000). Previous studies on copines in mammals showed that the majority of *copine* genes play a fundamental role in tumorigenesis and tumor migration, and suggest that they are potential drug targets for a range of different cancers including leukemia, osteosarcoma, esophageal squamous cell carcinoma and NSCLC (Sakthivel and Prabhu, 2017, Umeda *et al.*, 2018, Sun *et al.*, 2019).

All copines contain two C2 domains (C2A and C2B) at the amino end of the protein followed by a von Willebrand factor A (vWA) domain at the carboxyl end (Creutz *et al.*, 1998). The C2 domain, named after a second conserved domain in protein kinase C (PKC) (Parker *et al.*, 1986), is thought to be involved in Ca^{2+} -dependent phospholipid binding. Proteins containing C2 domains are abundant in eukaryotic cells. These include the proteins phospholipase C (Lars-Oliver Essen, 1996), rabphilin (Shirataki *et al.*, 1993), synaptotagmin (Grushin *et al.*, 2019) and Munc13 (Brose *et al.*, 1995), which are involved in cell signal transduction, membrane trafficking and GTPase activation (Nalefski and Falke, 1996, Rizo and Südhof, 1998). Interestingly, the number of copies of the C2 domain may be related to the biological function of the protein since many signaling C2 domain proteins have a single copy of the C2 domain, while many other membrane trafficking proteins have multiple C2 domains (Cho and Stahelin, 2006). Additionally, the C2 domains are typically combined with other domains, such as enzymatic or protein interaction domains, and contribute to the regulation of their function by Ca^{2+} and/or lipid binding. Many other proteins present in archaea, bacteria, and eukaryotes contain a vWA domain and are involved in cell adhesion and membrane transport (Whittaker and Hynes, 2002). Although most of the vWA domains are found in extracellular proteins, such as integrin α and β subunits, the majority of ancient vWA-containing proteins are intracellular proteins involved in transcription and DNA repair. Even though the roles of the vWA domains in these extracellular /intracellular proteins are not identical, the vWA domains seem to be involved in formation of multiprotein complexes and have the ability to bind divalent cations and go through

conformational changes (Tuckwell, 1999, Whittaker and Hynes, 2002). The vast majority of vWA domains have an important metal-ion-dependent adhesion site (MIDAS) (Lee *et al.*, 1995) which consists of three non-contiguous and highly conserved amino acid motifs (D-x-S/T-x-S. . . . T. . . . D, x represents any amino acid) and plays a central role in ligand binding (Whittaker and Hynes, 2002).

Copines were the first intracellular proteins to be reported to contain a vWA domain (Creutz *et al.*, 1998). It has been suggested that the C2 domains respond to the increase of intracellular calcium concentration, and direct copines to the plasma membrane (Hua *et al.*, 2001). The copines can combine with target proteins or multiprotein complexes through their vWA domain (Liu *et al.*, 2005). This would allow copines to have roles in different signaling pathways, depending on their sensitivity to calcium ions and the specificity for lipids and other target proteins.

Copines have been extensively studied from different eukaryotes, however their biological functions and detailed molecular mechanisms are still poorly understood. Current structural knowledge of copines has been limited to homology modelling based on the C2 domain or vWA domain of other protein families. Therefore, it is important to determine the structural characteristics of a full-length copine.

Here, we report the crystal structure of BON1 from *A. thaliana*, which is the first representative of a crystal structure from the copine family which has provided a prototype for in-depth investigation and characterization of other copine family members.

Results

Overall structure of copines

From the 20 family members of different eukaryotic copine proteins that were cloned and over-expressed many could not be obtained in a soluble form for crystallographic studies. A plant *A. thaliana* copine BON1 was however successfully crystallised. Optimal crystallization conditions are shown in Table S1. This plant copine shares 35% sequence identity with the copine CPNA (*Dictyostelium*), 36%

identity with GEM (*Caenorhabditis elegans*), 59% identity with OsBON1 (*Oryza sativa*) and 50% identity to copine-I (*Homo sapiens*). Comparison of the available copine amino acid sequences shows that the maximum degree of conservation is observed in residues characteristic of the C2 domains and the vWA domain (Figure S1). The structure BON1 (residues 30-572) was solved by molecular replacement at 2.50 Å resolution and refined to R_{work} of 0.227 and R_{free} of 0.266 with good geometry. The data collection and refinement statistics are given in Table S2. An asymmetric unit of the crystal contains eight molecules (BON1A-BON1H) and the subtle differences in conformation of each of the monomers was observed which could be due to crystal packing (the rmsd between different monomers is ranging from 0.25 to 0.62 Å) (Figure S2). Interestingly, within the same asymmetric unit, eight BON1 monomers bind Ca^{2+} in a different manner. The vWA domain of BON1D and BON1F molecules bind Ca^{2+} , which was not added to crystallization buffer, while the other molecules do not show Ca^{2+} binding (the binding of Ca^{2+} to these monomers is confirmed by the anomalous difference map shown in Figure S2). The BON1A molecule fits into the electron density better than other BON1 monomers and has a smaller contact area with other molecules. The BON1A 3D structure is shown in Figure 1A. The model includes the C2A domain (residues 48-188), the C2B domain (residues 194-326) and the vWA domain (residues 337-570) (Figure 1B). The BON1 overall structure contains twenty-three β -strands ($\beta\text{a1}-\beta\text{a8}$, $\beta\text{b1}-\beta\text{b8}$, $\beta\text{c1}-\beta\text{c7}$) with an accessory small two-stranded β -sheet and eleven α -helices (α1 , α2 , αa1 , αb1 , αb2 , $\alpha\text{c1}-\alpha\text{c6}$) as well as four 3_{10} helices ($\eta\text{1}-\eta\text{4}$) (Figure 1C). These secondary structure elements form the skeleton of the structure, while flexible loops are exposed on the protein surface. The overall fold of the structure adopts a butterfly shape, with the C2A and C2B domains resembling the ‘wings’. Two C2 domains fold into two individual structural modules and are connected by a 3_{10} helix (η3). The vWA domain forms the ‘tail’ of the butterfly and is connected to the C2B domains by a short helix (α2) that encompasses seven residues. The N- and C-termini of each domain, as well as the linkers between the domains are located in the center of the structure, forming

the ‘head’ and ‘abdomen’ of the butterfly. These regions play a central role in supporting the overall structural integrity and stabilizing the arrangement of the domains. Especially the linker ($\alpha 2$) plays a critical role in the folding of the protein, acting to stabilize the interaction between the C2 and vWA domains.

The structure of the BON1 C2A and C2B domains

The C2 superfamily includes two distinct families, the synaptotagmin-like variants also known as the type I topology and the PLC-like variants also referred to as the type II topology (Rizo and Südhof, 1998). The topology of C2 domains is not a vital factor for their function. Both the C2A and the C2B domains adopt the type II topology. The C2A and C2B domains are symmetrically distributed on both sides of the 3_{10} helix ($\eta 3$) and have a similar overall fold, consisting of a β -sandwich core formed by a pair of four-stranded anti-parallel β -sheets (Figure 1A). The compact β -sandwich has two sides, one side is composed of strands $\beta a3\backslash\beta b3$, $\beta a2\backslash\beta b2$, $\beta a5\backslash\beta b5$ and $\beta a6\backslash\beta b6$ that form a concave surface, and the other side contains strands $\beta a4\backslash\beta b4$, $\beta a1\backslash\beta b1$, $\beta a7\backslash\beta b7$ and $\beta a8\backslash\beta b8$ which form a convex shape. In addition, they contain three Ca^{2+} -binding regions (CBRs: CBRa1-a3, CBRb1-b3) located on the top and a conserved α -helix ($\alpha a1$, $\alpha b2$) at the bottom (Figure 2). The conserved helical part ($\alpha a1$ or $\alpha b2$) inserts vertically into the groove formed by the inter-strand regions between the $\beta a6\backslash\beta b6$ and $\beta a7\backslash\beta b7$ strands to “block” the bottom of the β -sandwich, thereby leaving only one active pocket at the top available for interactions with the membrane.

Although two C2 domains of BON1 share a similar structure, they differ by a rmsd of 2.8 Å, throughout the 123 equivalent α -carbon atoms (Figure 2B). The sequence similarity of the C2 family proteins is low, however the aspartate residues involved in Ca^{2+} regulation are strictly conserved in the family (Figure 2C). Analysis of the structures of other C2 domains in complex with Ca^{2+} and phospholipids has identified that there are two separate phospholipid binding regions, the Ca^{2+} -binding regions (LBR1) and the cationic concave face of the β -sandwich (LBR2) (Figure 2D). This suggests that these proteins could have the ability to reversibly interact with membranes.

A notable feature of the BON1 C2A domain is that the CBRA3 contains fifteen residues, making this region of the protein longer than the equivalent region of other known C2 domains. This extension results in the rearrangement of the structure into two 3_{10} helices (η_1 and η_2). Similarly to previously reported C2A domain of Piccolo (Garcia *et al.*, 2004, Corbalan-Garcia and Gomez-Fernandez, 2014), BON1 CBRA3 is positioned further away from the Ca^{2+} -dependent phospholipid binding region due to a sequence insertion. Therefore, even though the BON1 C2A domain retains the conserved aspartate residues (D61, D69, D122, D124) (Figure 2C, 2E), the different conformation of CBRA3 may affect the binding of Ca^{2+} to the BON1 C2A domain. Sequence alignment reveals that the long loop CBRA3 might not be conserved among other members of the copine family. For example, in *Dictyostelium*, the CBRA3 of the CPNA consists of only three residues, and the CBRA3 of human copine I is comprised of seven residues (Figure S1).

The structural differences between the C2B domain of BON1 and other C2 domains also involve insertions and deletions in flexible regions of the protein. These differences include the presence of a short α -helical turn (αb_1) between strands βb_6 - βb_7 of the C2B domain, and a protruding β -hairpin, composed of strands βb_7 and βb_8 , which may be involved in interaction with the membrane. Sequence comparisons suggest that a similar β -hairpin exists in C2B domains of other members of the copine family, however, no such β -hairpin is found in the BON1 C2A domain or in other classical C2 domains.

The BON1 vWA domain structure

The vWA domain adopts a characteristic α/β sandwich Rossmann fold, with a doubly wound, open, twisted β -sheet flanked by α -helices (Figure 3). It contains a six stranded β -sheet (βc_1 - βc_6) surrounded by six α -helices (αc_1 - αc_6), which are arranged alternately. The additional 3_{10} helix (η_4) links the first β - α segment which is the most conserved region of the Rossmann fold (Hanukoglu, 2015). Unlike the classical Rossmann fold, however, which consists of six parallel β -strands, the BON1 vWA is of mixed type. In addition, the vWA domain has two clefts, one of which is occupied

by the α -helix ($\alpha 2$), which separates the vWA domain and C2 domains. Another cleft is located at the bottom of the BON1 structure and contains long flexible inter-strand regions and the highly conserved MIDAS to facilitate ligand binding. Surprisingly, in the asymmetric unit, no divalent metal ions were detected in the MIDAS of the eight molecules, while far away from the MIDAS, strong globular electron densities were observed in BON1D and BON1F (Figure 3B). However, there were no metal ions other than Na^+ in the crystalline solution, so according to the average distance from the metal ion to coordinating ligands (2.42 Å) and backbone oxygen coordination, we have assigned Ca^{2+} at this position, which may be a trace contaminant coming with the polyethylene glycol (PEG) used for crystallization or from the water. The coordination mode of the Ca^{2+} sites are bidentate by the carboxyl oxygens of D388, monodentate by the hydroxyl oxygen of D390, backbone carbonyl coordination by R392 and by hydroxyl oxygen coordination by one water molecule (Figure 3B). By comparison with the structure of the Ca^{2+} free monomer, the BON1D monomer, had a conformational change upon Ca^{2+} binding, with the $\beta c2$ shortened and the $\beta c3$ absent (Figure 3C). In addition, when a sequence comparison was made between 16 typical copines from plants, animals and protozoa (Figure 3D), it showed that the two key aspartic acid residues that are important for Ca^{2+} binding are conserved in plant and animal copines, except for OsBON1. This would suggest that the new Ca^{2+} binding site observed in BON1 may be conserved in the vast majority of higher eukaryotic copines, but not in all members of the copine family.

Mn^{2+} binding to MIDAS motif of BON1

Similar to other members of the vWA domain, the vWA domain of BON1 has a MIDAS motif located at the bottom of the overall structure ((D347-x-T349-x-S351. . . T445. . . D477) (Figure 4C). To further study the structural features of this MIDAS motif for binding different divalent cations, the crystals were soaked in different buffers containing Ca^{2+} , Mg^{2+} or Mn^{2+} , and we have solved the BON1- Mn^{2+} crystal structure (Table S2). Similar to the BON1 crystals, the asymmetric unit contains eight molecules, which have slightly different conformations. In the BON1- Mn^{2+} structure,

strong globular electron densities were observed in every MIDAS site of the eight molecules in the asymmetric unit, and intriguingly, electron densities were also found around the newly identified Ca^{2+} binding sites (Figure S3). The coordination mode of the Mn^{2+} at the MIDAS sites are monodentate by the carboxyl oxygens of D347 and monodentate by the hydroxyl oxygen of T349, S351, T445 and D447 (Figure 4A). The average distance of the bound Mn^{2+} to the protein is 2.04 Å. Furthermore, by comparing the ion-free-BON1 structure with the BON1- Mn^{2+} structure (Figure 4B), the binding of Mn^{2+} with the MIDAS motif does not induce conformational changes in the BON1 vWA domain.

Modeling of C2 domains/PSF Complex Structure

It has been suggested that copines could be recruited to specific cellular membranes and regulate many important cellular events in eukaryotes via the Ca^{2+} -dependent lipid binding region (LBR1) in the C2 domain's CBRs (Li et al., 2010). In order to further understand the membrane docking mechanism of copines we have used molecular modeling to simulate a potential phospholipid binding model for copines based on the structures of C2A and C2B (Figure S5A, S5B). These modeling results have been verified by subsequent biochemical experiments.

In the computational simulation, the phospholipid 1,2-dihexanoyl-sn-glycer-3-phospho-L-serine (PSF) and Ca^{2+} were docked into the LBR1 of the C2A and C2B domains (Figure S4C, S4D). In the LBR1, Ca^{2+} provided a bridge between the C2 domains (C2A:D69, D122, D124 or C2B:D209, D215, D269, D271) and PSF, and the phosphate group of PSF occupied the incomplete coordination sites of C2-bound Ca^{2+} (Figure S4C, S4D). By comparison with the structure of C2 domains, the complex structures showed no significant change in conformation after binding PSF. As expected, the LBRb1 has a much higher affinity for PSF than the LBRa1 (Table S3).

Lipid selectivity and Ca^{2+} sensitivity of BON1 and two C2 Domains

To study whether the BON1 and its two C2 domains are capable of forming Ca^{2+} -dependent complexes with liposomes, two standard methods for verifying protein-phospholipid interactions were used: the vesicle-pelleting assay (Cho *et al.*,

2001) and the protein lipid overlay assay (Yu and Lemmon, 2001) (Figure 5). Additionally, on the basis of the BON1 structure and the generated docking models, we designed the following mutations to verify the Ca^{2+} -dependent properties of the C2A and C2B components. The D69, D122 and D124 located in the CBRa1 and CBRa3 were mutated to Ala (referred to as C2A-DA₃) and the double substitution of D215 and D269 located in CBRb1 and CBRb3 mutated to Ala (referred to as C2B-DA₂).

We firstly investigated the lipid selectivity of BON1, and two BON1 C2 domains and mutant proteins by analyzing their ability to bind different vesicles in 100 μM Ca^{2+} . Analysis of the pellets by dot-blot and by SDS-PAGE revealed they all bound phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylinositol (PI), but not phosphatidylcholine (PC) and phosphatidyl ethanolamine (PE) (Figure 5A 5C). The selectivity of BON1 and the two C2 fragments for phospholipids varied according to $\text{PG} > \text{PS} > \text{PI}$. As a control, the vWA fragment did not bind any lipids in the protein-lipid overlay assays (Figure 5A). In figure 5B, quantification of the intensity of the dot in the protein-lipid overlay assays revealed that the phospholipid binding activity of BON1 was significantly higher than that of the individual C2A and C2B components ($p < 0.05$)

Next, we studied the Ca^{2+} sensitivity of BON1, two C2 domains, and mutant proteins by analyzing their ability to bind to PG-vesicles (75% PC: 25% PG) at different concentrations of Ca^{2+} . In the vesicle-pelleting assay, analysis of the pellets by SDS-PAGE revealed that in the presence of EGTA, the C2A component was more strongly bound to PG-vesicles than the C2B component and BON1, and their binding ability increased with the increase of Ca^{2+} concentration. (Figure 5D). Remarkably, in the absence of Ca^{2+} , these proteins stayed mostly in the supernatant with a small fraction of them co-precipitating with the PG-vesicle, and in relation to the C2B fragment and BON1, the lipid pellet contained more of the C2A component. In addition, quantification of the protein content in the supernatant found that the Ca^{2+} sensitivity of BON1 was higher than that of its C2A and C2B components (Figure 5E).

In the protein-lipid overlay assays, the C2A, C2B components and BON1 bound to vesicles in 100 μM Ca^{2+} , and reduced their binding activity for the PG-vesicles in the absence of Ca^{2+} (Figure 5A). The vesicle-pelleting assay and protein-lipid overlay assays revealed that C2A, C2B, and BON1 are all partially bound to phospholipids in the absence of Ca^{2+} , especially the C2A component. In the presence of Ca^{2+} , the binding ability of C2A to phospholipids was slightly increased. That was in contrast to the binding ability of C2B and BON1 to phospholipids which was significantly enhanced ($p < 0.05$). Additionally, we observed a reduction of the Ca^{2+} -dependent phospholipid binding ability in the mutant proteins. (Figure 5A, 5D). Together, these results suggest that the BON1 and its two C2 domains are Ca^{2+} -dependent phospholipid binding proteins with a basic membrane binding capacity and that they have a preference to bind negatively charged liposomes over neutral liposomes.

Together, these results suggest that the BON1 and its two C2 domains are Ca^{2+} -dependent phospholipid binding proteins with a basic membrane binding capacity and that they have a preference to bind negatively charged liposomes over neutral liposomes.

BON1 mediates aggregation of liposomes in response to Ca^{2+}

To investigate the mechanism by which BON1 mediates vesicle aggregation, clustering of liposomes by BON1 were routinely monitored using turbidity measurements (Ohki S, 1982, Hui *et al.*, 2011) or dynamic light scattering (DLS) (Arac *et al.*, 2006) (Figure 6). We firstly used the turbidity measurement method to characterize the aggregation of BON1 mediated PG-vesicles by monitoring changes in turbidity over time after sequential addition of BON1, Ca^{2+} and excess EGTA (Figure 6A, black line). We found that BON1 did not affect the vesicles prior to the addition of Ca^{2+} , but induced rapid liposome aggregation with the increase of the Ca^{2+} concentration to a certain value (10-100 μM Ca^{2+}). The aggregation could be rapidly reversed by addition of excess EGTA (Figure 6A, black line). Notably, BON1 alone, in the absence of PG-vesicles, underwent Ca^{2+} -dependent aggregation at concentrations $\geq 100 \mu\text{M}$, and the aggregation did not reverse when excess of EGTA

was added (Figure 6A, red line). Therefore, in the presence of both vesicles and BON1, the increased turbidity was only partially reversed by addition of excess EGTA showing that the residual increase in turbidity is may be due to BON1 oligomerization (Figure 6A). In addition, we explored the vesicle aggregation activities of the C2A, C2B and C2AB components. Surprisingly, neither C2A, C2B nor the tandem C2 domain (C2AB) caused liposome aggregation even at 10 mM Ca^{2+} (Figure 6B, 6C). Remarkably, the tandem C2 domain did not cluster vesicles in the absence or presence of Ca^{2+} , but an increase in turbidity was observed at 10 mM Ca^{2+} , which appears to be caused by C2AB oligomerization (Figure 6B). Thus, these experiments show that the aggregation of BON1 mediated vesicles is strictly dependent on Ca^{2+} .

Next, we also verified the results by DLS. The PG-vesicles showed a relatively narrow size distribution (Figure 6D) with an average diameter ranging from 70 nm to 100 nm (Table S4). Notably, we found no significant change in particle size when we added BON1 (Figure 6E), but addition of BON1 and 100 μM Ca^{2+} resulted in a large increase in particle size (average radius > 900 nm) (Figure 6F). This increase was due to specific Ca^{2+} -dependent binding of the BON1 to the vesicles, as for the BON1 alone, the C2A, C2B, vWA or C2AB components the presence of 1 mM Ca^{2+} , or the BON1 in the presence of 1 mM Mg^{2+} , did not affect the particle size (Table S4). Subsequent addition of 2 mM EGTA returned the particle sizes to their original distribution (Figure 6G), showing that the size increase is reversible and arises from vesicle clustering rather than fusion.

Finally, we used negative stain transmission electron microscopy (TEM) to observe how BON1 interacts with the vesicles. Liposomes were incubated with BON1 in the absence or presence of Ca^{2+} and these preparations were analyzed by TEM (Figure 7). In the absence of Ca^{2+} , neither BON1 self-aggregation nor significant interaction of BON1 with the membrane was observed (Figure 7B). In addition, BON1 produced a roughness on the surface of the vesicles, indicating that BON1 still had weak membrane-binding activity in the absence of Ca^{2+} , which was consistent with the vesicle-pelleting assay and protein lipid overlay assay results presented

above. In the presence of Ca^{2+} , BON1 forms large Ca^{2+} -dependent linear formations on the membranes through interactions with the membrane and between individual BON1 molecules (Figure 7C, 7D). These linear formations can be envisioned as being similar to a cylinder, 90 nm high, 30 nm long, 30 nm wide, surrounded by clustered vesicles. This also indicates that vesicle aggregation is not caused by binding of a single BON1 molecule but by binding of an oligomeric state of BON1 (Figure 7C, 7D). However, when the Ca^{2+} ions were chelated by EGTA, the linear elements were still retained, but there were no vesicles around them (Figure 7F). These observed phenomena were consistent with the results of the DLS and the turbidity measurements, in that BON1-mediated vesicle aggregation is strictly Ca^{2+} dependent and can be readily reversed by the addition of excess EGTA. However, the aggregated BON1 did not revert to its monomeric form. Interestingly, the TEM experiments also showed that as the Ca^{2+} concentration increased to 1 mM, BON1 could still interact with the vesicles, but large aggregates of BON1 were formed around the vesicles (Figure 7E).

Additionally, we analyzed the influence of Ca^{2+} , Mg^{2+} and Mn^{2+} on the aggregation state of BON1 through DLS (Table S4). The particle size of the molecules of BON1 changed discontinuously with the increase of the concentration of the different divalent ions. At low concentrations of Ca^{2+} or Mn^{2+} ($\leq 100 \mu\text{M}$), there was no significant effect on the particle size of the BON1 samples (average diameter: 8-11nm) (Figure S5A). At high concentrations of the divalent ions the average diameter of the samples increases to 120-150 nm (Figure S5B). When the concentration reaches 1mM, the average diameter increased to over 1000 nm (Figure S5C), and the protein aggregation could not be reversed by the addition of excess EGTA (Figure S5D). However, Mg^{2+} had no significant effect on the change in particle size (Table S4). In addition, the BON1 vWA domain was found to be the main factor causing protein oligomerization. In the presence of 1 mM Ca^{2+} , the hydrodynamic diameter of the C2A, C2B and C2AB components did not change, but the particle size of vWA domain increased significantly, with the diameter of particles

increasing from 3 to 65 nm (Table S4). Unlike the irreversible BON1 oligomerization caused by Ca^{2+} , the change in particle size of the vWA samples was reversible. The aggregated vWA fragments returned to the monomeric form upon chelation of the Ca^{2+} ions with EGTA (Table S4).

Discussion

Copines contain a variable length N-terminal sequence which is required for plant copines plasma membrane localization (Li *et al.*, 2010).. Studies have shown that the second glycine residue which can potentially be myristoylated to confer tight membrane association of the protein (Guy A. Thompson, 2000). Indeed, BON1 is associated with the plasma membrane, and mutating the putative myristoylation residue glycine 2 abolishes its plasma membrane localization (Li *et al.*, 2010). The glycine 2 residue is found in all copines of green plants but not in any other human copines or copines from *C. elegans* or *D. discoideum* (Zou *et al.*, 2016). Therefore glycine 2 is a plant-specific feature, and it is possible that the tight association of plant copines with the plasma membrane can facilitate fast signaling and regulation at plasma membrane. Although the crystal structures did not contain the disordered N-terminal region, it is speculated from the 3D structures that the region is just like the ‘tentacles’ of the butterfly, sensing the changes in the intracellular microenvironment and helping BON1 to complete its localization.

C2 domains are unique among peripheral membrane proteins since they have a wide range of selectivity for the major phospholipids of the biological membrane (Nalefski *et al.*, 2001, Coudeville *et al.*, 2008, Diaz *et al.*, 2016, Brown *et al.*, 2018). They reversibly attach to phospholipids by forming a Ca^{2+} bridge between the acidic residues located on CBRs and phosphate groups of lipids. In the present study, we have compared the Ca^{2+} sensitivity of the C2A, C2B domains and BON1 and the selectivity of the phospholipids. There is no difference in the phospholipid selectivity between the two C2 domains, which is the same as that of BON1. Similar to human copine I and copine VI (Creutz *et al.*, 1998, Nakayama *et al.*, 1999a, Tomsig and

Creutz, 2000), BON1 and its two C2 domains prefer to bind to negatively charged phospholipids rather than to neutral phospholipids, which is consistent with the mechanism of membrane binding of C2 domains in other proteins such as PKC (Ochoa *et al.*, 2001) and synaptotagmin (Sutton *et al.*, 1999).

Although C2A, C2B domains and BON1 have the same phospholipid selectivity, their sensitivity for Ca^{2+} and phospholipid are different. The 3D-structural characteristics, biochemical experiments, and molecular simulations indicate that BON1 has a higher sensitivity for Ca^{2+} and phospholipids than the individual C2 domains, and the vWA domain does not interact with phospholipids, which indicates that the C2A and C2B may have cumulative effects. Nevertheless, increasing evidence has indicated that multiple C2 domains function more cooperatively than additively (Earles *et al.*, 2001, Bai *et al.*, 2002). However, Ca^{2+} may cause BON1 to partially oligomerize and precipitate which would cause errors in the results, so whether the C2A and C2B have synergistic effects will require further experiments and to extend these to other members of the copine family.

Studies have shown that the CBRs of the Ca^{2+} -dependent canonical C2 domains are typically involved in the coordination of multiple Ca^{2+} ions (Rizo and Südhof, 1998, Ochoa *et al.*, 2002, Shin *et al.*, 2010, Diaz *et al.*, 2016). The BON1 C2 domains are also involved in Ca^{2+} -dependent phospholipid binding, but no metal ions were bound to the CBRs in any of the BON1 crystal structures reported here. Further, even in the C2B crystal structures, no Ca^{2+} ions were observed with or without crystal soaking with Ca^{2+} or phospholipids (Table S2, Figure S6). Accordingly, we analyzed the possible reasons for the low affinity of the BON1 C2 domains for Ca^{2+} . Firstly, the long CBRa3 in the C2A domain results in a low affinity for Ca^{2+} and the CBRs of C2 domains contain a set of altered conserved acidic amino acids. For example, in the BON1 C2B domain, the residue 271 is Asn, while in human copines, the equivalent residues are all Asp (Figure 2C). Therefore, Asn instead of an Asp residue impedes the bipartite coordination with Ca^{2+} , resulting in a low affinity of the C2B domain for Ca^{2+} . Based on the computational simulation and the primary sequence of the C2

domains, we hypothesized that the C2A and C2B domains may bind only one Ca^{2+} ion, since compared with the classical C2 family members, they have lost the key aspartic acid residues (Q141, S277) that bind the second Ca^{2+} (Figure 2C). Conversely, in human copines (I-IX), the key aspartic acid residues are retained at the corresponding positions, and it is speculated that their CBRs are typically involved in the coordination of multiple Ca^{2+} . The two C2 domains of the copine family members contain an unequal number of highly conserved aspartate residues that are required for the copine's calcium regulatory function.

The mutational, vesicle-pelleting and protein lipid overlay data suggest that BON1 is a Ca^{2+} -dependent phospholipid binding protein with basic membrane binding capacity, which is consistent with previous studies showing that copines (Tomsig and Creutz, 2000, Perestenko *et al.*, 2010, Ilacqua *et al.*, 2018) and some multiple C2 domain proteins (Damer and Creutz, 1994) exhibit both Ca^{2+} -dependent and Ca^{2+} -independent binding to membranes. From the docking data, it was observed that the interactions between the proteins and phospholipids are established by the involvement of multiple amino acid residues. In addition to aspartic acid residues, additional residues in the CBRs also contribute to Ca^{2+} coordination, since the mutation of two or three aspartate residues only caused a decrease in interactions between the proteins and lipids, rather than the total disruption of the interaction. From the present data, electrostatic and hydrophobic interactions may contribute to the interaction between the BON1 C2 domains and the membrane. Indeed, accumulating evidence has previously indicated that even without well-defined conserved phospholipid binding sites, the C2 proteins may still share two separate phospholipid binding regions (LBR1, LBR2) (Verdaguer *et al.*, 1999, Ochoa *et al.*, 2001, Cho and Stahelin, 2006, Guillen *et al.*, 2013, Diaz *et al.*, 2016). Therefore, by using molecular docking, we have also obtained a computational model structure of LBRa2/LBRb2 and PSF complex, and found that the PSF binds weakly to the LBR2 of the C2A and C2B domains (Figure S7, Table S3). In addition, the morphology and composition of the vesicles also affects the binding of proteins to vesicles. A related

study demonstrated that for the hydra annexin, a Ca^{2+} -dependent phospholipid binding protein, binds to vesicles of high curvature in the absence of Ca^{2+} (Fischer *et al.*, 2007). All these results provide potential explanations for the weak binding of liposomes and BON1 or C2 domains in the absence of Ca^{2+} . Moreover, from the available data, we provide a possible model for the BON1 interaction with the membrane. When the Ca^{2+} concentration increases, LBR1- Ca^{2+} - negatively charged phospholipids form a ternary complex, and the LBR2 interact electrostatically and hydrophobically with the membrane, and some flexible loop may be inserted into the phospholipid bilayer (Figure S8).

BON1 promotes aggregation of lipid vesicles *in vitro*, and has been shown to achieve temperature homeostasis *in vivo* by increasing the fusion of vesicles with the membrane or associating with the plasma membrane to maintain membrane function at low temperature (Hua *et al.*, 2001, Jambunathan *et al.*, 2001). In other organisms, copines may function similarly to accelerate vesicle trafficking in response to specific environmental signals (Nakayama *et al.*, 1999a, Nakayama *et al.*, 1999b, Damer *et al.*, 2005, Damer *et al.*, 2007, Smith *et al.*, 2010, Ilacqua *et al.*, 2018). While the role of copines as a calcium-dependent membrane-binding protein has become generally accepted, the mechanism by which copines regulate aggregation of liposomes in response to Ca^{2+} remains poorly understood. By using the information obtained from the crystal structures, biochemical experiments and electron microscope results reported in this paper, we propose the potential mechanism of vesicle aggregation. BON1 respond to the increase of Ca^{2+} and direct the BON1 to the vesicles. At the same time, the BON1 monomer may form oligomers through its vWA domain, leading to vesicles aggregation (Figure S9). However, the aggregation mechanism of vesicles remains further research.

The vWA domain at the C terminus is responsible for interacting with the target proteins or multiprotein complexes through the regulation of divalent metal ions (Tuckwell, 1999, Whittaker and Hynes, 2002, Liu *et al.*, 2005). These targets include calcium-binding proteins, regulators of protein phosphorylation, transcription or

ubiquitination which suggests copines have roles in a wide variety of cellular processes (Whittaker and Hynes, 2002). In plants, yeast two-hybrid screens identified many target proteins interacting with BON1, such as BAP1 and BAP2 (Yang *et al.*, 2007). We were unable to co-crystallise the complex of BON1 and its target proteins so far.

Atomic force microscopy (AFM) results showed that human copine I formed linear structures approximately 45nm long on specific regions of the lipid bilayers (Creutz and Edwardson, 2009). However, the DLS experiment and TEM results show that BON1 formed linear structures with a length of about 90nm in the presence of Ca^{2+} ions. The differences in these results may be due to the limitations of the measurement methods and/or instruments, such as the interference of the finite geometry of the AFM probe tip to the image. On the other hand, it may be due to the differences in the biochemical properties of copine I and BON1. In addition, studies have shown that the vWA domain of BON1 seems to be involved in formation of multiprotein complexes (Liu *et al.*, 2005, Li *et al.*, 2010), so the formation of linear structures depends on the binding of the vWA domain of one BON1 to a section of another BON1 molecule. However, it is not clear whether BON1 also assembles into these linear structures when it binds to its target proteins. In this case, binding of the target protein to the vWA domain may make it impossible for BON1 to self-associate. Therefore, it will be important to determine the structural characteristics of complexes formed by BON1 and its target proteins when bound together with membranes.

As previously known, the binding of divalent cations to MIDAS is necessary for the vWA domain to coordinate ligand binding (Qu and Leahy, 1996, Luo *et al.*, 2007). In this study, we have not only determined the BON1 crystal structures of MIDAS combined with Mn^{2+} , but more importantly, we have found a new Ca^{2+} binding site away from the MIDAS. The protein conformation of BON1 changes slightly when Ca^{2+} binds to the new Ca^{2+} binding site when compared to the structure of BON1 without bound Ca^{2+} . We speculate that the new Ca^{2+} binding site may be involved in the transition between open and closed conformations of the vWA domain (Leitinger

et al., 2000, Chen *et al.*, 2003, Mould *et al.*, 2003). In addition, the vWA domain of BON1 has a weak sequence similarity to protein kinases and contains conserved kinase motifs including the ATP-binding glycine loop (A350-G353) and a putative catalytic lysine (K391). These motifs are highly conserved in copine family of proteins (Li *et al.*, 2010, Zou *et al.*, 2016). Human copine III has been shown to be a phosphoprotein with associated kinase activity (Caudell *et al.*, 2000). A previous study has shown that not all copines possess kinase activity, but all may possess ATP binding activity (Zou *et al.*, 2016). Therefore, based on the crystal structure of BON1, we have simulated a possible structure model of the interaction between ATP and BON1 with or without Mg^{2+} , and speculate on the potential critical residues (Figure S10, Table S5).

BON1 is a calcium-dependent membrane-binding protein which plays an important role in the inhibition of programmed cell death and other cellular defense responses in plant. Our studies resolve the three-dimensional crystal structure of BON1 from *A. thaliana*, which is the first representative of a crystal structure of the copine family protein, providing a high-resolution atomic architecture for in-depth investigation and characterization of other copine family members. Furthermore, we have demonstrated using *in vitro* studies that a ternary complex between BON1, Ca^{2+} and lipids is important for biological roles of BON1. These results provide a foundation for elucidating the mechanisms and biochemical function of copine family proteins.

Experimental procedures

Protein expression, purification and crystallization

The genes encoding BON1 (residues 30–572), C2A domain (residues 48-188), C2B domain (residues 194-326), C2AB domain (residues 48-326) and vWA domain (residues 337-570) were amplified using PCR from the complementary DNA (cDNAs) of *A. thaliana*. The amplified products were cloned into the modified pET-32a vector (Novagen) with a N-terminal 6His tag followed by a TEV protease cleavage site for the thioredoxin tag removal. The recombinant proteins were over-expressed in *E. coli* BL21 (DE3) grown in LB-medium supplemented with 50 μ g/ml ampicillin and the

protein induced by addition of IPTG (0.3 mM) for 12–16 h at 16°C. The cells were harvested by centrifugation and lysed by ultrasonication in a buffer containing 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.2% Tween 20, 2 mM β -mercaptoethanol and 5% glycerol. Proteins were purified on Ni-NTA affinity resin (GE Healthcare) followed by overnight TEV cleavage at 4 °C to remove the thioredoxin tag and N-terminal histidine tag. The protein was further purified by size-exclusion chromatography on a Superdex 200 HR 16/60 column (GE Healthcare) which was pre-equilibrated in a buffer containing 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% glycerol, and 2 mM DTT. The purified protein eluted predominantly as a monomer. The purified proteins were concentrated to about 10 mg/ml for crystallization. All protein crystals were produced by sitting drop vapor diffusion at 285K with 1 μ l protein mixed with 1 μ l of the crystallization solution. Optimal crystallization conditions are shown in Table S1. Crystals were flash-frozen in liquid nitrogen with an addition of 15%-25% glycerol in the crystallization mother liquid as a cryoprotectant.

Data collection and structure solution

All data were collected at 100 K at the Shanghai Synchrotron Radiation Facility. The native BON1 data were collected on beamline 17U1. The 360 degrees of data were collected with a rotational increment of 0.5°. The crystals were indexed in the space group $P2_1$ with unit cell parameters $a = 122.3$, $b = 118.7$, $c = 222.3$ Å, $\beta = 90.20^\circ$. Since the monoclinic angle is close to 90°, some crystals were non-merohedrally twinned and sometimes indexed in the wrong orthorhombic space group. The 360° of data for C2B domain crystals soaked in a range of Ca^{2+} concentrations (Table S1) were collected on beamline 19U1 with 1-degree increment. The 360 degrees of data of the Mn^{2+} soaks of native BON1 crystals were collected with 1-degree increment on beamline 17U1. All data were processed by XDS (Kabsch, 2010). The resolution cutoffs for all data sets were chosen where $\text{CC}1/2$ (Karplus and Diederichs, 2012) approaches 0.3, since weak anisotropic data were shown to be valuable for density modification (Sporny *et al.*, 2016).

The BON1 native structure was solved by the molecular replacement method (MR) using the structure the vWA domain of the *A. thaliana* RING domain ligase1 (PDB code 6K8B) as a model (34.5 % sequence identity). Eight copies of the search model were positioned by PHASER (McCoy *et al.*, 2007) and the structure was expanded using the AUTOBUILD option of PHENIX (Terwilliger *et al.*, 2008) (Adams *et al.*, 2010). The resulting partial model contained fragments of the N-terminal helix and both C2 domains and was refined to R_{free} of 0.377 at 2.9 Å resolution. Subsequent calculations were conducted using CCP4 software (Winn *et al.*, 2011). The refined partial model of each domain from the most complete BON1 monomer was positioned by conventional MR in MOLREP (Vagin and Teplyakov, 2010) and phased MR in MOLREP (Vagin and Isupov, 2001). Masks and NCS operators were calculated separately for the C2A, C2B, vWA domains and N-terminal helix. NCS averaging was conducted using DM (Cowtan, 2010). The density modification phases were used as an input for phased refinement (Pannu *et al.*, 1998) in REFMAC5 (Murshudov *et al.*, 2011). The model was manually rebuilt using COOT (Emsley *et al.*, 2010) and refined to R_{free} below 27 % at 2.5 Å resolution. The Mn^{2+} soaked model was refined using REFMAC5 and rebuilt using COOT and the Mn^{2+} soak was refined using the twin refinement option. The C2B structure was solved by MR using MOLREP and a model of the C2B domain of the native BON1 structure. The structure was refined in REFMAC5 and rebuilt in COOT.

Molecular docking and molecular dynamic (MD) simulations

Molecular dockings were performed to predict the binding mode between C2 domain protein and PSF as well as BON1 and ATP using AutoDock 4.2 (Morris *et al.*, 2009) and Glide module in Schrödinger 2017 (Morris *et al.*, 1998), respectively. The representatives with relatively low energy were chosen to be starting structures for MD simulations.

All the simulations were performed using the Gromacs-2018.3 (Pronk *et al.*, 2013) package. The starting structures obtained from molecular docking were first energy-minimized by applying steepest descent and followed by the conjugated

gradient method. Next, the minimized structures were heated from 0K to 300K and then equilibrated at 1 atm in an isochoric/isothermal (NPT) ensemble with periodic boundary conditions. A production run was carried out on each of the equilibrated system. Temperature and pressure controls were achieved by the Nosé-Hoover thermostat (Hoover, 1985) and the Berendsen barostat (Berendsen *et al.*, 1984) with a frequency of 2.0 ps, respectively. The LINCS algorithm (Hess, 2008) was applied on bond lengths during all of the MD runs.

Binding free energy calculation

The binding free energy of the complexes (ΔG_{bind}) were estimated using the Molecular Mechanics Poisson- Boltzmann Surface Area (MM-PBSA) method (Hayes *et al.*, 2011, Genheden and Ryde, 2015) using a series of snapshots from the stable MD trajectory of each system. Since the binding conformational entropy ΔS_{bind} is computationally expensive and of low accuracy, we neglect the effect of entropy contributions.

Liposomes

Synthetic liposomes were prepared by extrusion as described (Diaz *et al.*, 2016). Lipids were mixed and desiccated in a non-oxidizing atmosphere, then hydrated in HEPES buffer (50 mM HEPES pH 7.5, 100 mM NaCl) to a final concentration of 2 mg/ml. The sample was sonicated in a water bath for 20 min at 4°C. The liposomes were then extruded 30 times through 0.22 μ m filters. The PS-vesicle composition was 25% DPPS (1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine) and 75% DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine). The PG-vesicle composition was 25% DPPG (1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol)) and 75% DPPC. The PI-vesicle composition was 25% PI (L-A-phosphatidylinositol) and 75% PC. The PC-vesicle composition was 100% DPPC. The PE-vesicle composition was 100% DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine).

Vesicle-pelleting assays

Proteins (0.5 mg/ml) were mixed with the liposomes (1 mg/ml) in the presence of Ca^{2+} or EGTA, and then centrifuged at $12,000 \times g$ at 4°C for 30 min in a Hitachi

ultracentrifuge. The supernatant and/or pellet fractions from each sample were collected in equal volumes of buffer and subjected to 15% SDS-PAGE. Gels were stained with coomassie brilliant blue. To determine the percentage of protein bound/unbound to the liposomes, we measured the absorbance of the soluble fraction at 280 nm after pelleting the lipid and the Boltzmann equation was used to fit the data obtained

Protein lipid overlay assays

Lipids of the desired species were dissolved in TBS buffer and 2.5 µg of each lipid were spotted onto nitrocellulose membranes and dried in a desiccator for 1 h at 37°C. The membranes were blocked with 3% (w/v) non-fat dry milk in TBS-T (10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.2% (v/v) Tween 20 for 1 h at room temperature. The membranes were washed in TBS-T, followed by incubation with 2 µg/ml of purified His-proteins in TBS-T with either 100 µM CaCl₂ or 5 mM EGTA for 1 h at room temperature. Next, the membranes were incubated with the anti-His antibody (1:1,000) for 1 h and with the secondary anti-mouse IgG-HRP (1:5,000) for 1 h at room temperature. The chemiluminescent signal was detected using TMB. The bound protein was then analyzed using the Amersham Imager 600. All images were corrected for the background using the in-built ImageQuant TL, smoothed and the intensity of the dot was quantified using ImageQuant TL. The monoclonal antibodies were purchased from Beijing Solarbio Science & Technology Co., Ltd.

Liposome aggregation assays

In the presence of either EGTA or Ca²⁺, liposomes were mixed with 1 µM of the proteins in a total volume of 1 ml. Aggregation was observed by following the absorbance of the liposome suspensions at 400 nm (Hui *et al.*, 2011) in a ultraviolet spectrophotometer at room temperature. In order to obtain the pure aggregation signal caused by Ca²⁺, the turbidity of the protein-free vesicle suspension was subtracted from signals obtained in the presence of Ca²⁺.

Dynamic light scattering

DLS experiments were preformed using the Brookhaven Laser Particle Size and Zeta

Potential Analyzer. Samples contained 0.2 mg/ml of the lipid vesicles and/or 0.5 mg/ml of the proteins in 50 mM HEPES pH 7.5, 100 mM NaCl, with various additions as shown in Table S4. Each measurement was carried out as an average of 10 data points, and was measured 3 times.

Electron microscopy

Protein samples were prepared in the presence of 1 mg/ml of the vesicles composed of a 75:25 PC:PG ratio incubated with 0.2 mg/ml BON1, 0.2 mg/ml BON1 and 100 μ M Ca^{2+} , 0.2 mg/ml BON1 and 1 mM Ca^{2+} , and 0.2 mg/ml BON1, 100 μ M Ca^{2+} and 2 mM EGTA. The samples were negatively stained with uranyl acetate on carbon-coated grids and imaged using a Jeol 2100F electron microscope operated at 120 kV.

Abbreviations

NLR: Negative regulator of several R genes; AKT/mTOR: Protein kinase B / mammalian target of rapamycin; NSCLC: Oncogene in non-small cell lung cancer; VWA: von Willebrand factor A; PKC: Protein kinase C; MIDAS: Metal-ion-dependent adhesion site; RMSD: Root mean square deviation; CBR: Ca^{2+} -binding region; LBR: Ca^{2+} -dependent lipid binding region; PSF: Phospholipid 1,2-dihexanoyl-sn-glycerol-3-phospho-L-serine; PG: Phosphatidylglycerol; PS: Phosphatidylserine; PI: Phosphatidylinositol; PC: Phosphatidylcholine; PE: Phosphatidyl ethanolamine; DLS: Dynamic light scattering; TEM: Transmission electron microscopy; AFM: Atomic force microscopy; BIR1: BAK1-interacting receptor like kinase 1; BAP: BON association protein; IPTG: β -D-1-Thiogalactopyranoside; TEV: Tobacco etch virus; MR: Molecular replacement method; MD: Molecular docking and molecular dynamic

Author contributions

Q.W. and Y.W. conceived and designed the experiments. Q.W. performed sample preparation and most of the experiments. M.J., L.S., X.W., W.Y., Q.L., L.C. and Q.W.

performed crystallization and x-ray data collection. Y.C. performed computational simulation. M.I. and Q.W. performed structure determination. Q.W., M.I. and Y.W. performed the data analysis and drafted the manuscript. J.L. revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We thank beamlines (BL17U1, BL19U1) staff at Shanghai Synchrotron Radiation Facility for assistance in data collection. This work was financially supported by the National Nature Science Foundation of China (31270790, 31470741, and 31500218) and the National Thousand Talents Program of China.

Protein databank accession numbers

The atomic coordinates and structure factors for BON1 (PDB ID 6KXK), BON1-Mn²⁺ (PDB ID 6KXU) and C2B (PDB ID 6KXT) have been deposited in the RCSB Protein Data Bank.

Short legends for Supporting Information

Supporting figures :

Fig. S1 Multiple sequence alignment of AtBON1 with other copine family members

Fig. S2 Packing of the BON1 molecules in the crystal

Fig. S3 Packing of the BON1-Mn²⁺ molecules in the crystal

Fig. S4 Structure Model of the C2 domains LBR1/PSF Complex

Fig. S5 The influence of Ca²⁺ on the aggregation state of BON1

Fig. S6 A ribbon representation of C2B domain of in complex with SO₄²⁺ (orange spheres) and MES (violet sticks).

Fig. S7 Structure Model of the C2 domains LBR2/PSF Complex

Fig. S8 The Model of the BON1 interaction with the membrane

Fig. S9 Models of BON1 regulated liposomes aggregation

Fig. S10 Structure Model of the BON1/ATP Complex

Supporting tables :

Table S1 Crystallization and cryoprotectant reagent conditions

Table S2 Data collection and refinement statistics

Table S3 Binding free energy (ΔG_{bind}) calculated for C2 domain/PSF complexes

Table S4 Particle size observed for samples under various conditions

Table S5 Binding free energy (ΔG_{bind}) calculated for BON1/ATP complexes

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Figure legends

Fig. 1 Overall Structure, Secondary Structure, and Domains of BON1

The three distinct conserved domains are colored individually: C2A domain in cyan, C2B domain in yellow, and vWA domain in pink. (A) Ribbon diagram of BON1 structure; α -helices and β -strands are labeled with αa , αb , αc or βa , βb , βc respectively in C2A, C2B and vWA domains. Twisted helices are labeled with η . (B) Domain structure of BON1 with conserved domains and sequence range labeled. (C) Topological diagram of the secondary structure elements. Rectangles represent

β -strands, ovals represent α -helices and cones represent twisted helices. Parallel and antiparallel β -strands are represented with triangles facing different directions.

Fig. 2 Overall structures of C2A and C2B domain and comparison with the other C2 domains

(A) A ribbon representation of C2A (cyan) and C2B (yellow) domain. (B) Structural overlap of the C2A and C2B domains of BON1. (C) Structure-based sequence alignment of multiple C2 domains. The sequence alignment is annotated with arrows for β -strands and squiggles for α - and η -helices. Strictly conserved residues are shown on red background, highly conserved residues are underlined by blue boxes, and the main aspartate residues involved in calcium coordination are underlined by black boxes. (D) Structural comparison of the C2A and C2B domains with the indicated homologous C2 domains complexes with Ca^{2+} (limon spheres) or phospholipids (orange sticks) from the same perspectives. The LBR1 and LBR2 are pointed in the black arrows and red arrows respectively. (E) C2 protein ribbon structures in (D) are overlapped, and the color of each protein corresponds to (D). CBRa3 stays away from LBR1 due to additional insertion.

Fig. 3 Overall structures of the vWA domain and a new Ca^{2+} binding site

(A) A ribbon representation of the vWA domain. (B) Left, a ribbon representation of the BON1D in complex with Ca^{2+} . Right, close-up view of the new Ca^{2+} binding site. The ribbon representation of the new Ca^{2+} binding site of BON1. The key structural features of the BON1D fold are labeled and highlighted in different colors. The Ca^{2+} is shown as a limon sphere, one bound water molecule depicted as red sphere. Key residues at the Ca^{2+} binding sites are displayed in a ball-and-stick representation. (C) The structural alignment of the vWA domain of the BON1A and BON1D. $\beta\text{c}2$ and $\beta\text{c}3$ are indicated by black arrows. (D) Primary sequence alignment of the new Ca^{2+} binding sites. The key residues are included in the black arrows. Strictly conserved residues are shown on a red background, highly conserved residues are underlined by blue boxes. BON1, BON2, and BON3 come from *A. thaliana*, OsBON1 and OsBON3 come from *Oryza sativa*, CPNA comes from *Dictyostelium*, GEMA comes from *Caenorhabditis elegans*, and copine I-IX come from *H. sapiens*.

Fig. 4 Structure of MIDAS site binding Mn^{2+}

(A) Left, a ribbon representation of the BON1- Mn^{2+} in complex with Mn^{2+} in MIDAS. Right, close-up view of the Mn^{2+} binding MIDAS site. The key structural features of the BON1- Mn^{2+} folds are labelled and highlighted in different colors. The Mn^{2+} is shown as a purple sphere. Key residues constituting the MIDAS motif are displayed in a ball-and-stick representation. (B) The structural alignment of the BON1 and BON1- Mn^{2+} . (C) Primary sequence alignment of the MIDAS motif. The key residues are included in the black arrows. Strictly conserved residues are shown on a red background, highly conserved residues are underlined by blue boxes. BON1, BON2, and BON3 come from *A. thaliana*, OsBON1 and OsBON3 come from *O. sativa*, CPNA comes from *Dictyostelium*, GEMA comes from *C. elegans*, and copine

I-IX come from *H. sapiens*.

Fig. 5 Binding of BON1 to phospholipid vesicles: Ca^{2+} concentration dependence and phospholipid selectivity

(A) Protein lipid overlay assay of His-proteins binding PG, PS, PI, PC or PE. 2.5 μg of each lipid were spotted onto nitrocellulose membranes, and in the presence of 100 μM Ca^{2+} or 2 mM EGTA, the protein/antibody complex was incubated. The corresponding proteins and phospholipids are shown in the figure. Binding was detected with a secondary antibody conjugated to HRP and TMB color liquid. (B) Quantification of the intensity of protein-lipid overlay assays in the presence of 100 μM Ca^{2+} in (A). Binding to BON1 in the presence of 100 μM Ca^{2+} was assigned a value of 100 for PG. Comparisons between different data were performed by one-way analysis of variance (ANOVA) with LSD tests. Different letters indicates a significant effect differences in data in Figure 5E ($p < 0.05$). C, D, the phospholipid binding activity of proteins (BON1, C2A, C2B, C2A-DA3, C2B-DA2) was assayed by SDS-PAGE analysis of supernatants and pellets obtained by centrifugation of proteins/phospholipid vesicle mixtures. Liposomes were precipitated by centrifugation, and bound proteins were analyzed by SDS-PAGE. (C) Phospholipid selectivity. Purified proteins (0.5 mg/ml) were incubated with PG-vesicles, PS-vesicles, PI-vesicles, PC-vesicles, or PE-vesicles in 100 μM Ca^{2+} . (D) Ca^{2+} concentration dependence. Purified proteins (0.5 mg/ml) were incubated with PG-vesicles (1 mg/ml) in the presence of the indicated concentrations of Ca^{2+} or 2 mM EGTA. (E) Comparative analyses of Ca^{2+} sensitivity of BON1 and the two C2 domains. Protein quantifications of the soluble fraction after lipid pelleting on C2A fragment, C2B fragment and BON1 of (D) were performed by measuring the optical density at 280 nm on a spectrophotometer. B, E, error bars represent standard error of the mean (s.e.m.) from triplicate determinations.

Fig. 6 BON1 mediates aggregation of liposomes in response to Ca^{2+}

The BON1 aggregates PG-vesicles in a Ca^{2+} -dependent manner. (A) Kinetics of turbidity changes (absorbance at 400 nm, A_{400} nm) of the protein-free liposome suspension in response to sequential addition of BON1, Ca^{2+} , and EGTA. BON1 did not cluster vesicles in the absence of Ca^{2+} or Ca^{2+} concentration less than 10 μM , but induced rapid liposome aggregation with the addition of Ca^{2+} (up to 100 μM). When the Ca^{2+} concentration reaches 500 μM , BON1 forms suspended particles and increases the turbidity of the sample; only the liposome aggregation was rapidly reversed by chelation of Ca^{2+} with EGTA. (B) The same experiment was performed as shown in (A) except replace BON1 with C2AB fragments. C2AB fragments did not induce effective vesicle aggregation in the presence or absence of Ca^{2+} . High concentration of Ca^{2+} (10 mM) causes C2AB fragments to aggregate to form suspended particles, which significantly increases the turbidity of samples. (C) The same experiment was performed as shown in (A) except replace BON1 with C2A or C2B fragments. C2A or C2B fragments did not induce effective vesicle aggregation in

the presence or absence of Ca^{2+} . D-G, DLS measurements of particle sizes in samples containing 0.2 mg/ml PG-vesicles either without additions (D), adding 0.5 mg/ml BON1 (E), adding 0.5 mg/ml BON1 and 100 μM Ca^{2+} (F) or upon adding 2 mM EGTA (G).

Fig. 7 TEM images of liposomes incubated with BON1 in the absence or presence of Ca^{2+}

Negative-stain transmission electron micrographs of 1 mg/ml PG-vesicles (A), incubated with 0.2 mg/ml BON1 (B), 0.2 mg/ml BON1 plus 100 μM Ca^{2+} (C, D), 0.2 mg/ml BON1 plus 1 mM Ca^{2+} (E), and 0.2 mg/ml BON1 plus 100 μM Ca^{2+} and adding 2 mM EGTA after 10 mins incubation (F).

Figures and figure legends

Fig. 1 Overall Structure, Secondary Structure, and Connectivity of Domains of BON1

The three distinct conserved domains are colored individually: C2A domain in cyan, C2B domain in yellow, and vWA domain in pink. (A) Ribbon diagram of BON1 structure; α -helices and β -strands are labeled with αa , αb , αc or βa , βb , βc respectively in C2A, C2B and vWA domains. The 3_{10} twisted helices are labeled with as η . (B) Domain structure of BON1 with conserved domains and sequence range labeled. (C) Topological diagram of the BON1 secondary structure elements. Rectangles with arrows represent β -strands, ovals rectangles with rounded edges represent α -helices and ovals represent twisted 3_{10} helices. Arrows show directions of Parallel and antiparallel β -strands are represented with triangles facing different directions.

Fig. 2 Overall structures of C2A and C2B domain and comparison with the other C2 domains

(A) A ribbon representation of C2A (cyan) and C2B (yellow) domain. (B) Structural overlap of the C2A and C2B domains of BON1. (C) Structure-based sequence alignment of multiple C2 domains. The sequence alignment is annotated with arrows for β -strands and squiggles for α - and 3_{10} -helices. Strictly conserved residues are shown on with red background, highly conserved residues are underlined by shown in blue boxes, and the main aspartate residues involved in calcium coordination are underlined shown by in black boxes. (D) Structural comparison of the C2A and C2B domains with the indicated homologous C2 domains complexes with Ca^{2+} (greenlimon spheres) or phospholipids (orange sticks) from the same perspectives. The LBR1 and LBR2 are pointed in the black arrows and red arrows respectively. (E)

1183 C2 protein ribbon structures in (D) are overlappedsuperimposed, and the proteins are
1184 colored of each protein correspondsaccording to (D). CBRa3 stays awaydiffers from
1185 LBR1 due to additional insertion.

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Fig. 3 Overall structures of the vWA domain and a new Ca^{2+} binding site

(A) A ribbon representation of the vWA domain. (B) Left, a ribbon representation of the BON1D in complex with Ca^{2+} . Right, close-up view of the new Ca^{2+} binding site. The ribbon representation of the new Ca^{2+} binding site of BON1. The key structural features of the BON1D fold are labeled and highlighted in different colors. The Ca^{2+} is shown as a limongreen sphere, one bound water molecule depicted as red sphere. Key residues at the Ca^{2+} binding sites are displayed in a ball-and-stick representation. (C) The structural alignment of the vWA domain of the BON1A and BON1D. $\beta 2$ and $\beta 3$ are indicated by black arrows. (D) Primary sequence alignment of the new Ca^{2+} binding sites. The key residues are included in the black arrows. Strictly conserved residues are shown on a red background, highly conserved residues are underlined by blue boxes. BON1, BON2, and BON3 come from *A. thaliana*, OsBON1 and OsBON3 come from *Oryza sativa*, CPNA comes from *Dictyostelium*, GEM4 comes from *Caenorhabditis elegans*, and copine I-IX come from *H. sapiens*.

Fig. 4 Structure of MIDAS site binding Mn^{2+}

(A) Left, a ribbon representation of the BON1- Mn^{2+} in complex with Mn^{2+} in MIDAS. Right, close-up view of the Mn^{2+} binding MIDAS site. The key structural features of the BON1- Mn^{2+} folds are labelled and highlighted in different colors. The Mn^{2+} is shown as a purple sphere. Key residues constituting the MIDAS motif are displayed in a ball-and-stick representation. (B) The structural alignment of the BON1 and BON1- Mn^{2+} . (C) Primary sequence alignment of the MIDAS motif. The key residues are included in the black arrows. Strictly conserved residues are shown on a red background, highly conserved residues are underlined shown in by blue boxes. BON1, BON2, and BON3 come from *A. thaliana*, OsBON1 and OsBON3 come from *O. sativa*, CPNA comes from *Dictyostelium*, GEM4 comes from *C. elegans*, and copine I-IX come from *H. sapiens*.

Fig. 5 Binding of BON1 to phospholipid vesicles: Ca²⁺ concentration dependence and phospholipid selectivity

(A) Protein lipid overlay assay of His-proteins binding PG, PS, PI, PC or PE. 2.5 μg of each lipid were spotted onto nitrocellulose membranes, and in the presence of 100 μM Ca²⁺ or 2 mM EGTA, the protein/antibody complex was incubated. The corresponding proteins and phospholipids are shown in the figure. Binding was detected with a secondary antibody conjugated to HRP and TMB color liquid. (B) Quantification of the intensity of protein-lipid overlay assays in the presence of 100 μM Ca²⁺ in (A). Binding to BON1 in the presence of 100 μM Ca²⁺ was assigned a value of 100 for PG. Comparisons between different data were performed by one-way analysis of variance (ANOVA) with LSD tests. Different letters indicates a significant effect differences in data in Figure 5E (p < 0.05). C, D, the phospholipid binding activity of proteins (BON1, C2A, C2B, C2A-DA3, C2B-DA2) was assayed by SDS-PAGE analysis of supernatants and pellets obtained by centrifugation of

1241 proteins/phospholipid vesicle mixtures. Liposomes were precipitated by
1242 centrifugation, and bound proteins were analyzed by SDS-PAGE. (C) Phospholipid
1243 selectivity. Purified proteins (0.5 mg/ml) were incubated with PG-vesicles,
1244 PS-vesicles, PI-vesicles, PC-vesicles, or PE-vesicles in 100 μ M Ca^{2+} . (D) Ca^{2+}
1245 concentration dependence. Purified proteins (0.5 mg/ml) were incubated with
1246 PG-vesicles (1 mg/ml) in the presence of the indicated concentrations of Ca^{2+} or 2
1247 mM EGTA. (E) Comparative analyses of Ca^{2+} sensitivity of BON1 and the two C2
1248 domains. Protein quantifications of the soluble fraction after lipid pelleting on C2A
1249 fragment, C2B fragment and BON1 of (D) were performed by measuring the optical
1250 density at 280 nm on a spectrophotometer. B, E, error bars represent standard error of
1251 the mean (s.e.m.) from triplicate determinations.

Fig. 6 BON1 mediates aggregation of liposomes in response to Ca^{2+}

The BON1 aggregates PG-vesicles in a Ca^{2+} -dependent manner. (A) Kinetics of turbidity changes (absorbance at 400 nm, $A_{400 \text{ nm}}$) of the protein-free liposome suspension in response to sequential addition of BON1, Ca^{2+} , and EGTA. BON1 did not cluster vesicles in the absence of Ca^{2+} or in Ca^{2+} concentration less than 10 μ M, but induced rapid liposome aggregation with the addition of Ca^{2+} (up to 100 μ M). When the Ca^{2+} concentration reaches 500 μ M, BON1 forms suspended particles and increases the turbidity of the sample; only the liposome aggregation was rapidly reversed by chelation of Ca^{2+} with EGTA. (B) The same experiment was performed as shown in (A) except replace BON1 with C2AB fragments. C2AB fragments did not induce effective vesicle aggregation in the presence or absence of Ca^{2+} . High concentration of Ca^{2+} (10 mM) causes C2AB fragments to aggregate to form

suspended particles, which significantly increases the turbidity of samples. (C) The same experiment was performed as shown in (A) except replace BON1 with C2A or C2B fragments. C2A or C2B fragments did not induce effective vesicle aggregation in the presence or absence of Ca^{2+} . D-G, DLS measurements of particle sizes in samples containing 0.2 mg/ml PG-vesicles either without additions (D), adding 0.5 mg/ml BON1 (E), adding 0.5 mg/ml BON1 and 100 μM Ca^{2+} (F) or upon adding 2 mM EGTA (G).

Fig. 7 TEM images of liposomes incubated with BON1 in the absence or presence of Ca^{2+}

Negative-stain transmission electron micrographs of 1 mg/ml PG-vesicles (A), incubated with 0.2 mg/ml BON1 (B), 0.2 mg/ml BON1 plus 100 μM Ca^{2+} (C, D), 0.2 mg/ml BON1 plus 1 mM Ca^{2+} (E), and 0.2 mg/ml BON1 plus 100 μM Ca^{2+} and adding 2 mM EGTA after 10 mins incubation (F).