Insights into the mechanism of membrane fusion induced by the plant defense element, plant-specific insert

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Xiaoli Zhao, Jenny (Jinxing) Tian, Hua Yu, Brian C. Bryksa, John H. Dupuis, Xiuyuan Ou, Zhaohui Qian, Chen Song, Shenlin Wang, and Rickey Y. Yada

From the 1College of Chemistry and Molecular Engineering and Beijing NMR Center, the 3Center for Quantitative Biology, Academy for Advanced Interdisciplinary Studies, and the 4Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China, the 5Food, Nutrition, and Health Program, Faculty of Land and Food Systems, University of British Columbia, Vancouver, British Columbia, Canada, the 6MOH Key Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Science, Beijing, China, and the 7Beijing National Laboratory for Molecular Sciences, Beijing, China

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In plants, many natural defense mechanisms include cellular membrane fusion as a way to resist infection by external pathogens. Several plant proteins mediate membrane fusion, but the detailed mechanism by which they promote fusion is less clear. Understanding this process could provide valuable insights into these proteins’ physiological functions and guide bioengineering applications (i.e. the design of antimicrobial proteins). The plant-specific insert (PSI) from Solanum tuberosum can help reduce certain pathogen attack via membrane fusion. To gain new insights into the process of PSI-induced membrane fusion, a combined approach of NMR, FRET, and in silico studies was used. Our results indicate that (i) under acidic conditions, the PSI experiences a monomer-dimer equilibrium, and the dimeric PSI induces membrane fusion below a certain critical pH; (ii) after fusion, the PSI resides in a highly dehydrated environment with limited solvent accessibility, suggesting its capability in reducing repulsive dehydration forces between liposomes to facilitate fusion; and (iii) as shown by molecular dynamics simulations, the PSI dimer can bind stably to membrane surfaces and can bridge liposomes in close proximity, a critical step for the membrane fusion. In summary, this study provides new and unique insights into the mechanisms by which the PSI and similar proteins induce membrane fusion.

Unlike mammals, plants do not have mobile immune cells and have evolved several natural defense mechanisms in response to pathogen attack (1–4) (i.e. expression of certain proteins with antimicrobial activity). Many of those antimicrobial proteins are able to mediate membrane fusion to facilitate the translocation of hydrolytic enzymes, aiding in the resistance to pathogen resistance to pathogen attack (5–7). In Solanum tuberosum, infection by the pathogenic fungus Phytophthora infestans results in late blight disease. The pathogen infection leads to the expression of plant aspartic proteases (AP) as part of a host-defense response (8). Between the N and C termini of the plant aspartic protease, a unique domain exists—the plant-specific insert (PSI)—which is involved in host immunity, functioning independently of the parent enzyme (9–11). In vitro studies have shown that recombinantly expressed PSI is cytotoxic to both human and plant pathogens (11). The transgenic expression of PSI confers enhanced resistance upon Botrytis cinerea in Arabidopsis thaliana, thus confirming in vivo antimicrobial activity (12). Understanding the mechanism of PSI could provide valuable insights into its physiological functions and aid in the design of antimicrobial proteins or endogenously pathogen-resistant transgenic plants.

The PSI protein belongs to a large group of proteins—the saposin-like protein (SAPLIP) family (13). The SAPLIPS share similar structure and functions, some of which are involved in host defense in other organisms (i.e. the innate animal immune system proteins NK-lysin and granulysin) (14, 15). The structure of the PSI has been determined previously (9), showing a topology of symmetric dimers with a characteristic saposin-like fold; the N and C termini of saposins are swapped relative to other SAPLIPS (9, 16, 17). The functional roles of the PSI have been postulated to be related to its ability to bind phospholipid membranes and catalyze membrane fusion (9, 10, 14) by interacting with phospholipid membranes and causing liposome leakage in a pH- and lipid composition-dependent manner (9, 10, 14). The AP from S. tuberosum, of which the PSI is a part, also exhibits pH-dependent activities and has optimal antimicrobial function around pH of 2–5 (18, 19). In vivo characterization also has suggested that the PSI is toxic to certain plant and human pathogens by permeabilizing their plasma membranes (9, 12).

Although the structure of the PSI is known, detailed information regarding the role and mechanism of the PSI in membrane fusion is still lacking. It is possible that the various SAPLIP proteins could have very different sequence-dependent mechanisms, despite having similar structures (17). Earlier work has indicated the pH-triggered dimerization of PSI and suggested that dimeric PSI is the active form for membrane fusion (9). Although it is known that the acidic condition is mandatory for PSI activity, the relationship between the formation of the PSI dimer and the pH-triggered activity remains unclear. It is possible that (i) the acidic pH induces the dimers that can fuse the membrane or (ii) low pH neutralizes the protein surface...
chambers, which is the factor critical to the PSI functions. Further, characterizing the topology of a membrane fusion protein on a membrane is often challenging. In the present study, an integrative approach using NMR, FRET, and in silico methods demonstrated the details of the mechanisms of PSI. We show that under acidic conditions, the PSI undergoes monomer-dimer equilibrium in a pH-dependent manner. However, the ability of PSI dimers is insufficient to induce membrane fusion; the PSI interacts strongly with the phospholipid membranes and induces full membrane fusion only at pH values below a certain critical pH. Upon binding with membranes, the PSI resides in a highly dehydrated environment, suggesting an ability to drive membrane fusion by reducing the dehydration energy barrier between liposomes. Furthermore, molecular dynamics simulations revealed the most likely configurations of dimeric PSI on model membranes. These results provide evidence that it is possible that the PSI could interact with two liposomes at the same time and bring them into close proximity, which may be a critical step in the early stages of membrane fusion, therefore providing new insights into the mechanisms by which the PSI functions in a defensive role in plants to disrupt the pathogen membrane.

Results

**pH dependence of PSI fusion activity**

The PSI was reported to trigger membrane fusion in a pH-dependent manner (9, 14, 16, 20). In this study, a fluorescence-based fusion assay was used to study the PSI activity at various pH values. Two different types of liposomes were prepared, including nonfluorescent liposomes (containing egg phosphatidylcholine (PC), egg phosphatidyethanolamine (PE), and brain phosphatidylserine (PS) phospholipids) and fluorescent liposomes containing the aforementioned phospholipids, as well as N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and fluorescent liposomes containing the aforementioned phospholipids, as well as N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rho-PE). Rho-PE and NBD-PE serve as the fluorescence acceptor and donor, respectively. The close proximity of fluorophore groups quenches the FRET resonance at 535 nm, with excitation at 465 nm. The fluorescent and nonfluorescent liposomes were mixed at a ratio of 1:9, with the lipid mixing being expected to result in a significant increase in the fluorescent resonance due to dilution of fluorophore lipids by nonmodified lipids.

The fluorescence-based fusion assays were conducted over a range of pH values from pH 7.0 to 2.0. Upon the addition of the PSI into the liposome solution at pH 7.0, no fluorescence intensity changes were observed, indicating no membrane fusion. In contrast, the addition of the PSI to liposome in acidic conditions resulted in a significant increase in fluorescence intensity, suggesting the occurrence of membrane fusion by PSI (Fig. 1A). The fusion efficiency at different pH values was measured by comparing the fluorescence intensities at 1200 s after mixing the PSI and the liposomes with those obtained by the addition of Triton X-100 to the liposome at the same pH. Fig. 1B plots the fusion efficiency against pH, showing a sharp increase in fusion activity within a narrow range of pH 5.0 to 4.5. This result indicates that PSI effectively acts as a membrane fusion protein below a critical pH around 4.5, in agreement with the physiological findings that the PSI is functional under acidic conditions (18, 19).

**pH dependence of PSI aggregation states**

Earlier studies demonstrated that the PSI experiences pH-dependent monomer-dimer equilibrium (9). The saposin-family proteins commonly form dimers to act as membrane fusion proteins (17). Here, solution NMR was used to study the pH dependence of aggregation states of the PSI. Comparing the $^{1}H$-$^{15}N$ heteronuclear single quantum coherence (HSQC) of PSI at pH values from 2.0 to 7.0 showed a single set of cross-peaks at pH 7.0. At pH 2.0, more than 80% of the population of the major state that showed distinct spectral patterns of PSI at pH 7.0 exists, whereas a minor state has a spectral pattern close to that at neutral pH, which suggests the detection of a new state of PSI at pH 2.0. In addition, two sets of cross-peaks were observed at pH values between 2.0 and 7.0 (Fig. 2 and Fig. S1), suggesting the presence of a pH-dependent monomer-dimer equilibrium. To confirm the aggregating states, $^{15}N$ relaxation parameters were measured at pH values of 2.0 and 7.0. At pH 2.0, the correlation time $\tau_c$, fitted from the average $T_1^{(15}N)/T_2^{(15}N)$ ratio of the cross-peaks of the major components, was found to be approximately double that at pH 7.0 (Fig. S2 and supporting Results), which is consistent with the expected increase in molecular tumbling upon dimerization. Thus, these results verify that the PSI experiences a monomer-dimer equilibrium at acidic pH. At pH 2.0, PSI exists primarily as a dimer.

The NMR resonance assignments were obtained both for the monomeric PSI at pH 7.0 and the dimeric PSI at pH 2.0 (Fig. 2B and Figs. S3 and S4). The amide cross-peaks of G40 were used as a reference to follow the monomer-dimer equilibrium, because the cross-peaks of G40 corresponding to both species were obtained, with well-resolved resonances. Changes in pH altered the relative intensities of G40 cross-peaks corresponding to the monomeric and the dimeric species, thus allowing for the determination of the pH-dependent monomer-dimer equilibrium (Fig. 1, C and D). The percentages of the dimeric PSI were obtained as a function of pH values, showing that decreasing pH substantially increases the percentage of the dimeric species. A nearly linear correlation was found for the percentage of the dimeric PSI against the pH value.

The crystal structure of the dimeric PSI has been reported (9), sharing a saposin-like fold with a boomerang shape (Fig. 2A). Although our earlier work of CD spectra on PSI indicated the major $\alpha$-helical structures of PSI in solution, site-specific secondary structure elements of PSI in solution are still unknown. On the other hand, the monomer structure of PSI has not been determined; thus, a study of its secondary structure in solution is needed. The NMR resonance assignments and relaxation analysis allow for the study of the secondary structures under different pH values. Chemical shift index (CSI) analysis from NMR assignments revealed predominantly $\alpha$-helical structures for both species, which is in agreement with the known PSI crystal structures (21). In solution, a flexible unstructured loop (residues 40–72) connecting helix II and helix III in both species was identified by the CSI and the low
1H-15N NOE values (Figs. S2–S5). Consistently, those segments do not appear in the crystal structure of PSI (PDB entry 3RFI), due to their low electron density, further demonstrating its dynamic nature.

The pH dependence of PSI-liposome interactions by NMR spectroscopy

Further solution NMR experiments were used to gain additional information regarding PSI-liposome interactions. The PSI-liposome interactions were characterized at pH 2.0, 3.4, 5.4, and 7.0 to determine the nature of PSI-membrane interactions as a function of pH values. The 1H-15N HSQC spectra were collected for the liposome-free PSI and the PSI after the addition of liposomes (Fig. 3). Upon binding of the liposomes, the PSI signals are expected to experience broader line width as being attached to the liposome with a radius of a few hundred nanometers.

At pH 5.4 and 7.0, no substantial spectral changes were observed (no cross-peak shifting or line broadening), thus indicating the absence of interactions between PSI and liposomes. In contrast, at pH 2.0 and 3.4, the intensity of most cross-peaks in the 1H-15N HSQC spectra of dimeric PSI was largely reduced in the presence of liposome. The greater breadth in line width of the PSI after the addition of liposomes indicated strong interaction between the PSI with a particle with a much larger size.

As both the monomer and the dimers are present at acidic pH values, the disagreement in terms of PSI-liposome binding at pH 3.4 and 5.4 suggests that the formation of the PSI dimer is not sufficient to induce PSI-liposome interactions. The PSI-liposome binding is strongly regulated by the pH, a pH below a critical value needed for PSI-liposome interactions. The decrease in pH neutralizes the negative charges of PSI, thus effectively reducing the repulsive forces between the protein and the negatively charged liposome, leading to effective PSI-

**Figure 1.** pH-dependent PSI-induced membrane fusion and monomer-dimer equilibrium of PSI in solution. A, the fluorescent to nonfluorescent liposome molar ratio is 1:9, and the protein/liposome molar ratio is 1:15. Lipid-mixing curves induced by PIS are shown at pH 3.4 (red), 4.5 (blue), 5.4 (magenta), and pH 7.0 (green). The control experiments with Triton X-100 and buffer are shown in black and blue, respectively. B, membrane fusion efficiency by PSI plotted against pH, showing a sharp increase around pH 4.5. C, pH-dependent 1H-15N HSQC showing an expanded region for G40 to demonstrate the pH-dependent monomer-dimer equilibrium of PSI in solution. The cross-peaks of G40 corresponding to the residues of the monomer and the dimer are highlighted. D, dimer content obtained by cross-peak intensities plotted against pH values.
liposome binding and membrane fusion. Indeed, the pI of the PSI is also around 4.5, near the critical pH at which PSI induces membrane fusion. It thus confirms the need to neutralize the negative charges on the protein surface to perform membrane-binding and fusion functions.

**Topology of the PSI in post-fusion liposomes**

It is commonly accepted that the membrane fusion process involves a dehydration process. Solid-state NMR (SSNMR) is powerful in studying the topology of membrane fusion proteins (22–28). In particular, the SSNMR-based H/D exchange experiments would provide insights to study the solvent accessibility of the protein, thus suggesting the level of hydration. Here, 2D $^{15}$N-$^{13}$C spectra of the PSI-liposome complexes were recorded to monitor backbone signals, the cross-peaks of which represent the correlation between the $^{15}$N and the $^{13}$C atom from the same residue. The magnetization transfer pathway of the $^{15}$N-$^{13}$C experiment starts from the amide proton and transfer to the covalently attached $^{15}$N atom and further to the $^{13}$C atom. By incubating the PSI-liposome in D$_2$O-based buffer, the H/D exchange between the amide proton and D$_2$O would lead to a reduction in the intensities of the cross-peaks on the $^{15}$N-$^{13}$C spectra. Thus, the measurement of changes to peak intensities could provide the information regarding solvent accessibility of the protein.

In this study, two sets of $^{15}$N-$^{13}$C spectra were collected on two PSI-liposome complex samples. The first sample, termed the $^1$H-based sample, was prepared by mixing the $^{15}$N, $^{13}$C-labeled PSI in H$_2$O-based buffer with liposomes in H$_2$O-based buffer. The $^{15}$N-$^{13}$C spectra of the $^1$H-based sample were collected before and after a 24-h incubation in D$_2$O-based buffer. The second sample, termed the $^2$D-based sample, was prepared by dissolving PSI in D$_2$O-based buffer, followed by incubation for 24 h in solution, and mixed with liposomes in D$_2$O-based buffer. The $^{15}$N-$^{13}$C spectra were recorded on the $^2$D-based sample before and after 24-h H$_2$O back-exchange. Comparison of the four spectra indicated the solvent accessibility of the PSI in liposomes. In addition, the $^1$H-$^{13}$C cross-polarization (CP) experiments are not affected by amide exchange and were used to evaluate the sample amounts between different samples (Fig. S5).

Fig. 4A and Fig. S6 compare the 2D $^{15}$N-$^{13}$C of the $^1$H-based sample before and after incubation in D$_2$O-based buffer for 24
h, showing that the H/D exchange in D$_2$O led to $\sim$50% reduction in the overall spectral intensity of 2D $^{15}$N-$^{13}$C$_{a}$ spectra of PSI (Fig. S5, A and B), whereas the overall spectral patterns were not altered significantly (Fig. 4A and Fig. S6 (A and B)). These results indicate that a 24-h period of incubation is not sufficient to achieve a complete exchange and hint that the lipid protects the PSI from solvent accessibility.

To confirm this issue, the 2D $^{15}$N-$^{13}$C$_{a}$ of the 2D-based sample was collected to compare with those of the 1H-based sample. As shown in Figs. S5 and S6, the overall intensity of the 15N-$^{13}$C$_{a}$ spectrum of the 2D-based sample before H back-exchange was only $\sim$30 and $\sim$60% of that of 1H-based sample before and after D$_2$O exchange, respectively, showing a more complete exchange for the 2D-based sample. Moreover, spectral patterns are largely different between the two samples (Fig. S6); many cross-peaks are missing in the spectra of the 2D-based sample (Fig. 4 and Fig. S6). Consistently, the H$_2$O back-exchange of 2D-based sample did not result in large enhancement of spectral intensities, showing again the slow H/D exchange in lipid environments. Considerably fewer cross-peaks were observed for the 15N-$^{13}$C$_{a}$ spectra of the H$_2$O-back-exchanged 2D-based sample than for the PSI in liposomes with H$_2$O-based buffer (Fig. 4C), which is in agreement with the protective effect of lipids on the PSI. Overall, these results showed that for some residues, complete H/D exchange could be achieved in solution within 24 h, but not in the lipid environment, confirming that the lipids protect the PSI.

Further, the H/D exchange experiments of PSI in solution were also conducted at the same pH to investigate the level of H/D exchange in solution within a 24-h period, as a control experiment. After the lyophilized $^{15}$N-$^{13}$C-labeled PSI was dissolved in a pH 2.0 D$_2$O-based buffer, a series of HSQC spectra were collected over 24 h. Unlike those obtained with PSI in liposomes, most amide signals of the PSI in solution were largely diminished (Fig. 4D), with a few exceptions including residues 22–36 and 78–84, near the connecting turns between helix I-II and helix III-IV, respectively. These results demonstrate that the PSI’s amide protons are generally exchangeable in solution and achieve complete exchange within 24 h (Fig. 4D), which in turn confirms the additional protective effect of the PSI by lipids in post-fusion PSI-liposome complexes. Taken together, these results demonstrate that in post-fusion membrane systems, the PSI interacts strongly with liposomes and resides in a dehydrated environment, owing to a protective effect by the phospholipids.

**Simulation of PSI-membrane interactions**

The topology of the PSI in the post-fusion membrane systems remains unclear, as does the detailed role of the PSI dimer in facilitating membrane fusion. The PSI could either insert
into the hydrophobic center of the membrane or interact with the surface of lipid bilayers. In both cases, the lipids could interact strongly with proteins, leading to solvent inaccessibility. The detailed roles of the monomer and the dimer in the membrane fusion process still remain elusive. To address these questions, molecular dynamics simulations were performed to study the detailed interactions between the PSI and the phospholipid molecules/bilayers (1:1:1, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)/1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE)/1-palmitoyl-2-oleoylphosphatidylserine (POPS) in concert with the NMR measurements) under various conditions.

To study the pose of the PSI dimer in the lipid bilayer system, we performed coarse-grained self-assembly MD simulations, in which the PSI dimer was initially positioned in randomly mixed POPC/POPE/POPS (1:1:1) and water molecules, and then the whole system self-evolves into a stable configuration. The results showed that PSI dimer could attach to and interact with the surface of a lipid bilayer with two stable orientations (Fig. 5A). The orientation of Mode A exhibited a $\sim 29^\circ$ angle between the first principal axis of the PSI dimer and the $z$ axis, which was found in 4 of 20 simulations. Mode B exhibited an orientation angle of $\sim 64^\circ$, which was the major orientation and was observed in 15 of 20 simulations. Thus, the most probable orientation is Mode B, as shown in Fig. 5A. It should be noted that no membrane insertion of the PSI dimer was observed in the simulations, which suggested that it may be energetically unfavorable for the PSI dimer to be embedded into a lipid bilayer using the current simulation time scale with the coarse-grained force field.

To study the position and pose of the PSI dimer in post-fusion liposomes, coarse-grained self-assembly simulations of the PSI dimer and more densely distributed 1:1:1 POPC/POPE/POPS in a simulation cell were conducted. These simulations were to simulate a scenario in which there are more lipid molecules around the PSI dimer, simulating the environment during and after membrane fusion. The results showed that the PSI dimer was surrounded by two phospholipid bilayers (Fig. 5B, inset), and took similarly stable orientations as when interacting with one lipid bilayer. Mode A binding, with an orientation angle of $\sim 23^\circ$, was observed in one of the five simulations; Mode B binding, with an orientation angle of $\sim 55^\circ$, was the main orientation and was observed in four of five simulations. These are close to the orientations of the PSI dimer on a lipid bilayer (Fig. 5A).
Furthermore, a coarse-grained simulation of one PSI dimer placed between two preformed vesicles was also run. Results showed that the PSI dimer can spontaneously bind to the surfaces of the two vesicles and form a bridge between them. Only Mode B binding (orientation angle $\sim 63^\circ$) was observed in all of the four simulations (Fig. 5C). It was suspected that Mode B...
would need to transform into Mode A to bring the two vesicles closer to each other before fusion could proceed. Unfortunately, a complete fusion process was not able to be observed due to the limited simulation time in the current study (15 μs).

To study the orientation and pose of the PSI monomer in or around lipid bilayers, coarse-grained self-assembly simulations of one PSI monomer in the densely distributed 1:1:1 POPC/POPE/POPS were conducted. The results showed that the PSI monomer was located between two phospholipid bilayers, interacting with only one or both of the bilayers during the simulations. Although we can roughly categorize the orientation into three clusters (Fig. 5D), no strongly preferred orientation was observed, in contrast to the PSI dimer in similar simulations (Fig. 5B). Interchanges among different orientations were observed in four of seven trajectories, which were not seen in the dimer systems (Fig. S8). Among the two PSI interfaces in close contact with the two bilayers, there was always one that was unstable (Fig. 5E (interface A) and Fig. S9) and the other one stable (Fig. 5E (interface B) and Fig. S9) in all of the simulation trajectories of the PSI monomer. The probability of the PSI monomer not embedded into the bilayer was about 41% for the unstable interaction interface (Fig. 5E, green histogram), whereas for the stable interface, the probability of being embedded was nearly 99% (Fig. 5E, black histogram). The probability

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**Figure 5. The orientations and the interface stability PSI dimer and monomer on lipid bilayer surfaces composed of 1:1:1 POPC/POPE/POPS.** Depicted are the orientation angle between the first principal axis of the PSI dimer and the normal axis in one lipid bilayer (A), the post-fusion state (B), and the vesicle system (C). Monomers A and B of the PSI dimer are shown with red and yellow van der Waals spheres, respectively. D, orientation angle between the first principal axis of the PSI monomer and the normal axis of the two lipid bilayers. The mauve surface represents the backbone of the PSI monomer. The yellow arrows represent the first principal axis, the blue dots represent the PO₄ groups, and the gray lines constitute the tails of the phospholipid molecules. E, stability of the interactions between the two lipid bilayers and the PSI dimer or monomer. Interfaces A and B represent the unstable and stable interfaces, respectively.

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**Figure 6. Interaction interfaces of the PSI dimer and monomer with the lipid bilayers.** A, key interface residues of the PSI dimer within a distance of 1 nm from the center of mass of the nearest phospholipid molecule in the hemifusion state. Red and yellow licorice lines represent the backbones of the monomer A and B, respectively. The key interface residues are shown as van der Waals spheres. Basic, polar, and nonpolar residues are shown in blue, green, and white, respectively, and the protonated Glu and Asp are shown in cyan. The lipid bilayer surface is shown as a gray background. Residues belonging to monomer A and B are labeled without and with underlines, respectively. B, basic, acidic, polar, and nonpolar residues are shown in blue, red, green, and white, respectively, and the protonated Glu and Asp are shown in cyan. The interaction interfaces are denoted with dashed lines.
of the PSI dimer embedding into the bilayer in both of the interaction interfaces was nearly 100% (Fig. 5E (blue histogram) and Fig. S8). The latter suggested that the interactions between the PSI dimer and both of the lipid bilayers were very stable, indicating that the PSI dimer may play a positive role in binding and bridging two bilayers. On the contrary, the existence of the unstable interface between the PSI monomer and bilayer, together with the relatively even distribution of the orientation angle of the monomer, suggested that the PSI monomer is probably not as effective as the dimer in bringing two lipid bilayers together and then inducing membrane fusion.

The combination of our in silico and experimental results led to the proposal of a potential binding pathway: (i) the PSI dimer is able to bind to two nearby vesicles by favorable interactions with their membrane surfaces, whereas the PSI monomer only binds stably with one vesicle on its membrane surface; (ii) the PSI formed a bridge between them via Mode B binding, thereby tethering them together in close proximity, and (iii) through various intermolecular and external forces, the vesicles are pulled even closer by altering the orientation to the metastable Mode A, which may eventually facilitate membrane fusion. Therefore, the simulation data indicated that the dimeric PSI is the component that induces membrane fusion.

To identify the key residues on the interaction interfaces, the minimum distances between the centers of mass of each residue of the PSI dimer and the phospholipid molecule were measured. The results showed that the interacting residues were almost identical for Mode A in one lipid bilayer and Mode A in the post-fusion state (Fig. S7). Similar results were obtained for Mode B in different aggregation states of the phospholipid molecules (Fig. S7). Taking Mode A and Mode B in the post-fusion state as an example, the residues with a distance of <1 nm from the lipid bilayers, which we view as indicating interaction with the lipid bilayers, were mostly hydrophobic. As shown in Fig. 6A, the close interaction of Mode A involved the helix I ( Ala-0, Ile-1, Val-2) and C-terminal residues (Leu-98, Lys-101, Ile-102, Pro-103) of one monomer and helix III (Pro-66, Thr-69, Glu-72, Met-73, Val-76, Trp-77, Asn-80) and one loop residue (Glu-64) of the other monomer. The short-range interaction with lipid bilayers in Mode B involved helix III (Pro-66, Leu-67) and several loop residues (Glu-35, Leu-36, Phe-38, Glu-64, Ala-65) of one monomer and helix I ( Ala-0, Ile-1, Met-4, Glu-5, Thr-8, Gln-12) of the other monomer. The orientations of Mode A and Mode B obtained in the present study were consistent with our previous all-atom simulation results (20). In addition, our results showed that the two monomers of the PSI dimer are interacting with the two surfaces of the lipid bilayers in a symmetrical way, which is natural, considering the fact that the sequence and structure of the two monomers are identical and the two monomers are arranged symmetrically when forming the dimer. The key residues involved in the interactions also explained the difference in vesicle binding between monomer and dimer PSI. As the hydrophobic residues of monomer align on the same side of the protein (Fig. 6B), the interactions between the monomeric PSI with one vesicle could be strong, whereas it lacks the capability of bringing two vesicles into close proximity. In contrast, the PSI dimer has hydrophobic interfaces on both sites, each of which can bind strongly with the one of the two vesicles, thus forming a bridge between the two vesicles.

**Discussion**

**The mechanism of membrane fusion by PSI**

The fusion of two liposomes requires overcoming the dehydration energy and repulsion forces between the two liposomes with negatively charged phospholipids. Membrane fusion proteins can accelerate this process by interacting with the liposomes and neutralizing charge-charge repulsion locally between the liposomes. This allows for the liposomes to come into close contact, leading to dehydration and ultimately membrane fusion.

With a single technique, it is difficult to obtain all of the details of the membrane fusion. In this study, FRET, solution NMR, SSNMR, and in silico studies were combined to elucidate the detailed mechanism by which the PSI induces membrane fusion. Each technique could provide an aspect of the mechanism; thus, the joint approach is likely to provide a more comprehensive picture. The pH-dependent FRET and solution NMR studies confirmed the pH dependence of the monomer-dimer equilibrium, of the activity as a membrane fusion protein, and of the PSI-liposome binding. Neutralizing the negative charges at low-pH conditions is critical in reducing the repulsion force between the protein and the liposome, thus leading to PSI-mediated membrane fusion. The NMR data also suggested the local environment of the PSI after fusion is dehydrated, confirming that dehydration involves the PSI. In silico studies indicated that the two monomers of the PSI dimers interact symmetrically on opposing sides with different lipid bilayers through residues of helix I and helix III, which bridge the liposomes, forcing them into close proximity, and eventually leading to membrane fusion. In the post-fusion complex, the PSI is proposed to bind to the surface of the liposome, either through a multilayered topology or by disturbing the shape of the membrane to surround itself (Fig. 5B). The conclusions of the coarse-grained molecular dynamics simulation here are also consistent with our earlier all-atom simulations of the protein topology on membranes (16).

Taken together, the mechanism of membrane fusion process induced by PSI involves a complex set of conditions and events, including pH-dependent monomer-dimer equilibrium, PSI interactions with membrane and the formation of a stable binding mode, transition of the binding mode to bring the two membranes closer, and eventually membrane fusion (Fig. 7). This mechanism could serve as the natural defense process used by the PSI, which occurs under acidic conditions, resulting in physiological activities in fighting against the external pathogens.

**Biological implications**

PSI is similar in sequence, structure, and function to proteins in the SAPLIP family, many of which are involved in the innate immune systems of animals (15). Although saposin proteins share similar structures, they differ in the lipid-binding targets and the mechanisms by which they catalyze membrane fusion (17). Compared with other SAPLIPs, PSI is unique to plants,
although it exhibits membrane fusion activities similar to those proteins from animals.

The plant cells may experience different physiological pH conditions (i.e., the acidic conditions in vacuole) (29). This work revealed that PSI experiences monomer-dimer equilibrium at a broad range of pH values, whereas the dimers induce membrane fusion at pH values below a critical pH. It implies that under physiological conditions, PSI in plant cells forms inactive monomer or dimers, whereas a decrease in pH could trigger the PSI dimer into an active resistant state. Indeed, in vivo data have shown that upon infection by P. infestans, the AP, the “parent” proenzyme of PSI, was found to be overexpressed and had a pH optimum from 2 to 5 (18, 19), consistent with the active pH for induction of membrane fusion observed in this study.

The cellular location of the S. tuberosum AP is still unclear, whereas earlier studies on the phytepsin, a homologue of AP from barley grains, indicated that the PSI sequence has a role in vacuolar sorting (15, 30). It is highly possible that the S. tuberosum AP localizes in the vacuole. The physiological condition of the vacuole is acidic and could vary the pH depending on the life cycles (29). The membrane fusion of the vacuole with the plasma membrane has been thought to be a critical step in releasing antimicrobial proteins and hydrolytic enzymes to attack pathogens (30, 31). Therefore, the studies of PSI regarding membrane fusion in acidic conditions could provide evidence in understanding its physiological roles. The S. tuberosum PSI may function in facilitating the sorting of the parent AP to the vacuole and working together with the AP in anti-pathogen activities. For instance, the PSI domain of AP induces membrane fusion at acid conditions, whereas the mature AP degrades the pathogen proteins as a “toxic” cargo that is transported to the intercellular space to attack pathogens. It is also worth noting that the phospholipids used in the present study are negatively charged. Detailed biophysical and biochemical characterization of the PSI fusion activity upon different lipid compositions is anticipated to reveal how PSI selects its binding targets.

Conclusions

In conclusion, this work reported a systematic investigation on the mechanism of PSI-induced membrane fusion whereby a combined approach of NMR, FRET, and in silico methods was used. Results showed that under acidic conditions, PSI experiences a monomer-dimer equilibrium and the dimeric form of PSI induces membrane fusion below a critical pH. After membrane fusion, the phospholipids protect PSI, leading to limited solvent accessibility, thus suggesting its capability in reducing repulsive dehydration forces between liposomes to drive the occurrence of the fusion. Molecular dynamics simulations indicated that the PSI dimer was found to bind stably to membrane surfaces and bridge liposomes in close proximity. In summary, this study, which used a combined approach, sheds light on the mechanisms by which the PSI and similar proteins induce membrane fusion.

Experimental procedures

**PSI expression and purification**

Isotope-labeled compounds ($^{15}$NH$_4$Cl and $^{13}$C$_6$-glucose) were obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Uniform $^{15}$N,$^{13}$C-labeled PSI was produced according to a protocol reported previously (9). The genes that encode thioredoxin-fused PSI protein with a His$_6$ tag at the C-terminal of thioredoxin were incorporated into the pET32b (+) plasmid. The constructed vector was transformed into Escherichia coli Rosetta-gami B (DE3) pLysS. The 2-ml cell culture was incubated at 37 °C with vigorous agitation at 250 rpm for 12 h. The overnight culture was inoculated in a 500-ml baffled flask in sterilized lysogeny broth medium. When the cell density reached an $A_{600}$ of 0.8, the cells were collected by centrifugation at 4000 × $g$ for 5 min. The biomass was washed and resuspended with 250 ml of sterilized M9 minimal medium for growth at 37 °C with vigorous agitation. Isopropyl β-D-1-thio-galactopyranoside was added to a final concentration of 0.5 mM to induce cell expression. After overnight growth at 30 °C, the cells were harvested by centrifugation at 5000 × $g$ for 5 min at 4 °C and frozen at −20 °C for later use. To produce $^{15}$N,$^{13}$C-labeled samples, $^{15}$NH$_4$Cl and $^{13}$C$_6$-glucose were used as the sole sources of nitrogen and carbon, respectively. The expression and purification approach was identical to that of natural abundance PSI.

To purify the PSI protein, frozen cells were thawed at room temperature and resuspended in 5 ml of 20 mM Tris buffer at pH 7.4 for every 100 ml of culture. The resuspension was incubated at room temperature for 15 min, and 1 µl of Benzonase nuclease was added for every 5 ml of lysate. The suspension was left incubated on ice for 1 h for lysis to fully occur, and the cell lysates were centrifuged at 5000 × $g$ for 20 min at 4 °C. The supernatant was further centrifuged at 20,000 × $g$ for 30 min at 4 °C to remove insoluble cell debris.

Protein purification was performed using a peristaltic variable-speed pump (Watson Marlow Inc.). The solution was applied to a 5 ml of HisPur nickel-nitrioltriacetic acid resin column (Pierce) and equilibrated with a pH 7.4 buffer (20 mM Tris, 300 mM NaCl, and 10 mM imidazole), followed by washing with buffer (20 mM Tris, 300 mM NaCl, and 30 mM imidazole at pH 7.4) for 10 column volumes. The thioredoxin-PSI fusion protein was eluted with elution buffer (20 mM Tris, 300 mM NaCl, and 300 mM imidazole at pH 7.4). The purified thioredoxin-PSI

**Figure 7.** Schematic representation of the membrane fusion mechanism of the PSI

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fission protein was then dialyzed against digestion buffer (20 mM Tris at pH 7.4). Thrombin was added to the solution at a 1:1000 mass ratio and incubated at 37°C for at least 12 h with mild agitation for hydrolysis of the fusion protein to separate thioredoxin from the PSI. The PSI was collected in the flow via reaplication of the affinity chromatography at room temperature using HisPur nickel-nitrilotriacetic acid resin columns. The purified PSI was then dialyzed against water. The final yield of purified PSI was ∼10 mg/liter of cell culture.

Preparation of liposome and lipid-mixing experiments

The lipid-mixing experiments were performed using a protocol reported previously (20). Two types of liposomes, nonfluorescent and fluorescent, were prepared to perform the lipid-mixing experiments. Nonfluorescent liposomes were composed of equimolar amounts of egg PC, egg PE, and brain PS. The fluorescent liposomes were composed of PC, PE, PS, Rhodamine lipids, and NBD-PE at a molar ratio of 1:1:1:1:0.6:0.6.

To prepare the liposomes, the lipid mixtures were stirred for 2 h at room temperature and dried under a nitrogen gas flow. The residual solvent was removed by vacuum for 3 h. The lipid film was further rehydrated in a reconstitution buffer (27 mM NaH2PO4 and 140 mM NaCl) with the desired pH values. The liposomes were extruded 20 times through two stacked polycarbonate membranes with a pore size of 100 nm after 10 freeze-thaw cycles.

To study the lipid-mixing experiments, the fluorescent and nonfluorescent liposomes, which were prepared in buffer with the desired pH, were mixed at a molar ratio of 1:9; the final concentration of the mixed liposomes was 300 μM. The PSI was added to the liposome mixtures to a final concentration of 15 μM to trigger membrane fusion in the pH range of 2.0–7.0. The fluorescent intensity of the fluorescent liposomes without proteins was set as baseline (0 fluorescence value, f0). The fluorescence value from mixing the fluorescent liposomes with a Tri-ton X-100 solution to a final concentration of 0.2% was set as 1.0 fluorescence value (f100). The fusion efficiency of the PSI-induced lipid mixing was determined with the equation,

\[ \% f_i = (f_i - f_0) / (f_{100} - f_0) \times 100 \]

Solution NMR spectroscopy

All solution NMR data were collected on a 500- or 600-MHz Bruker Avance III spectrometer equipped with a cryogenic triple-resonance TXI probe. All experiments were carried out at 298 K. The 3D triple-resonance experiments, including HNCA, HNCOCA, CBCACONH, HNCACB, and HNCO, were collected to perform resonance assignments. The triple-resonance NMR spectra were collected both at pH 2.0 and 7.0. At neutral pH, the 3D experiments were collected on a sample of 0.5 mM 15N,13C-labeled PSI dissolved in a pH 7.0 buffer (20 mM Tris and 10% D2O), whereas a 0.3 mM 15N,13C-labeled PSI in a pH 2.0 buffer (27 mM NaH2PO4 and 10% D2O) was used to record the 3D experiments in acidic conditions.

To analyze the aggregation states and site-specific dynamics of PSI in solution, the site-specific 15N relaxation parameters, including 15N–1H and H–1H, and steady-state heteronuclear 1H–15N NOE were measured for the PSI at both pH 2.0 and pH 7.0. The experiments were conducted using the standard pulse sequences in the Bruker sequence library. Relaxation experiments were performed with 0.3 mM 15N-labeled PSI. All relaxation experiments were performed at 298 K on a Bruker spectrometer at 500 MHz. For 15N–T1, 15N–T2 measurements, complex data points of 1024 (1H) and 80 (15N) were collected with 16 transients/increment and a recycle delay of 3.0 s. The relaxation delays used in the 15N–T1 experiments were 20, 100, 200, 300, 400, 600, 800, 1000, 1600, and 2000 ms. For the 15N–T2 experiments, the relaxation delays were 7.94, 15.88, 31.76, 47.64, 63.52, 95.28, 127.04, 158.80, 190.56, and 238.20 ms. The residue-specific relaxation times were fitted with a monoeponential function. The 1H–15N NOE experiments were performed with a 3.0-s presaturation period before a 15N excitation pulse with recycle delays of 5.0 s. A reference spectrum of the 1H–15N NOE experiment (non-NOE) was collected in a manner identical to that of the NOE experiments with the exception of running the presaturation pulse trains. Each experiment was collected with 32 transients.

To analyze the site-specific solvent accessibility, the H/D exchange experiments were performed on the dimeric PSI. The purified 15N-labeled PSI in water was lyophilized. The lyophilized PSI powders were dissolved in D2O-based buffer (27 mM NaH2PO4, pD 2.0) to a final concentration of 0.1 mM. A series of 1H–15N HSQC spectra were recorded to examine the H/D exchange.

All of the PSI-liposome titrations were carried out on 0.1 mM 15N-labeled PSI samples. For the PSI-liposome interaction at pH 2.0 and 3.4, the PSI was dissolved in 27 mM NaH2PO4 adjusted to the target pH values. 20 mM sodium acetate and 20 mM Tris buffers were used for titrations at pH 5.4 and 7.0, respectively. Liposomes with a composition of PC/PE/PS with a ratio of 1:1:1 were prepared to a concentration of 0.3 mM and suspended in the same buffer as used for the PSI. The lipidosome stock was added directly to the PSI solution in the NMR tube to reach a PSI-liposome molar ratio of 1:1. The 1H–15N HSQC spectra of PSI were recorded before and after the addition of liposomes. The number of scans was adjusted according to the concentration of the PSI before and after the addition of liposome.

Solid-state NMR spectroscopy

To investigate the topology of the PSI in post-fusion PSI-liposome complexes, a 0.3 mM 15N,13C-labeled PSI at pH 2.0 (27 mM NaH2PO4, 140 mM NaCl) was mixed with liposome (PC/PE/PS at a molar ratio of 1:1:1) at a molar ratio of 1:30 for 1 h at room temperature with gentle agitation. The PSI-liposome complexes were then ultracentrifuged at 900,000 × g at 4°C for 3 h. The collected complexes for SSNMR were center-packed in a 3.2-mm SSNMR rotor.

Approximately 4.0 mg of 15N,13C-labeled PSI in PSI-liposome complex was used in the SSNMR studies. All SSNMR measurements were acquired on a 600-MHz Bruker Avance III spectrometer equipped with a 3.2-mm E-free 1H/13C/15N probe. The rotor spinning frequency was set to 12.0 kHz, and the effective sample temperature was maintained at 298 K for all experiments. The temperature was calibrated externally by...
using this system. Another was a starting simulation box of Twenty independent production simulations were conducted last 500 ns of the trajectories were used for data analysis. MD 1:1:1 POPC/POPE/POPS was used in all of the systems. The same phospholipid combination of our previous work (16). The same phospholipid combination of states of the amino acids to pH 3.0, which was determined by was included in the simulations by assigning the protonation starting structure for all of the simulations. The effect of pH was implemented with a contact time of 5.5 ms, with a nitrogen radiofrequency field of ~42 kHz and carbon radiofrequency field ramped (10% ramp) linearly around ~30 kHz. The 1H CW decoupling during 13C-13C CP transfer was 100 kHz. The time domain matrix of 2D 15N,13C CP experiments was 160 (t1) × 2048 (t2), with total acquisition times of 15 and 20 ms for t1 and t2, respectively. The carrier frequency was set to 120 and 58 ppm for the 15N and the 13C dimensions, respectively. Two hundred scans were recorded with a recycle delay of 1.8 s for each experiment, resulting in a total NMR time of 20 h.

The SSNMR-based H/D exchange experiments were performed to examine the solvent accessibility of the PSI in PSI-liposome complex, following the published protocol (35). After centrifugation, the PSI-liposome complex was incubated in a D2O-based buffer for 24 h, followed by collection of 2D 15N-13C band-selective SPECIFIC CP (21) was implemented with a contact time of 5.5 ms, with a nitrogen radiofrequency field of ~42 kHz and carbon radiofrequency field ramped (10% ramp) linearly around ~30 kHz. The 1H CW decoupling during 13C-13C CP transfer was 100 kHz. The time domain matrix of 2D 15N,13C experiments was 160 (t1) × 2048 (t2), with total acquisition times of 15 and 20 ms for t1 and t2, respectively. The carrier frequency was set to 120 and 58 ppm for the 15N and the 13C dimensions, respectively. Two hundred scans were recorded with a recycle delay of 1.8 s for each experiment, resulting in a total NMR time of 20 h.

The SSNMR-based H/D exchange experiments were performed to examine the solvent accessibility of the PSI in PSI-liposome complex, following the published protocol (35). After centrifugation, the PSI-liposome complex was incubated in a D2O-based buffer for 24 h, followed by collection of 2D 15N-13C spectrum. In a 15N-13C experiment to study the H/D exchange, the 1H-15N CP was set with a mixing time of 300 μs to minimize the remote 1H-15N CP transfer. The other parameters were set identically as for the 15N-13C of PSI in H2O-based buffer.

All SSNMR data were processed using the NMRpipe program (36). The 13C chemical shift was calibrated externally to DSS (37). The data were analyzed using the CARA program.

Simulations

Simulation parameters—All coarse-grained molecular dynamics (MD) simulations were performed with Gromacs 5.1.3 (38) with the Martini2.2 (39–42) force field with extension to proteins and utilizing an elastic network (43). The crystal structure of the PSI dimer (PDB entry 3RFI, residue number in each monomer: from 0 to 39 and from 64 to 103) (9) was used as the starting structure for all of the simulations. The effect of pH was included in the simulations by assigning the protonation states of the amino acids to pH 3.0, which was determined by our previous work (16). The same phospholipid combination of 1:1:1 POPC/POPE/POPS was used in all of the systems. The last 500 ns of the trajectories were used for data analysis. MD Analysis (44) was used to calculate the principal axis. VMD (visual molecular dynamics) (45) was used to view trajectories and render figures.

For the coarse-grained self-assembly molecular dynamics simulations of the lipid molecules and the PSI dimer, two different sizes of simulation box were used. One was an initial simulation box of 36 × 36 × 15 nm with 4097 lipid molecules. Twenty independent production simulations were conducted using this system. Another was a starting simulation box of 48 × 48 × 24 nm with 11,090 lipid molecules (Fig. S10). Five independent production simulations were conducted using this system. In each system, one PSI dimer, water, and the lipid molecules were randomly placed in the simulation box. Then energy minimization (200,000 steps) and NVT (canonical ensemble) equilibration (500 ps) with position restraints on backbone beads (force constant 1000 kJ/mol/nm²) were performed. Following this, 1000-ns production simulations under the NPT ensemble (isothermal-isobaric ensemble) were conducted. In all simulations, the periodic boundary conditions were applied, and the time step was 10 fs. The V-rescale algorithm (46) with a time constant of 1.0 ps was used to maintain the temperature at 320 K. The Berendsen algorithm (47) with a time constant of 10 ps was used to maintain the pressure at 1.0 bar. The reaction-field method with a cut-off of 1.1 nm was used to calculate electrostatics, and a cut-off of 1.1 nm was used to calculate the van der Waals interactions.

Coarse-grained molecular dynamics simulations of vesicles and PSI—For the coarse-grained molecular dynamics simulations of vesicles and the PSI dimer, Martini Vesicle Maker (48, 49) in CHARMM-GUI (50) was used to build a vesicle with a radius of 7 nm. One PSI dimer was inserted between two vesicles immersed in water in the simulation box. The distance between the two vesicles was 4.5 nm, −0.1 nm shorter than the greatest length of the PSI dimer in its initial state. This system was energy-minimized for 200,000 steps. Then a 1000-ps NVT equilibration with position restraints on backbone beads (force constant 1000 kJ/mol/nm²) with a time step of 1 fs was performed. Following this, two 1000 ps NPT equilibrations with a time step of 1 fs and 10 fs were performed, respectively. Then eight independent NPT production simulations were conducted. The simulation time was 1000–15,000 ns. The other simulation parameters were the same as the self-assembly molecular dynamics simulations described above.

Molecular dynamics simulations for the PSI monomer—MODELLER 9.20 (51) was used to do the homology modeling of the PSI monomer. The crystal structure of the plant aspartic proteinase prophytepsin (30) (PDB entry 1QDM) was used as the template for homology modeling, which has a sequence identity of 52.29% with our target PSI (Fig. S11), much larger than the 30% that is required for a reasonable homology modeling (52) and ensuring that the homology model was accurate enough for MD simulations. The monomer model was equilibrated for 100 ns with the default protonation states, and then the protonation states at pH 3.0 were calculated with PROPKA 3.0 (53). Subsequently, another 400-ns all-atom simulation was conducted for the structure of the PSI monomer in the protonation states of pH 3.0 to relax the protein. The obtained structure was used as the starting structure of the coarse-grained self-assembly simulations.

For the all-atom molecular dynamics simulations, Gromacs with the charmm36m (54) force field and Tip3p (55) water model was used. After a 2000-step energy minimization with steepest descent, a 500-ps NVT equilibration was conducted followed by a 500-ps NPT equilibration. Then production simulation was conducted for 100 or 400 ns. Position restraint with a force constant of 1000 kJ/mol/nm² was applied on all of the heavy atoms for the equilibration simulations. The periodic
boundary conditions were applied with a time step of 2 fs. The temperature (310 K) was maintained with the V-rescale algorithm [46] with a time constant of 0.5 ps, and the pressure (1 bar) was maintained with the Berendsen algorithm [47] with a time constant of 1.0 ps. The particle mesh Ewald method [56] was used to calculate electrostatics, and the cut-off of the van der Waals interaction was 1.2 nm.

For the coarse-grained self-assembly simulations of the lipid molecules and the PSI monomer, the elastic network was only applied on the nonloop residues (residues 9–39 and 70–108) to ensure the flexibility of the loop regions (residues 1–8 and 40–69). Seven 4000-ns independent simulations were conducted, and the last 3000 ns of each trajectory were used for data analysis. All of the other simulation parameters were the same as described above for the coarse-grained self-assembly simulations of the PSI dimer.

Data availability

All data discussed is contained with the article or the supporting material. The $^1$H, $^{15}$N, and $^{13}$C chemical shifts of PSI were deposited in BMRB with accession codes 27771 for PSI at pH 7.0 and 27772 for PSI at pH 2.0, respectively.

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Abbreviations—The abbreviations used are: AP, aspartic protease; PSI, plant-specific insert; SAPLIP, saposin-like protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; NBD-PE, N-(7-nitro-2,1,3-benzoazadiazol-4-yl)-phosphatidylethanolamine; Rho-PE, N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; HSQC, heteronuclear single quantum coherence; CSI, chemical shift index; PDB, Protein Data Bank; SSNMR, solid-state NMR; 2D, two-dimensional; 3D, three-dimensional; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; MD, molecular dynamics.

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