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Title

Inhibition of cryoaggregation of phospholipid liposomes by an *Arabidopsis* intrinsically disordered dehydrin and its K-segment

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Total number of words

5310 words

Total number of tables/figures

no tables and 8 figures

Highlights

1. An intrinsically disordered dehydrin inhibited the cryoaggregation of liposomes.
2. The K-segment was the cryoprotective site of dehydrin.
3. The K-segment protected liposomes without binding them.
4. Liposomes affected the secondary structures of the K-segment.

Abstract

Dehydrin is an intrinsically disordered protein involved in the cold tolerance of plants. Although dehydrins have been thought to protect biomembranes under cold conditions, the underlying

protective mechanism has not been confirmed. Here we report that *Arabidopsis* dehydrin AtHIRD11 inhibited the aggregation of phospholipid liposomes after freezing and thawing. AtHIRD11 showed significantly greater cryoaggregation-prevention activity than cryoprotective agents such as trehalose, proline, and polyethylene glycols. Amino acid sequence segmentation analysis indicated that the K-segment of AtHIRD11 inhibited the cryoaggregation of phosphatidylcholine (PC) liposomes but other segments did not. This showed that K-segments conserved in all dehydrins were likely to be the cryoprotective sites of dehydrins. Amino acid replacement for a typical K-segment (TypK for short) sequence demonstrated that both hydrophobic and charged amino acids were required for the cryoaggregation-prevention activity of PC liposomes. The amino acid shuffling of TypK remarkably reduced cryoprotective activity. Although TypK did not bind to PC liposomes in solution, the addition of liposomes reduced its disordered content under crowded conditions. Together, these results suggested that dehydrins protected biomembranes via conserved K-segments whose sequences were optimized for cryoprotective activities.

Keywords: Cryoaggregation; Cryoprotection; Dehydrin; Intrinsically disordered proteins; Liposome; Phosphatidylcholine

1. Introduction

Environmental temperature is a cardinal factor determining the growth and development of organisms. Tolerance to extreme cold (i.e., freezing) influences not only the survival of individual organisms but also the formation of ecosystems [1, 2], and cold stress affects plant production and vegetation dynamics. The appropriate management of cold stress is important for crop production [3], postharvest technologies [4], and seed storage [5]. Some plant species are known

to withstand freezing after cold acclimation, and many orthodox seeds also exhibit high freezing tolerance [6]. Freezing tolerance is based on the prevention of damage symptoms induced by freezing and thawing (designated hereafter as F/T), such as membrane destabilization, protein denaturation, macromolecule precipitation, and the destruction of cellular components due to ice formation [7]. Among these symptoms, the protection of membranes has been described as the most crucial for freezing tolerance because the plasma membrane is the initial site of freezing injuries in plants [7, 8]. In nonacclimated plants, freezing induced the reorganization of membranes, such as through membrane invagination and lipid aggregation, which leads to the hexagonal II phase [8]. These lesions damage cells severely because membrane disorder excessively enhances membrane permeability to water and solutes [9]. Molecules that prevent damage to plant membranes have been identified. Soluble sugars, such as sucrose, trehalose, and sorbitol, protected membranes by preventing stress-induced membrane fusion [10]. Proline and glycine betaine protected chloroplast membranes against freezing [11, 12]. Several proteins, such as cold-regulated proteins (e.g., COR15), osmotin-like protein, β-1,3-glucanase, lectins, and lipid transfer proteins, are known to stabilize membranes during F/T [13]. Some late embryogenesis abundant (LEA) proteins, particularly group 2 and group 3 LEAs, have been frequently described as membrane-stabilizing proteins [14, 15].

LEA proteins are expressed in seeds at the late stage of embryogenesis and in stressed plants. LEA protein expression was responsive to various stresses (e.g., cold and drought) and abiotic stress-related hormones such as abscisic acid. Many genetic studies have indicated correlations between LEA accumulation and both seed longevity and stress tolerance in plants [14, 16]. Group 2 LEAs, designated as dehydrins, are intrinsically disordered proteins (IDPs) that are widely found in the plant kingdom. Dehydrins are known to consist of characteristic segments called K-segments (e.g., EKKGIMEKIKEKLPG), S-segments (e.g., LHRSGSSSSSSSEDD), Y-segments (e.g., DEYGNP), and Φ-segments, which are rich in G and polar amino acids that connect K-, S-, and Y-segments [17]. ChP-segments containing K and E repeats [18] and F-segments

105 (DRGLFDFLGKK) [19] have also been reported. Among the segments, K-segments are
106 conserved in all dehydrins, whereas other segments are present in some dehydrins but not in others.
107 Namely, dehydrins are defined by the presence of K-segments. Dehydrins were ubiquitously
108 found in various subcellular localizations in plants, especially near the plasma membrane [20].
109 Since cold is a major cue for the expression of dehydrins, the proteins have been thought to be
110 related to cold tolerance in plants [21]. Many transgenic studies have demonstrated that the
111 expression of dehydrin genes enhanced the cold tolerance of plants [22-25]. In vitro investigations
112 have shown that dehydrins have multiple functions, such as preventing enzyme inactivation by
113 freezing, binding to water and ions, and associating with anionic phospholipids and nucleic acids
114 [26]. Cumulatively, these functions may efficiently mitigate cellular damage due to cold.
115 Electrolyte leakage is a typical symptom of freeze damage in plants. It has been reported that
116 the expression of dehydrins reduced freeze-induced electrolyte leakage [e.g., 22], suggesting that
117 dehydrins may stabilize plasma membranes. From this context, the association between dehydrins
118 and membranes has been studied. Dehydrins bound to liposomes containing anionic
119 phospholipids such as phosphatidic acid (PA) [27-29], phosphatidylserine (PS) [30], and
120 phosphatidylglycerol (PG) [31] but showed little affinity for neutral phospholipid liposomes such
121 as phosphatidylcholine (PC) [27-29].
122 Dehydrins affected the aggregation of anionic phospholipid liposomes. A dehydrin from *Vitis*
123 *riparia* (K_2) inhibited the aggregation of PC liposomes containing PA, PG, or PS after the F/T
124 process [29]. *Thellungiella salsuginea* dehydrins TsDHN-1 and TsDHN-2 stabilized the lipid
125 monolayers, which consisted of lipid mixtures containing PS [32]. *Arabidopsis thaliana* dehydrin
126 Lti30 stabilized the lamellar structures of anionic phospholipids [33]. However, the mechanisms
127 by which dehydrins protect anionic phospholipid liposomes have not been confirmed, and the
128 activities by which they protect neutral phospholipid liposomes have not been studied yet.
129 In this paper we report that *Arabidopsis* dehydrin AtHIRD11 prevented the cryoaggregation of
130 PC liposomes via its K-segment, and we discuss the putative mechanism underlying this

cryoprotection of PC liposomes.

2. Material and methods

2.1. Chemicals

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (PG) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Trehalose, sucrose, glycine betaine, proline, and polyethylene glycol (PEG) with an average molecular weight of 7,500 were purchased from Wako (Osaka, Japan). PEG with an average molecular weight of 1,450 was from Sigma-Aldrich (Tokyo, Japan).

2.2. Peptides

AtHIRD11 was chemically synthesized by the 9-fluorenylmethyloxycarbonyl-based long-peptide synthetic system (Biosynthesis, Lewisville, TX, USA). AtHIRD11 was purified and identified by a Voyager DE-RP mass spectrometer (Applied Biosystems, Foster City, CA, USA). AtHIRD11 segments (i.e., NK1-6 and Kseg), a typical K-segment (TypK), and TypK-related peptides were prepared by the solid-phase procedure (Tetras, Advanced ChemTech, Louisville, KY, USA) and purified by reversed-phase chromatography (UFLC-20AB, Shimadzu, Kyoto, Japan). A linear gradient (acetonitrile from 5% to 95% in 0.05% trifluoroacetic acid solution) for 25 min was conducted in the reversed-phase column (Alltima C18, 4.6 × 250 mm, Alltech Associates, Deerfield, IL, USA). The synthesized peptides were identified by mass spectrometry (LCMS-2020, Shimadzu) and lyophilized. When the peptides were used for experiments, the concentrations of peptide solutions were determined from the gross weight of the lyophilized powder.

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158 *2.3. Liposome preparation*

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160 The phospholipids used were PC, PG, and a mixture of PC and PG at a molar ratio of 3:1
161 [designated below as "PC:PG (3:1)"]. Phospholipid powders were dissolved in chloroform at a
162 total lipid concentration of 10 mM (routinely 1-2 mL) in a glass vial. After the chloroform was
163 evaporated, the residue was dried under a stream of nitrogen gas. The phospholipid film was
164 covered with 10 mM sodium phosphate buffer pH 7.0 (designated below as "NaPi buffer") at a
165 concentration of 10 mM and incubated for 1 h at 45℃. The phospholipid dispersion was gently
166 pipetted and transferred to a 1.5-mL plastic tube. A sample (1 mL) was treated with 5 cycles of
167 F/T (in liquid N_2 for 1 min and then in a water bath at 25℃ for 4 min) and then extruded 21 times
168 using a Mini Extruder (Avanti Polar Lipids, Alabaster, AL, USA) through 100-nm-pore-size
169 polycarbonate membranes (Avanti Polar Lipids). The distribution of particle sizes was checked
170 out by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments,
171 Malvern, UK). The liposome sample (70 μL) was transferred to a cuvette (ZEN0040, Malvern
172 Instruments) and analyzed under the following conditions: refractive index detector (1.450),
173 absorption coefficient (0.001), dispersant (water), temperature (25°C), and measurement angle
174 (173°). The liposome dispersion was used for the following experiments.

175

176 *2.4. Cryoaggregation of liposomes*

177

178 The liposome dispersions (10 mM) prepared as described above were diluted to 1.9 mM with
179 NaPi buffer. The sample (500 μL) was subjected to F/T (in liquid N_2 for 1 min and then at 25℃
180 for 3 min) for 1-10 cycles. Turbidity was measured at 415 nm in 96-well microplates with a reader
181 (Varioskan Flash, Thermo Fisher Scientific, Tokyo, Japan) at 25 °C. The particle sizes were
182 monitored by the Malvern Zetasizer Nano ZS as described above.

Inhibition of liposome cryoaggregation was measured as follows. The mixtures (320 μL with NaPi buffer) containing test compounds (i.e., AtHIRD11, segments, and cryoprotective agents) and liposome dispersions (1.9 mM) were prepared. The concentrations of test compounds differed from assay to assay. The mixtures were divided into two equal portions. One portion was used as a control (before F/T) and the other was proceeded to F/T (in liquid N_2 for 1 min and then at 25℃ for 3 min, 3 cycles) (after F/T). The turbidities of the samples were measured as described above. In some cases, the values of relative cryoaggregation (RCA) were determined. Protective compounds reduced the F/T-promoted increase in turbidity. The magnitude of the increase in turbidity without protectant was set to 100% RCA. The concentration of the compound at which the RCA value reached 50% was represented as IC_{50}.

In this work, liposomes that contained only buffer were used because they were easily prepared with high yield and uniform quality. However, it should be noted that an osmotic imbalance between the inside and outside of a liposome may occur, depending on the additives.

2.5. Size exclusion chromatography (SEC)

SEC was applied to analyze whether TypK could bind to PC liposomes. For specific detection, fluorescein isothiocyanate (FITC)-labeled TypK (i.e., FITC-TypK) was used. An open column (15 mm diameter x 28 mm thickness) of Bio-Gel P-60 (Bio-Rad Laboratories, Tokyo, Japan) was prepared. NaPi buffer was a running buffer. Samples (20 mM PC liposome and/or 50 μM FITC-TypK, 200 μL) were loaded. The flow rate was 1 mL min^{-1}, and the fraction size was 0.2 mL. The fractions were analyzed by fluorescence (Ex 494 nm and Em 520 nm, Varioskan Flash) for FITC-TypK and by a PC assay kit (LabAssay™ Phospholipid, Wako, Osaka, Japan) for PC liposomes.

2.6. Circular dichroism (CD)

The secondary structures of TypK were analyzed by CD. TypK (40 μM) with PC liposomes (0 and 0.4 mM) was prepared in NaPi buffer. Samples were applied to a spectropolarimeter (J-820, Jasco, Tokyo, Japan) under the following analytical conditions: scan range from 195 to 250 nm, scan speed 100 nm min^{-1}, resolution 1 nm, and cell width 2 mm. The CD data were transformed into the contents of secondary structures using K2D3 software (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/) [34].

2.7. Statistical analysis

Data for *P* values were analyzed by Dunnett's test at a significance level of 0.05.

3. Results

3.1. Inhibition of liposome cryoaggregation by AtHIRD11

It has been reported that phospholipid liposomes, especially those of neutral phospholipids such as PC, were aggregated and fused when they were treated with F/T [35-37]. Cryoprotective agents have been used to prevent liposome cryoaggregation [35, 38]. K_2 dehydrin from *V. riparia* inhibited the increase in the particle sizes of negatively charged phospholipid liposomes after F/T [29]. However, it has not been investigated whether dehydrins prevent the cryoaggregation of neutral phospholipid liposomes. Here the effect of AtHIRD11 on the cryoaggregation of neutral phospholipid liposomes was assessed.

In general, treatment of liposome dispersions with F/T promoted aggregation (Fig. 1A). The turbidities of PC liposome dispersions increased as the number of F/T cycles increased (Fig. 1B, circles). Transmission electron microscopy (TEM) indicated the apparent aggregation of PC

liposomes after F/T, in which the original shapes of liposomes were visually indistinguishable (Supplementary Fig. 1). The increase in turbidity was greater in PC liposomes than in PC:PG (3:1) liposomes or PG liposomes (Fig. 1B). DLS indicated that the distribution of particle sizes of the three kinds of liposomes [PC, PC:PG (3:1), and PG] was centered around 100 nm diameter (Fig. 1 C, red lines). After three cycles of F/T, the distribution of particle sizes became broader (Fig. 1 C, blue lines). The upsizing of particles after F/T was more remarkable in PC liposomes than in PC:PG (3:1) and PG liposomes.

AtHIRD11, an *Arabidopsis* KS-type dehydrin (AGI locus code, At1g54410), was used to investigate dehydrin's effect on the cryoaggregation of liposomes. AtHIRD11 is the smallest of the 10 dehydrin genes in *Arabidopsis* [39]. The orthologues in *Medicago sativa* [40] and *Hordeum vulgare* [41] were highly induced by cold stress. However, the elevation of AtHIRD11 content in *Arabidopsis* due to cold stress was slight, because AtHIRD11 accumulated abundantly in the whole plant before the cold treatment [39]. AtHIRD11 was added to the liposome dispersions, and then the mixture was treated with three cycles of F/T. AtHIRD11 inhibited the cryoaggregation of all three kinds of liposomes in a dose-dependent manner (Fig. 2). The IC_{50} values for the aggregations of PC, PC:PG (3:1), and PG liposomes were 47.3 μM, 21.7 μM, and 12.8 μM, respectively. General cryoprotective agents, such as saccharides (trehalose and sucrose), compatible solutes (glycine betaine and proline), and polyols (PEGs), were tested for the inhibition of the cryoaggregation of PC, PC:PG (3:1), and PG liposomes (Fig. 3). The IC_{50} values were represented as mass concentrations (g L^{-1}) to compare the inhibitory activities of compounds whose molecular weights were highly different from each other. Although trehalose, sucrose, glycine betaine, and proline apparently inhibited liposome cryoaggregation, their inhibitory activities were lower than that of AtHIRD11. PEGs inhibited the cryoaggregation of liposomes less than the other agents did. These results suggested that AtHIRD11 showed higher cryoprotective activities for both neutral and negatively charged liposomes than general cryoprotectants.

3.2. Cryoprotective activities of K-segments

Since plant membranes are composed mainly of neutral phospholipids including PC [42, 43], PC liposomes were used for the following analysis. We determined which segments were involved in the cryoprotection of PC liposomes. AtHIRD11 was 98 amino acids in length (Fig. 4A). Its amino acid sequence was divided into seven segments (NK1 to 6 and Kseg), each consisting of 15 amino acids. Here, Kseg indicates a K-segment conserved in all dehydrins and NK is an abbreviation for non-K-segment. Kseg apparently reduced the increase in turbidity of PC liposome dispersions, but NKs did not (Fig. 4B). This was supported by DLS analysis. Only Kseg inhibited the increase in particle diameters of PC liposome dispersions after F/T (Supplementary Fig. 2). These results suggested that Kseg is a cryoprotective site for PC liposomes. The dose-dependent inhibition by Kseg is shown in Fig. 4C. The IC_{50} value was 162 μM.

AtHIRD11's Kseg sequence (H_{41}KEGIVDKIKDKIHG$_{55}$) is not identical to the general sequence of the K-segment found in various plant species, which is EKKGIMEKIKEKLPG. That general sequence was designated as a typical K-segment (TypK) [44]. To elucidate the general cryoprotective effects of K-segments, TypK was used for the following experiments. The preliminary results indicated that the IC_{50} value of TypK (around 200 μM) was somewhat higher than that of Kseg (162 μM), suggesting that TypK and Kseg had similar degrees of cryoprotective activity for PC liposomes.

3.3. TypK did not bind to PC liposomes

Since dehydrins and K-segments have been reported not to bind to PC liposomes [27, 45], we confirmed that TypK unbound from PC liposomes by using SEC (Fig. 5). TypK was labeled with fluorescein isothiocyanate (FITC) at the N-terminus (FITC-TypK) for sensitive fluorescence

detection. When the mixture of PC liposomes and FITC-TypK was subjected to SEC, they were eluted separately. The elution fractions of PC liposomes and FITC-TypK were nearly identical to the corresponding fractions when they were applied separately to SEC, showing that TypK did not bind to PC liposomes. The isothermal titration calorimetry (ITC) analysis also suggested that TypK did not interact with PC liposomes (Supplementary Fig. 3).

CD analysis indicated that the structure of TypK was highly disordered because a dominant negative peak was observed around 200 nm (Fig. 6A, blue line). The negative peak was also observed when PC liposomes were added (Fig. 6A, red line). This structural feature was quantified by using K2D3 software (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/) [34] that predicts secondary structures of peptides from the CD data (Fig. 6B). The structure of TypK was predominantly disordered (Fig. 6B, TypK - PC liposome), because "others" (interpreted as disordered) accounted for approximately 90% of the composition. The addition of PC liposomes did not change the percentage of disordered content (Fig. 6B, TypK + PC liposome). However, when TypK and PC liposomes were more concentrated in solution, the disordered state of TypK was reduced in the presence of liposomes (Supplementary Fig. 4).

To investigate whether TypK's amino acid order influenced its cryoaggregation-prevention activity, we produced 20 mutant peptides by randomly changing its sequence order. The shuffling peptides were generated by the RAND function in Excel 2016. The results showed that all 20 mutant peptides had remarkably lower activity levels than TypK (Supplementary Fig. 5), demonstrating that TypK's activity depended on its amino acid sequence but not on its amino acid composition.

TypK is known to have four hydrophobic amino acids (I5, M6, I9, and L13), three negatively charged amino acids (E1, E7, and E11), and five positively charged amino acids (K2, K3, K8, K10, and K12). To test whether the hydrophobic residues were related to the inhibition of PC liposome cryoaggregation, we prepared a mutant peptide (pG_TypK_Φ) in which the amino acids other than hydrophobic residues were changed to G (Fig. 7). Similarly, pG_TypK_E and

313 pG_TypK_K were produced to investigate the respective effects of negatively and positively
314 charged residues on the inhibitory activities. The IC_{50} values showed that pG_TypK_Φ,
315 pG_TypK_E, and pG_TypK_K had low activity levels. pG_TypK_EK, pG_TypK_ΦE, and
316 pG_TypK_ΦK also showed low activity. However, pG_TypK_ΦEK exhibited potent activity.
317 These data demonstrated that all of the hydrophobic, negatively charged, and positively charged
318 residues were required for the cryoprotection of PC liposomes by TypK.
319
320 **4. Discussion**
321
322 Plasma membrane is the primary site of freezing injuries in plants. After F/T, phospholipid
323 membranes are mechanically damaged and incorrectly fused [8]. The disruption of plasma
324 membrane results in electrolyte leakage from cells; if the magnitude of the leakage is significant,
325 the plant will die. Dehydrins are IDPs that accumulate in mature seeds and plants exposed to cold
326 and drought stresses. Although dehydrins have been found to protect cells from stresses, the
327 mechanism underlying this protection has not been fully elucidated.
328 K_2, a dehydrin from *V. riparia*, was shown to prevent the cryoaggregation of liposomes
329 containing anionic phospholipids [29]. Similar protective effects of dehydrins on anionic
330 phospholipid membranes have been documented elsewhere [32, 33]. In these cases, dehydrins
331 have been thought to protect the membranes by binding to anionic phospholipids, whereas the
332 details of cryoprotective mechanisms have been under discussion. Besides that, the effects of
333 dehydrins on the cryoaggregation of neutral phospholipid liposomes have not been reported even
334 though neutral phospholipids are major components of plant membranes [42, 43].
335 Here, we demonstrated that AtHIRD11, Kseg, and TypK inhibited the cryoaggregation of PC
336 liposomes. This indicated that K-segments are sites in dehydrins that inhibit liposomal
337 cryoaggregation. Because TypK (molecular weight: 1728) inhibited PC liposome aggregation at
338 an approximately 100-fold lower concentration than did PEG 1450 (average molecular weight:

1450), the molecular weight (i.e., the hydrodynamic radius) of a cryoprotective polymer was not the sole determinant of the polymer's cryoprotective activity. Since TypK's cryoprotective activity depended heavily on its amino acid sequence (Fig. 7), the results suggest that the TypK sequence is optimized for the cryoprotection of liposomes.

We next discuss how TypK prevented the cryoaggregation of PC liposomes. It has been well documented that dehydrins could bind to acidic phospholipid liposomes [27, 29, 30, 32, 45]. K-segments might bind to acidic phospholipids and sodium dodecyl sulfate [28, 29, 45, 46]. On the other hand, dehydrins did not bind to neutral phospholipids [27, 29]. TypK also did not bind to PC liposomes (Fig. 5 and Supplementary Fig. 3). In addition, the structure of TypK was highly disordered, and PC liposomes did not alter the disordered state (Fig. 6). Intriguingly, however, the strong negative peaks around 200 nm (showing disorder) in CD were reduced when TypK and PC liposomes were highly concentrated (Supplementary Fig. 4). This suggests that TypK may change the structure from disordered to ordered when TypK and PC liposomes are crowded together.

Combining these results, in Fig. 8 we show the putative cryoprotective mechanism of TypK for PC liposomes. F/T treatment resulted in mechanical damage to phospholipid bilayers after the formation of ice crystals [38] (Fig. 8A, B). The damaged sites of the membranes were associated together via hydrophobic interactions (Fig. 8C), and then the membranes were fused and aggregated (Fig. 8D). Indeed, TEM observation indicated that the interfaces of PC liposomes were not clear in the fused aggregates (Supplementary Fig. 1B). TypK added to the liposome dispersions might form an ordered structure adjacent to the membranes without binding to the liposomes (Fig. 8E). After F/T, the hydrophobic area of the ordered TypK might facilitate the repair of the damaged membranes by hydrophobic attractions (Fig. 8F). The cryoprotection of lactate dehydrogenase by TypK was previously found to depend on the hydrophobic residues [44]. Thus, the importance of hydrophobic residues might be a common feature for the cryoprotective activities of K-segments for biomembranes and enzymes. On the other hand, it is noteworthy that AtHIRD11's cryoprotective activity did not depend only on the activity of Kseg, because the IC_{50}

value of Kseg (162 μM) (Fig. 4) was remarkably higher than that of AtHIRD11 (47.3 μM) (Fig. 2). This suggests that dehydrin's large hydrodynamic radii, which are due to the intrinsic disorder, may be related to the efficient prevention of collision between biomembranes. Reports that the large hydrodynamic radii of dehydrins were crucial for the inhibition of enzyme cryoaggregation [47, 48] support this idea.

Considering that a wheat dehydrin accumulated close to plasma membrane [20] and that dehydrin-expressing plants showed less electrolyte leakage than wild-type plants after cold stress [e.g., 22, 25], we can infer that dehydrins may protect plant plasma membranes by the above mechanism in vivo. Intriguingly, AtHIRD11 potently inhibited the cryoaggregation of soybean phospholipid liposomes (Supplementary Fig 6). In conclusion, dehydrins can be efficient cryoprotectants in plants. Further studies will facilitate the breeding of stress-tolerant plants and the development of cryopreservation technologies.

Funding

This work was supported by Grants-in-Aid (18H02222 and 19K22274) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

CRediT authorship contribution statement

Y. Kimura: Experimentation and original manuscript writing. **T. Ohkubo:** Experimentation. **K. Shimizu:** Conceptualization and editing of the manuscript. **Y. Magata:** Conceptualization. **E.Y. Park:** Supervision. **M. Hara:** Supervision, conceptualization, and writing and editing of the manuscript.

Declaration of competing interest

All the authors declare no conflict of interest.

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464
465 **Figure legends**
466
467 **Fig. 1.** Turbidities of liposome dispersions after F/T. (A) Illustration of liposome cryoaggregation.
468 (B) Changes in turbidities of PC, PC:PG (3:1), and PG liposome dispersions during F/T cycles.

469 Liposomes were dispersed in NaPi buffer at a concentration of 1.9 mM. Turbidity was monitored
470 at 415 nm. Circles, triangles, and squares correspond to PC, PC:PG (3:1), and PG, respectively.
471 Values represent means and standard deviations of three independent experiments. (C) Particle
472 sizes of PC, PC:PG (3:1), and PG liposomes. A DLS system (Malvern Zetasizer Nano ZS) was
473 used. Liposome dispersions were treated with three cycles of F/T. Measurements were done
474 before F/T (red lines) and after F/T (blue lines). Values shown are the means of three
475 measurements.
476
477 **Fig. 2.** AtHIRD11's effect on the aggregation of liposomes after F/T. (A) Reduction of the
478 cryoaggregation of liposomes by AtHIRD11. White and gray bars represent before and after F/T,
479 respectively. (B) Dose-dependent inhibition of the cryoaggregation of liposomes by AtHIRD11.
480 RCA and IC_{50} values are shown. For (A) and (B), PC, PC:PG (3:1), and PG liposomes (1.9 mM
481 with NaPi buffer) were used. Values shown represent the means and standard deviations of three
482 independent experiments. Asterisks indicate significant differences ($p < 0.05$) between before and
483 after F/T.
484
485 **Fig. 3.** Effects of cryoprotective agents on the cryoaggregation of liposomes. PC, PC:PG (3:1),
486 and PG liposomes (1.9 mM with NaPi buffer) were used. Liposome dispersions were subjected
487 to three cycles of F/T after the addition of different concentrations of cryoprotective agents. The
488 IC_{50} values were determined. Values represent means and standard deviations of three
489 independent experiments. Asterisks indicate significant differences ($p < 0.05$) from AtHIRD11.
490 For IC_{50} values greater than 50 g L^{-1}, the tops of the columns are shown as broken. In these cases,
491 the asterisks are not labeled.
492
493 **Fig. 4.** Effects of AtHIRD11 segments on PC liposome cryoaggregation. (A) Amino acid sequence
494 of AtHIRD11. Yellow, blue, and red residues are hydrophobic, positively charged, and negatively

charged, respectively. (B) Effects of segments on the cryoaggregation of PC liposomes. Segments (500 μM) and liposomes (1.9 mM) were prepared in NaPi buffer. White and black bars represent before and after F/T, respectively. Values indicate means and standard deviations of three independent experiments. Asterisks indicate significant differences ($p < 0.05$) between before F/T and after F/T. (C) Dose-dependent inhibition of PC liposome cryoaggregation by Kseg. RCA and IC_{50} values are shown. Means and standard deviations of three independent experiments are represented.

Fig. 5. SEC analysis. (A) PC liposomes and FITC-TypK were loaded separately into the column. (B) The mixture of PC liposomes and FITC-TypK was loaded. Orange and green bars represent approximate peaks of liposomes and FITC-TypK, respectively.

Fig. 6. Secondary structures of TypK. (A) CD spectra of TypK (40 μM) with NaPi buffer. PC liposomes (0.4 mM) were added (TypK + PC liposome) or not (TypK − PC liposome). (B) Secondary structures of TypK were analyzed by K2D3 software. Means and standard deviations of three independent experiments are shown.

Fig. 7. Inhibition of cryoaggregation by TypK and its mutants. Peptides (500 μM) and PC liposomes (1.9 mM) were mixed in NaPi buffer. Yellow, blue, and red residues are hydrophobic, positively charged, and negatively charged, respectively. Values represent means and standard deviations of three independent experiments. Asterisks indicate significant differences ($p < 0.05$) from TypK. For IC_{50} values greater than 500 μM, the tops of the columns appear as broken. In these cases, the asterisks are not labeled.

Fig. 8. A putative scheme of membrane cryoprotection by TypK. Phospholipid membrane (A) was damaged by F/T (B). The damaged membrane was fused and aggregated (C, D). TypK formed

an ordered structure (E) and protected the damaged membrane (F). Symbols Φ, -, and + represent hydrophobic amino acids, negatively charged amino acids, and positively charged amino acids, respectively.

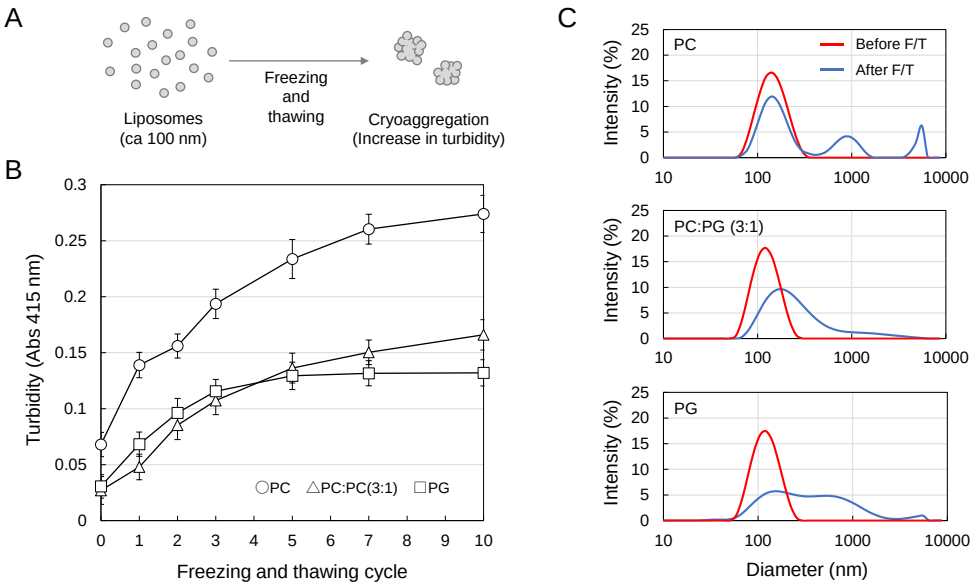
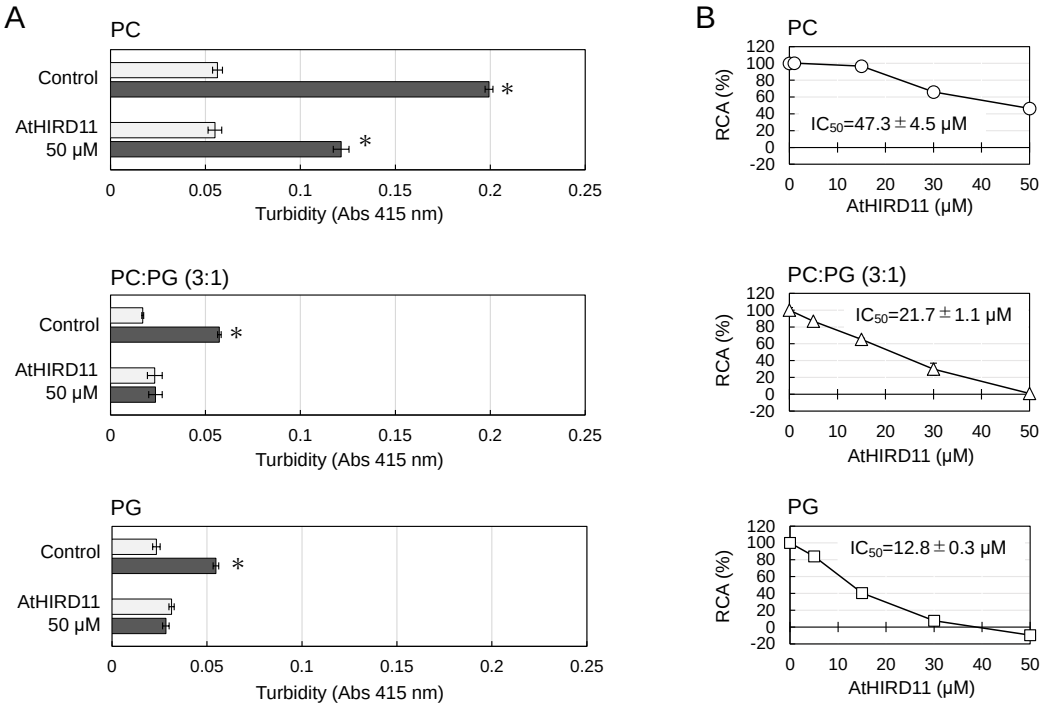


Fig. 1 Kimura et al.



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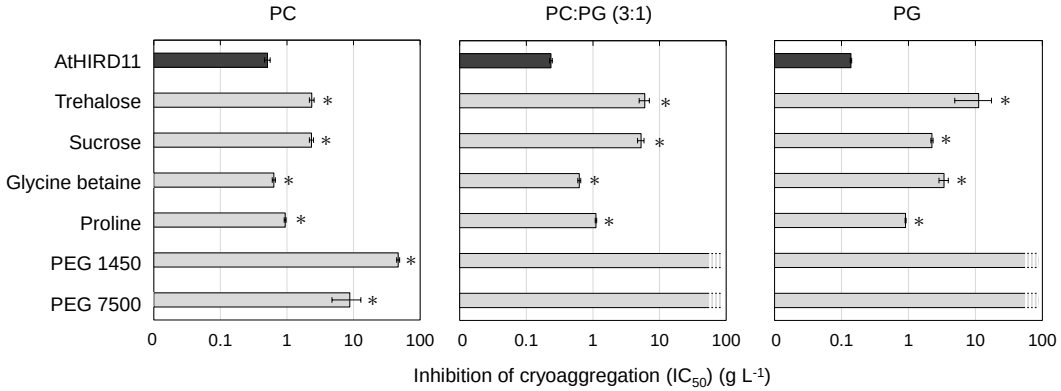

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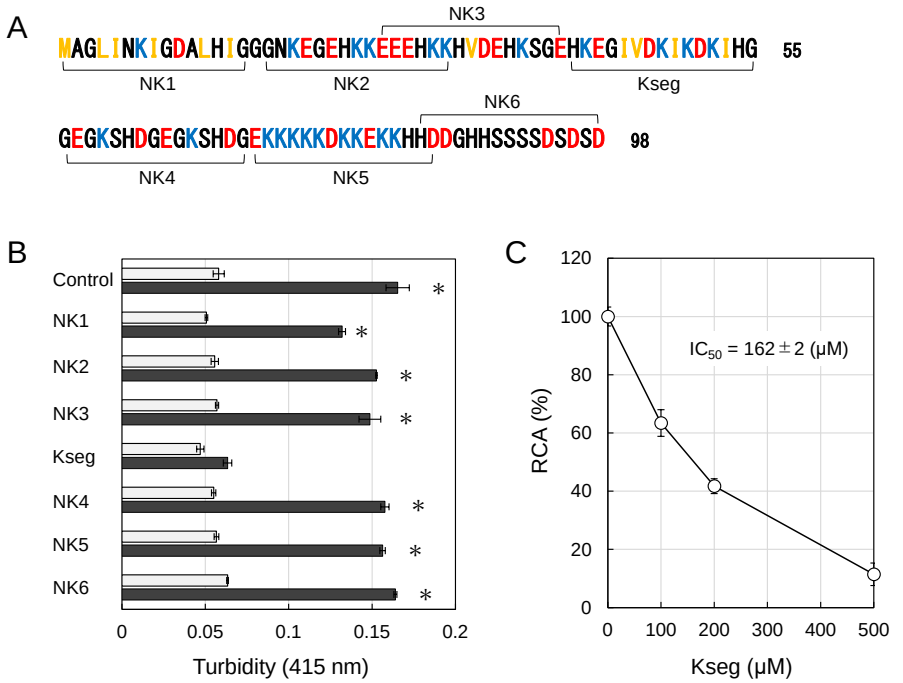

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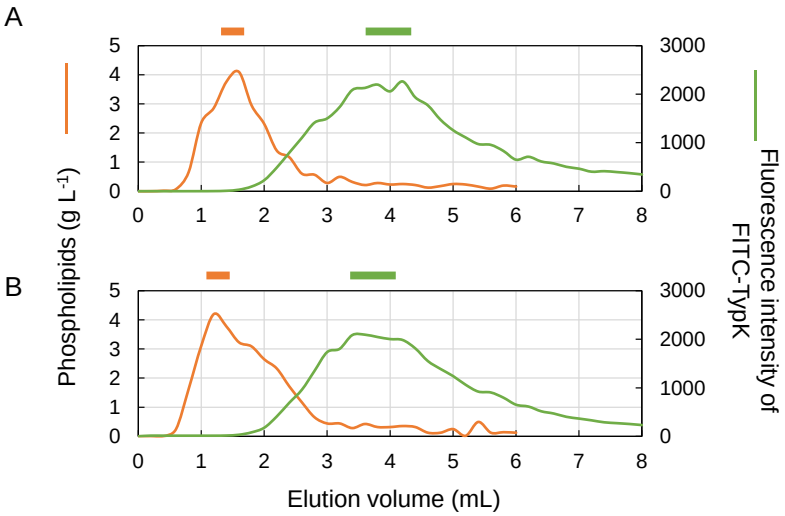

Fig. 5 Kimura et al.

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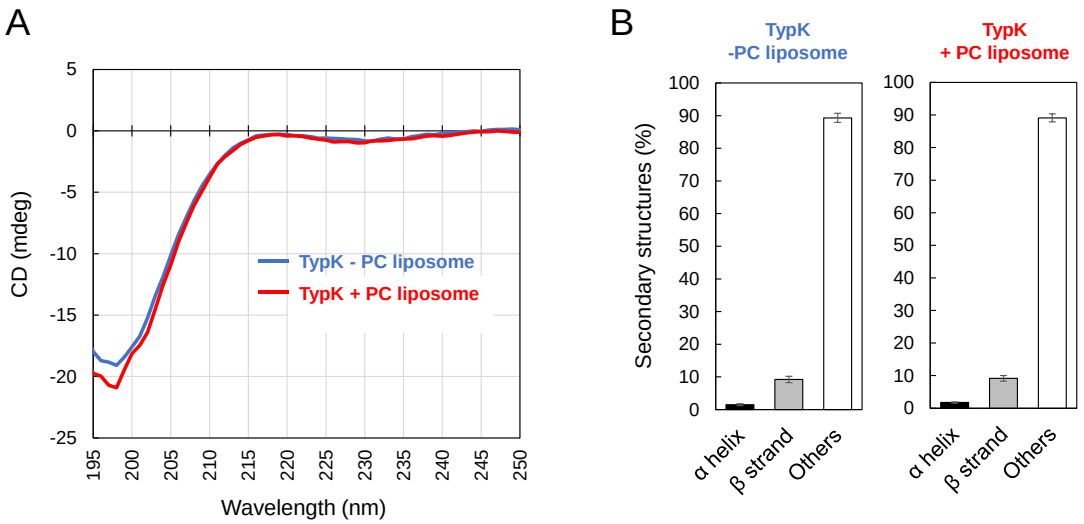

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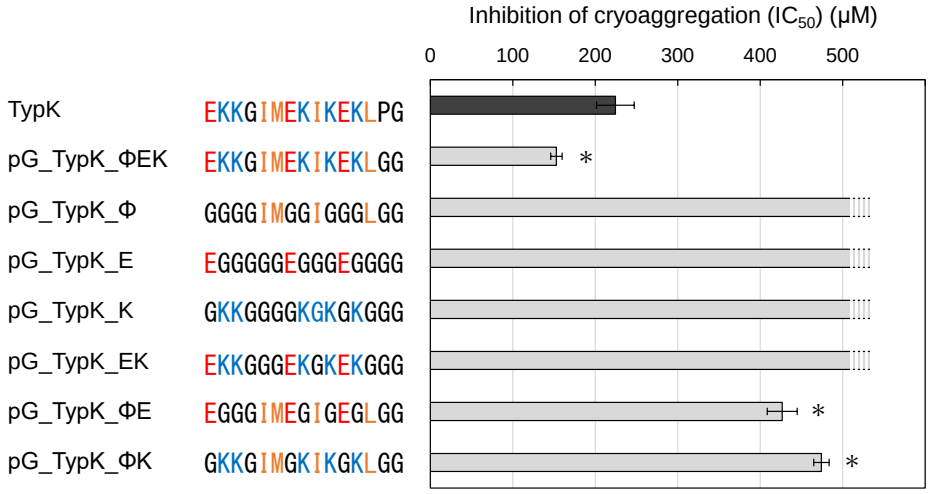

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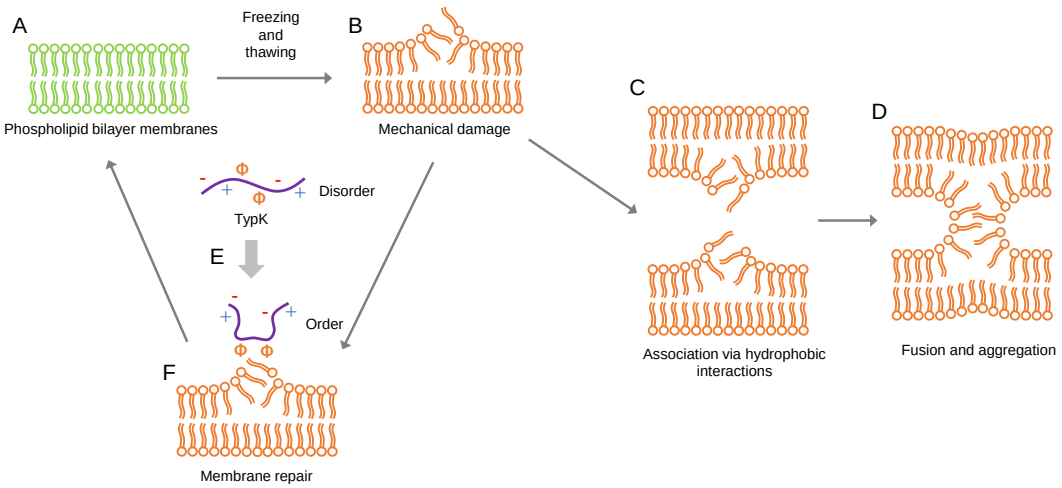

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Fig. 8 Kimura et al.