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Palmitoylation mediates the proteolysis of seed storage proteins during the cooling process in hydrated lettuce seeds (*Lactuca sativa*)

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Research Paper

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Abstract

Palmitoyl-protein thioesterase (PPT), involved in the fatty acid synthesis and the de-palmitoylation of protein, was induced under ecological cooling treatment in hydrated lettuce seeds. However, there was no significant difference in fatty acid levels between the control and the cooled samples. To further study the function of PPT, 2-bromopalmitic acid (2-Bp), an inhibitor of protein palmitoylation, was applied during the imbibition of hydrated lettuce seeds, which was followed by slow-cooling treatment ($-3°C\ h^{-1}$). The application of 2-Bp (1 mM) significantly increased the survival rate of seeds from 6.70% (control imbibition) to 22.67% (2-Bp imbibition) after slow cooling to −20°C. Differential scanning calorimetry (DSC) analysis indicated that 2-Bp led to earlier onset of ice crystals in the endosperm than the control group. Two-dimensional electrophoresis (2D) confirmed that 2-Bp could promote the hydrolysis of seed globulins and the accumulation of globulin peptides with small molecular weights. High-efficiency hydrolysis of globulin induced by mercaptoethanol improved the freezing tolerance of hydrated lettuce seeds and led to the accumulation of small globulin peptides, which further proved the positive function of small globulin polypeptides in enhancing the freezing tolerance of hydrated lettuce seeds. DSC of small globulin peptides showed that the smaller the molecular weight, the earlier the appearance of ice crystals and the higher the enthalpy of heat release. For the smallest peptides, the 2-Bp-4 in 2-Bp group exhibited higher enthalpy in exothermic peak than the control group (c-4). In conclusion, the hydrolysis of seed globulins and accumulation of small-molecule globulin peptides could be the major reason for improving the freezing tolerance of hydrated seeds after de-palmitoylation treatment.

Introduction

The low-temperature storage of seeds is plausibly the best available method to preserve plant germplasm (Berjak and Pammenter, 2008; Wesley Smith et al., 2014). Seeds can be divided into three types based on their ability to withstand drying: orthodox, intermediate and recalcitrant (Roberts, 1973; Ellis et al., 1990). Orthodox seeds can be dried and stored at seed bank condition, i.e. dry to 3–7% moisture content and −18°C or low temperatures for a long time (Ellis and Roberts, 1980). Intermediate seeds can tolerate dehydration but cannot survive low-temperature storage (Ellis et al., 1990). Recalcitrant seeds are sensitive to dehydration, therefore cannot be stored at ideal seed bank conditions (Ellis and Roberts, 1980). Almost 36% of the highly endangered plants are recalcitrant (Wyse et al., 2018). The higher water content challenges the storage of recalcitrant seed (Benson, 2008; Wilms et al., 2020).

A better understanding of hydrated orthodox seeds is expected to provide insights into recalcitrant seed storage. Lettuce (*Lactuca sativa*) seeds are orthodox that have been studied as model (Juntilla and Strushnoff, 1977; Keefe and Moore, 1983; Jaganathan et al., 2016). They are widely distributed, from tropical to cold zones and plain to alpine life zones. Therefore, hydrated lettuce seeds are becoming a model to study the relationship between seed moisture content and freezing tolerance. Furthermore, the genome of lettuce has been sequenced (Svanella Dumas et al., 2018), which strengthens the usage of lettuce in freezing tolerance studies (Jaganathan et al., 2016). Previous studies have shown that hydrated lettuce seeds adopt several strategies to avoid low-temperature damage based on different cooling rates (Keefe and Moore, 1983; Jaganathan and Liu, 2014; Jaganathan et al., 2017). During fast-cooling conditions ($-60°C\ h^{-1}$), a super-cooling mechanism inhibits ice crystal formation (Juntillla and Strushnoff, 1977). However, under slow-cooling conditions (less than $-4°C\ h^{-1}$), the water available in the extracellular space freezes first, causing the moving out of free water from the embryo and, thus, the desiccation of the embryo. Consequently, ice formation in the embryo is avoided, and any injury from freezing water in the embryo is minimized.

Palmitoylation is an essential post-translational modification of proteins (Hornemann, 2015). It is the only reversible lipid modification of proteins, enabling proteins to interact with the membrane transportation system (Batistic et al., 2012; Mohammed et al., 2013). Protein acyltransferase (PAT) is a vital transferase that can quickly and accurately modify target proteins with palmitoylation and provide the protein with high membrane affinity (Hornemann, 2015; Li et al., 2016). Palmitoylation of proteins plays the function of protein-specific membrane targeting, protein transportation and preventing degradation of protein (Blaskovic et al., 2013). Previous studies indicated that protein palmitoylation is involved in multiple abiotic stresses (Qi et al., 2013; Zhou et al., 2013; Li et al., 2016). However, the correlation between protein palmitoylation and seed freezing tolerance has not been reported.

In this study, the palmitoyl modification inhibitor 2-bromopalmitic acid (2-Bp) was applied to explore the effects of palmitoyl modification on the freezing tolerance of hydrated lettuce seeds and to find new targets of palmitoylation, which can deepen the understanding of cold resistance mechanisms of hydrated seeds. These could provide a new basis for the cryogenic preservation of recalcitrant seeds. 2-Bp and its targets can become potentially protective agents for the preservation of recalcitrant seeds.

Materials and methods

Seed material

Seeds of a *Lactuca sativa* hybrid cultivar (Luoshalv) were purchased from Fengmingyashi seed company (Beijing) and stored at 15% RH at 15°C until being used in the experiments. The viability of the seeds was tested before the experiments.

Seed imbibition

2-Bp and mercaptoethanol (S-h) were dissolved in dimethyl sulfoxide-acetic anhydride (Me_2SO) and diluted to a final concentration of 0.1, 1 and 10 mM in 1% agar medium (1 g agar was boiled in 100 mL de-ionized water and solidified), respectively. Thus, there were seven agar medium types: the control group and the 2-Bp group: with concentrations of 0.1, 1 and 10 mM and S-h group with concentrations of 0.1, 1 and 10 mM. Four replicates of 25 seeds were used for each treatment, and imbibition was carried out at 21 ± 1°C for 11 h, by which the seeds reached full imbibition but did not germinate visibly (Jaganathan et al., 2017).

Programmed cooling of fully hydrated seeds and survival rate tests

The fully hydrated but non-germinated seeds were surface dried by placing them between soft tissue pads. These seeds were cooled in a programmable freezer (Kryo series III) at rates of −3°C h^{-1} (slow cooling) and −60°C h^{-1} (fast cooling) to a final temperature of −20°C. The cooled seeds were incubated in 1% agar medium at 21°C, and germination was monitored for 5 d. The seeds with radicles extending more than 2 mm were scored as germinated. Three replicates of 50 seeds were used for each treatment.

RNA-Seq and real-time PCR analysis

RNA-Seq analysis was performed as described in our previous study (Jaganathan et al., 2017). For the real-time PCR, total RNA was extracted using the Trizol agent (Takara, Japan) from the fully hydrated seeds (imbibed in 1% agar medium, i.e. control medium) after slow (−3°C h^{-1}) and fast (−60°C h^{-1}) cooling to −20°C (Rio et al., 2010). Then cDNA was synthesized using reverse transcriptase and real-time PCR (Takara, Japan). Primers were designed with Primer5 software and then synthesized by Shanghai Sangon Bioengineering Co., Ltd. PPT-Forward primer: 5′AAGGCAAATT CGTAAAT GTG3′; PPT-Reverse primer: 5′ATTGAAGGTCTTGTTGTCC3′; Actin was used as internal control, Actin-Forward primer: 5′CTGGT GTGATGGTAGGTATGG3′; Actin-Reverse primer 5′CTCGTTG TAGAAAGTGTGATGC3′.

Fatty acid extraction and determination of palmitic acid content and linoleic acid

Approximately 0.2 g of the seeds imbibed with control medium (1% agar) and cooled with slow (−3°C h^{-1}) and fast (−60°C h^{-1}) cooling to −20°C, were added to a mortar with 4 mL pre-cooled hexane solution. After grinding thoroughly, samples were transferred to tubes and vortexed for 30 s. Then, the sample was incubated at 90°C for half an hour. During incubation, vortexing was carried out for 15 s at 20 min intervals. Then the sample mixture with hexane was centrifuged at 10,000 rpm at 4°C and the supernatant was obtained to be analysed by gas chromatography (GC).

The conditions for GC were as follows. An Agilent HP-5 MS gas capillary column (5% Phenyl Methyl Silox, 19091S-4333, 30 m × 250 μM × 0.25 μM), high-purity helium (99.99%) as the carrier gas, a flow rate of 1.0 mL min^{-1}, pre-column pressure of 8.23 psi. The split ratio was 10:1, the injection volume was 1 μL and the programmed temperature conditions were 15°C min^{-1} to 180°C for 1 min and 10°C min^{-1} to 270°C for 5 min, respectively.

For mass spectrometry, the electron impact ion source was EI (Electron Bombardment Source), the ion source temperature: 230°C, the quadrupole temperature: 150°C, the ionization voltage: 70 eV and the solvent delay was 2.5 min. Full-scan mass spectrometry was used.

Ice formation in the hydrated seeds during the cooling process

The heat release during ice formation in fully hydrated seeds in the cooling process was measured differential scanning calorimetry (DSC; Perkin-Elmer DSC8500, Shelton, CT). Before the experiments, the temperature scale of the DSC was calibrated following the methods described by a previous study (Mori et al., 2012). The DSC thermal programs calculated latent heat values concerning a sigmoidal baseline and normalized them to the total sample weight.

For the DSC experiments, one intact seed that had been fully hydrated with imbibition of 2-Bp (1 mM), was placed in the sample lid and cooled to −30°C at the rate of −3°C h^{-1}, then held at −30°C for 1 min, followed by warming to 20°C at a rate of −60°C h^{-1}. The heat released during the cooling process was measured and calculated to understand the freezing behaviour of water in hydrated seeds including the starting temperature of ice formation and the enthalpy of heat release.

Protein identification by 2D electrophoresis

Total protein was extracted by TCA/acetone precipitation from hydrated seeds in 1% agar medium with or without 2-Bp (1 mM) or S-h (1 mM) after cooling to −20°C with a slow-cooling rate (−3°C h^{-1}). Two-D electrophoresis was carried out according

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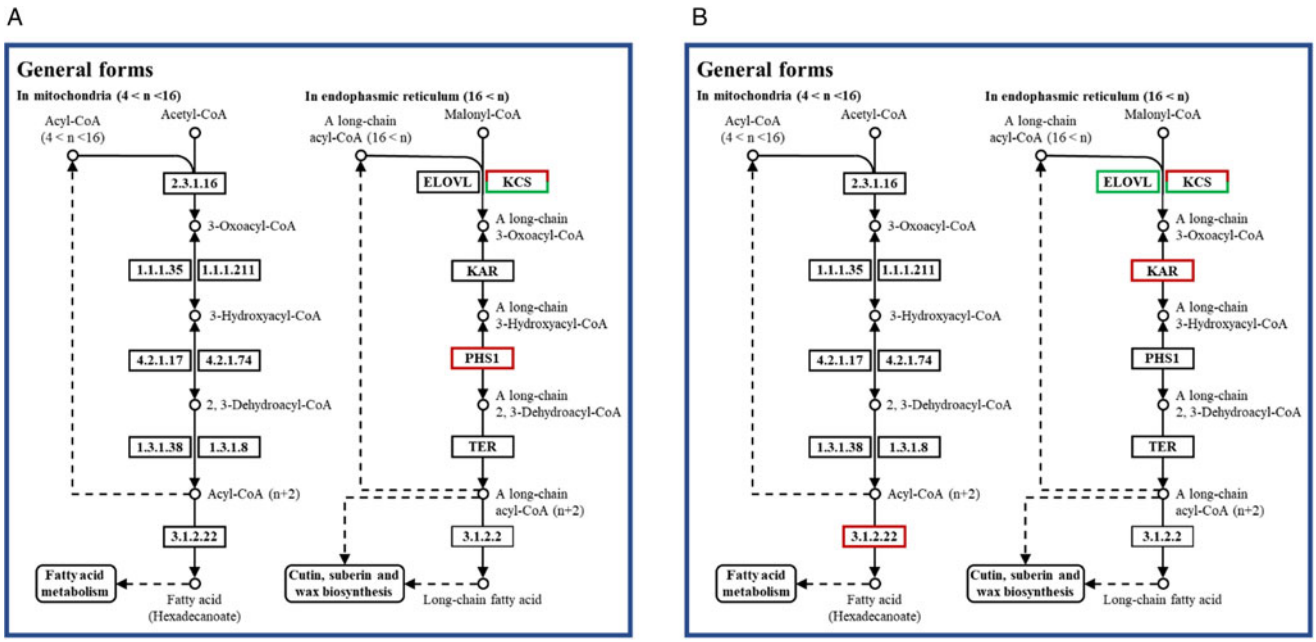

Figure 1. Fatty acid synthesis pathway was enriched after the slow-cooling treatment. RNA-Seq for fast (−60°C h^{-1}) (A) and slow (−3°C h^{-1}) (B) cooling to −20°C. The coloured rectangles represent a change in gene expression. Red indicates that the gene expression was upregulated compared with non-freezing treatment while green indicates down-regulation compared with non-freezing treatment; 3.1.2.22 represents the gene encoding the palmitoyl-protein thioesterase (PPT) protein.

to a previous study (Krishnan et al., 2009) with some modifications. The isoelectric focusing (IEF) method was as follows: 100 V, 0.5 h, linear ramp; 1000 V, 1 h, linear ramp; 4000 V, 1 h, linear ramp; 4000 V, rapid ramp 1 h; 500 V, rapid, 24 h and was performed using the Protean II IEF (BioRad). Separation of protein polypeptides according to isoelectric point (IP) was completed when the current was at or below 65 μA per strip and steady for 1 h. SDS-PAGE was conducted for the separation of the second dimension. The gel was dyed with Coomassie brilliant blue. Experiments were repeated three times independently and the differentially detected protein spots were characterized by mass spectrometry (Sangon, Shanghai).

Palmitoylation prediction

The amino acid sequences of the differently expressed proteins were downloaded from NCBI, and CSS-Palm 4.0 software was conducted to predict palmitoylation sites.

Extraction of seed storage globulins and hydrolytic peptides and ice formation mode of polypeptides with different molecular weights

Seed storage globulins and the hydrolytic peptides were extracted using the following method. 0.2 g cooled seeds were grinded into powder. Then the powder was mixed with 4% NaCl with a volume ratio of 1:1 (powder:liquid) and adding PSMF (1 mM) to the solution to avoid protein degradation. After incubation at 50°C for 2 h, the supernatant was obtained by centrifugation at 12,000 rpm at 4°C for 30 min.

Then the supernatant was mixed with SDS sample buffer (1:1) and the mixture was boiled for 5 min, and placed on ice. SDS-PAGE was conducted under a constant current of 20 mA for 30 min. The gel was stained with Coomassie brilliant blue

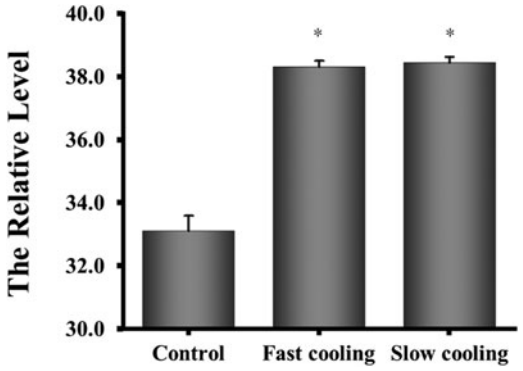

Figure 2. The mRNA level change of PPT under different cooling treatments. The expression of PPT in the control group was set as 1, and the fast and slow-cooling groups were calculated as relative values, $^{*}P < 0.05$. The starting temperature was 20°C. Control: non-freezing; Fast cooling: cooled to −20°C with −60°C h^{-1}. Slow cooling: cooled to −20°C with −3°C h^{-1}.

R-250. The molecular weight of independent bands was determined based on a standard protein marker (Sangon, Shanghai, China). The peptides with different molecular sizes were extracted by a Gel recovery kit (Sangon, Shanghai, China).

The concentration of all the extraction samples was determined by Nanodrop. Then the polypeptides were cooled with a rate of −60°C h^{-1} to −40°C, and the ice formation during the cooling process was detected with DSC. Other conditions were the same as the detection of crystal formation of seeds.

Superoxide dismutase (SOD) activity determination

SOD activity was determined by the NBT light reduction method (Sripichitt et al., 1988). 0.2 g of cooled seeds were weighed for each treatment, then the seed sample was ground to powder

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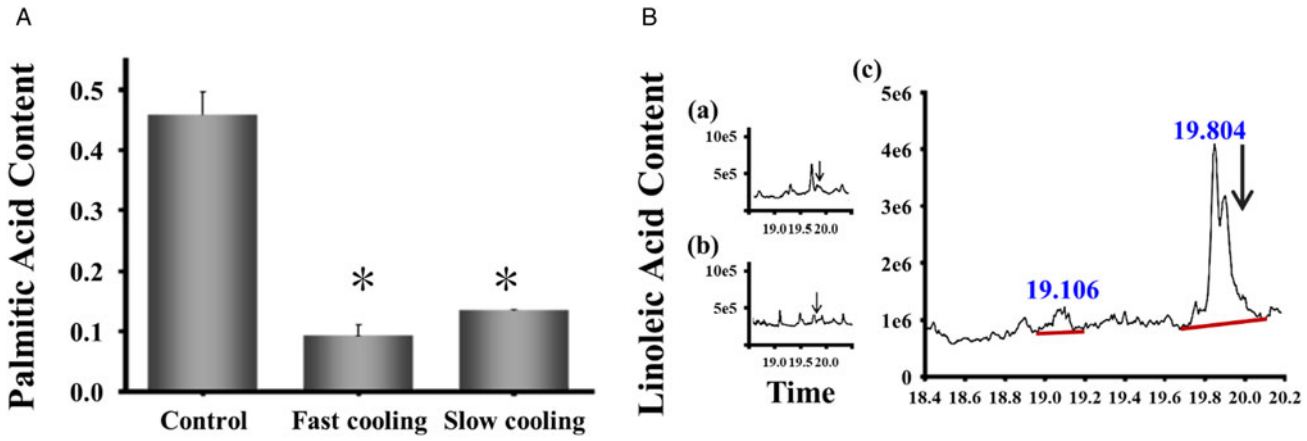

Figure 3. The content of palmitic acid (A) ($P < 0.05$) and linoleic acid (B) after cooling treatment. *Note*: Fast cooling: cooled by −60°C h^{-1}; Slow cooling: cooled by −3°C h^{-1}. In B, (a) control (non-freezing) treatment; (b) fast-cooling treatment and (c) slow-cooling treatment. The arrows indicate the position of linoleic acid.

Table 1. The water content and germination rate after adding 2-Bp at room temperature

2-Bp	0	0.1 mM	1 mM	10 mM
Water content (%)	49.02 ± 0.7	49.04 ± 0.4	47.73 ± 3.3	47.11 ± 0.5
Germination rate (%)	96.00 ± 3.27	92.00 ± 0.00	92.00 ± 3.27	93.33 ± 1.89

Table 2. The effects of 2-Bp on the survival rate of hydrated lettuce seeds after cooling treatment

	0	0.1 mM	1 mM	10 mM
Slow cooling (%)	6.70 ± 2.89	6.04 ± 1.50	22.67 ± 2.31*	10.35 ± 3.00
Fast cooling (%)	94.67 ± 1.89	94.67 ± 3.77	97.33 ± 1.89	89.33 ± 3.77

*The survival rate was significantly increased after applying 2-Bp (1 mM) in imbibition medium after slow cooling, $P < 0.05$. Slow cooling: cooled by −3°C h^{-1} to be −20°C; Fast cooling: cooled by −60°C h^{-1} to be −20°C.

followed by the addition of 10 mL of pre-cooled phosphate buffer. The mixture was centrifuged at 4°C and 10,000 rpm for 20 min and the supernatant was obtained. After mixing the supernatant with the reaction buffer (Supplementary Table S1), the mixtures were placed under a 4000 lx fluorescent lamp to react for 20 min (each tube received the same light). Taking an un-illuminated tube as a control, the absorbance value of each tube at 560 nm was determined. The SOD activity unit is an enzyme activity unit that inhibits 50% of the photochemical reduction of NBT:

$$\text{SOD activity} = \frac{(A_c - A_t) \times V_s}{0.5 \times A_c \times W_t \times V_t},$$

where A_c is the absorbance of the control; A_t is the absorbance of the treatment; V_t is the total volume of the treatment; W_t is the fresh weight of the treatment and V_s is the volume of the treatment detected.

Determination of scavenging power of hydroxyl radicals

The Fenton reaction was performed to generate hydroxyl radicals (Chen and Schopfer, 1999). For this, 0.26 g of cooled seeds were weighed for each treatment, then the seed sample was ground to a powder followed by adding 10 mL of pre-cooled phosphate buffer, and thoroughly mixed into a slurry, centrifuged in a high-speed refrigerated centrifuge at 4°C and 10,000 rpm for 20 min. The supernatant is the sample solution which was mixed with the reaction buffer (Supplementary Table S2). Then the mixture was quickly transferred to a 37°C water bath for 30 min. Using de-ionized water as the blank, the absorbance value of the sample solution was measured at a wavelength of 510 nm.

Statistical analysis

Statistical analyses were performed using SPSS version 15.0. For comparison of all data groups, the control imbibition group was used as control, and the treatment with 2-Bp and Mercaptoethanol (S-h) were used as the treatment group. For all the statistical analyses, the P-value was set as 0.05.

Results

PPT was induced under both slow-cooling and fast-cooling treatments

Transcriptome sequencing (RNA-Seq) and real-time PCR performed on hydrated lettuce seeds that had been treated with

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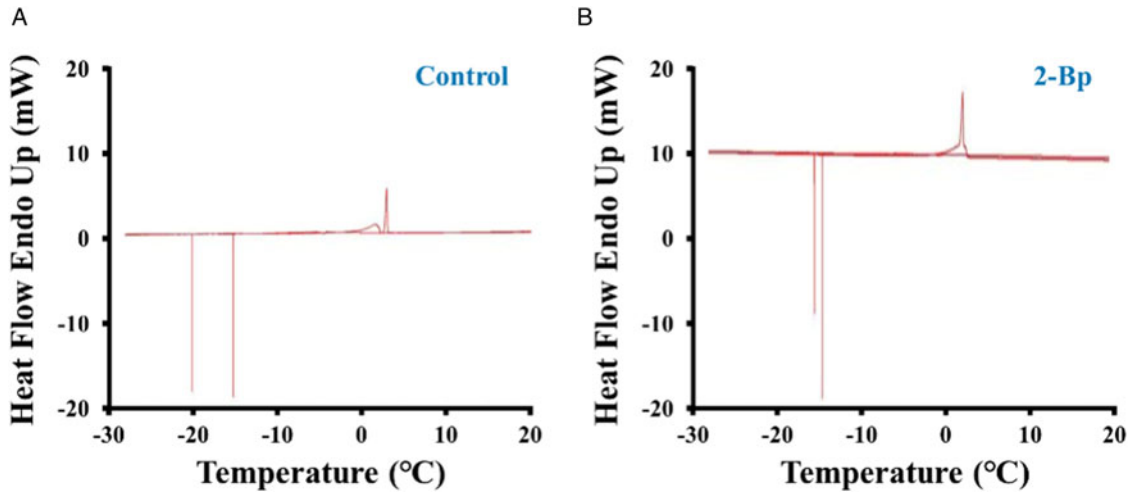

Figure 4. Differential scanning calorimeter (DSC) analysis of hydrated lettuce seeds with slow-cooling treatment. (A) Control imbibition and (B) 2-Bp imbibition. The area of downward peaks indicates the enthalpy of the exotherm, and the area of upward peaks indicates the enthalpy of the endotherm. The detailed data are shown in Table 3.

Table 3. The exotherm during the slow-cooling treatment

Type	Valve	ONSET1 (°C)	$\Delta H1$ (J/g)	ONSET2 (°C)	$\Delta H2$ (J/g)	Total ΔH (J/g)
Control slow-cooling	Mean	−15.71	−68.997	−18.56	−32.867	−101.864
	SD	0.834	15.289	2.036	6.303	8.986
2-Bp slow-cooling	Mean	−13.71	−43.214	−14.55	−64.887	−108.101
	SD	1.216	11.859	1.315	68.462	56.603

ONSET1: The starting temperature of the first exothermal peak; $\Delta H1$: the enthalpy of heat release during the first exotherm; ONSET2: The starting temperature of the second exothermal peak; $\Delta H2$: the enthalpy of heat release during the second exotherm; Total ΔH: the total enthalpy of heat release; SD: standard deviation; Slow cooling: −3°C h^{-1}.

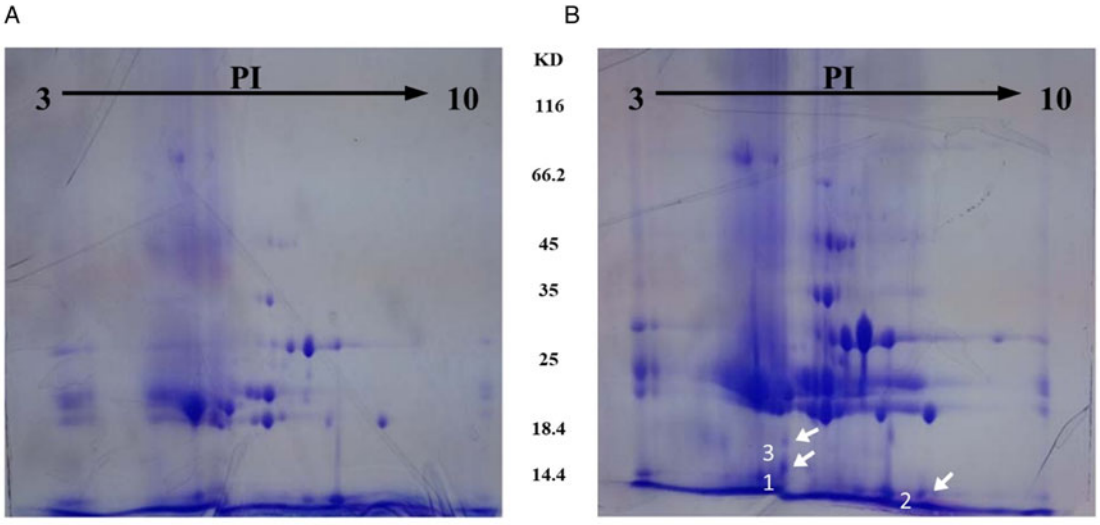

Figure 5. The two-D electrophoresis of protein from seeds with control (A) and 2-Bp (B) imbibition after slow-cooling treatment. The hydrated seeds were cooled with −3°C h^{-1} to −20°C followed by the extraction of total proteins. The arrow indicates the differentially accumulated protein and peptides.

fast cooling (−60°C h^{-1}) and slow cooling (−3°C h^{-1}) to −20°C revealed that PPT (3.1.2.22) was significantly upregulated under both fast- and slow-cooling treatments (Figs. 1 and 2, $^*P <$ 0.05). Although the level of palmitic acid was significantly reduced after fast and slow cooling, compared with the control (Fig. 3A, $^*P < 0.05$), there was no significant upregulation in

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Table 4. Mass spectrometric identification of different protein spots specifically accumulated in 2-Bp treatment and prediction of palmitoylation sites

Treatment		2D result[a]					Palmitoylation modification site prediction[b]				
Spots	Protein Accession Number	Name	pI	Position	Matched score	*P*-value	Position	Peptide	Score	Cut-off	Cluster
1	XP023770765.1	13S globulin seed storage protein 1-like	7.64	468–482	15	$1.7 * 10^{-2}$	11	RLLSLGL**C**FLVLFHG	37.449	4.222	Cluster C
	XP023769778.1	11S globulin seed storage protein 2-like	5.5	199–216	18	$4.4 * 10^{-2}$	13	LVLGFFL**C**LLGTTAL	30.094	4.222	Cluster C
2	XP023743389.1	11S globulin seed storage protein G3-like	6.09	423–435	13	$4.6 * 10^{-3}$	18	FLLLFST**C**LAHHQQQ	26.381	4.222	Cluster C
				441–455	15	$2.9 * 10^{-2}$					
				463–478	16	$3.10 * 10^{-9}$					

[a]The total protein was extracted from seeds that imbibed with or without 2-Bp 1 mM and cooled to be −20°C with −3°C h^{-1}. Then the total protein was analysed by 2D electrophoresis.
[b]The palmitoylation of protein sequences was predicted by CSS-Palm 4.0. The 'C' in bold italics indicates the sites modified by palmitoylation.

linoleic acid under either fast-cooling or slow-cooling treatment (Fig. 3B). Then we focused on the involvement of palmitoylation regulation in seed freezing tolerance using 2-bromopalmitic acid (2-Bp), the inhibitor of palmitoylation, as the treatment group.

2-bromopalmitic acid (2-Bp) enhanced the freezing tolerance of hydrated lettuce seeds during the slow-cooling treatment

At room temperature, 2-Bp did not alter water content and germination percentage compared with control imbibition (Table 1). During the slow-cooling treatment, compared with the control imbibition group, the exogenous application of 2-Bp (1 mM) significantly improved the germination percentage from 6.70% (control group) to 22.67% (2-Bp group) after cooling to −20°C (Table 2, $^{*}P < 0.05$). However, under the fast-cooling treatment, 2-Bp showed no effect on the survival rates of hydrated seeds (Table 2).

2-Bp treatment altered the ice crystal formation of hydrated seeds after the slow-cooling treatment

Based on the DSC analysis with intact seeds, there were two exothermic peaks in both control and 2-Bp treated (1 mM) seeds in the process of cooling to −30°C at the rate of −3°C h^{-1} (Figure 4; Table 3). But different treatments affect the temperature at which the exotherm occurs. In the control treatment, the hydrated seeds had two exothermic peaks, at approximately −15°C which might be resulted from ice formation in the endosperm, whereas the second peak at −18°C resulted from ice formation in the embryo (Figure 4; Table 3). The 2-Bp treatment group had two close exothermic peaks at approximately −15°C (Figure 4; Table 3). Compared with the control group, ice crystal formation at −18°C did not appear in the 2-Bp treatment.

2-Bp enhanced the degradation of seed storage globulins after the slow-cooling treatment

After the slow-cooling treatment, the protein samples were subjected to two-dimensional (2D) electrophoresis, and the differentially accumulated protein spots were detected. In the 2-Bp (1 mM) treatment, three differentially accumulated protein spots with small molecular weights were detected (between 14.4 and 18.4 kDa) (Figure 5B) compared to the control imbibition (Figure 5A). According to mass spectrometric data (Table 4), protein spot 1 was identified as a mixture of 13S globulin seed storage protein 1-like (XP_023770765.1) and 11S globulin seed storage protein 2-like (XP_023769778.1). Protein spot 2 was identified as 11S globulin seed storage protein G3-like (XP_023743389.1). The proteins in protein spot 3 could not be identified due to the small quantity.

13S globulin seed storage protein 1-like, 11S globulin seed storage protein 2-like and 11S globulin seed storage protein G3-like were all seed storage proteins since the molecular weight of the mono subunit for seed globulins was more than 27 kDa and the detected protein spots were between 14 and 18 kDa. The spots detected might be the hydrolytic peptide of corresponding globulins.

The palmitoylation prediction of the three seed storage proteins is summarized in Table 4. The prediction analysis demonstrated that these three proteins have palmitoylation sites (cysteine residue), which indicated that the functions of these three proteins might be regulated by palmitoylation.

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Table 5. The effect of mercaptoethanol (S-h) on the survival rate of hydrated lettuce seeds after slow-cooling treatment

Imbibition	Control	S-h (0.1 mM)	S-h (1 mM)	S-h (10 mM)
Slow-cooling survival rate (%)	11.00 ± 3.83	21.33 ± 4.62*	46.00 ± 12.44*	12.00 ± 3.27

*The survival rate was significantly increased after applying S-h in imbibition medium, $P < 0.05$. Slow cooling: cooled to −20°C by −3°C h^{-1}.

Table 6. Mass spectrometric identification of differentially accumulated protein spots with S-h imbibition after cooled to −20°C with −3°C h^{-1}

Protein	Name	pI	Position	Matched score	*P*-value
XP_023743392.1	Legumin B-like	6.05	362–380; 425–439	173	$2.60 * 10^{-10}$
XP_023743389.1	11S globulin seed storage protein G3-like	6.29	310–329; 465–480	129	$3.25 * 10^{-8}$
XP_023743391.1					
XP_023771234.1					
XP_023770765.1	13S globulin seed storage protein 1-like	7.64	468–482; 468–486	125	$4.59 * 10^{-8}$

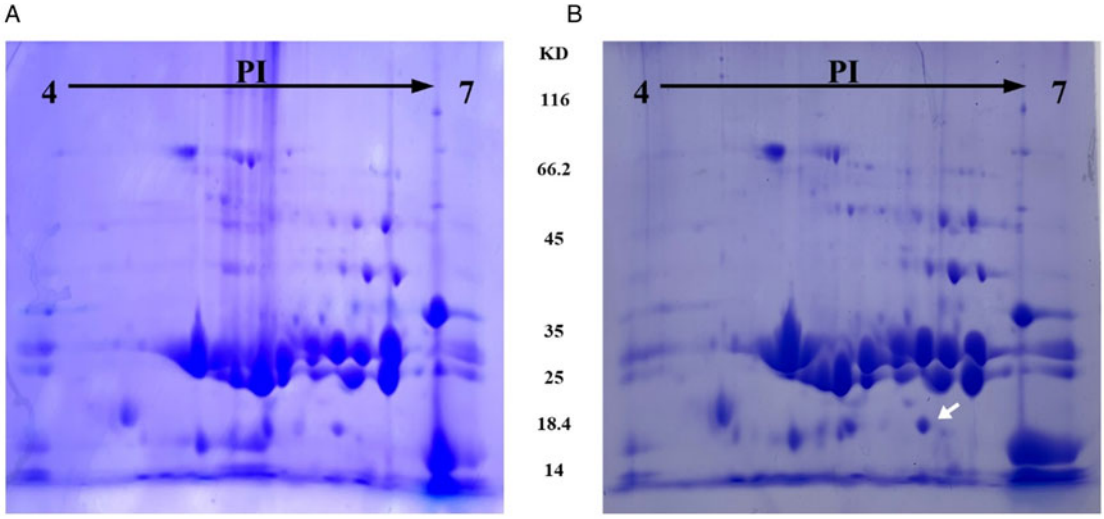

Figure 6. 2D electrophoresis of protein from seeds with control (A) and S-h (B) imbibition after slow-cooling treatment. The hydrated seeds were cooled at −3°C h^{-1} to −20°C. The arrow indicates the differentially accumulated protein and peptides.

High-efficiency hydrolysis of globulin improved the freezing tolerance of hydrated lettuce seeds

Upon adding mercaptoethanol (S-h) (with the final concentration of 1 mM) to the imbibition medium, the survival rates of hydrated seeds significantly increased from 11% (control group) to 46% (S-h group) under the slow-cooling condition (−3°C h^{-1}) (Table 5, $P < 0.05$). Two-D electrophoresis was performed with S-h (1 mM) treatment and differential protein spots were detected (Figure 6). The detected spot in the S-h group had a molecular weight similar to those in the 2-Bp group, i.e. molecular size was less than 18.4 kDa. Mass spectrometry was performed to identify the protein spot, which was determined to be globulin proteins, including legumin B-like (XP_023743392.1), 11S globulin storage protein G3-like (XP_023743389.1), 11S globulin seed storage protein G3-like (XP_023743391.1), 11S globulin seed storage protein G3-like (XP_023771234.1) and 13S globulin seed storage protein 1-like (XP_023770765.1) (Table 6).

Ice formation of globulin peptides under programmed cooling treatment

The seed storage globulins and their hydrolyzed peptides were extracted and separated using SDS-PAGE (Figure 7). Based on SDS-PAGE, the globulins peptides were distributed on the gel according to their molecular weights (Figure 7). Globulin peptides with different molecular sizes (the peptides indicated by arrows including c-1, c-2, c-3, c-4, 2-Bp-4 and S-h-4; Figure 7) were extracted by gel recovery. The ice formation of the above peptides with different molecular weights was examined by DSC (Figure 8). In the process of cooling to −40°C at a rate of −60°C h^{-1}, each detected peptide showed only one exothermic peak. With the decrease of the molecular weight for globulin peptides, the onset temperature of ice formation and exothermic enthalpy increased (Figure 8A, B). The exothermic peak of c-1 (the largest peptide) showed the lowest onset temperature and exothermic enthalpy, while other smaller peptides (c-2, c-3 and c-4 peptide)

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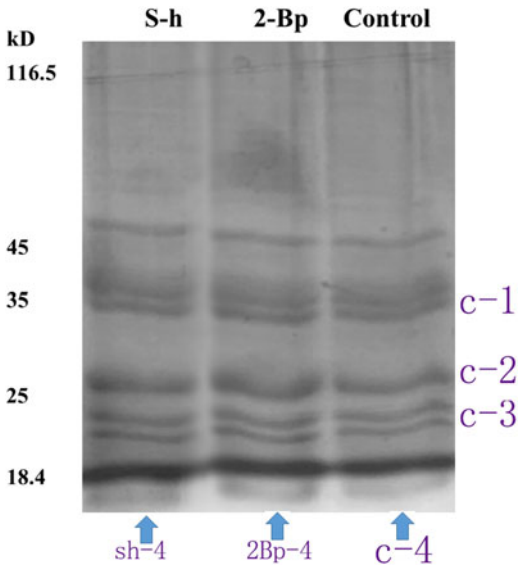

Figure 7. SDS-PAGE analysis of seed storage globulins and their polypeptides. S-h, S-h imbibition; 2-Bp, 2-Bp imbibition; Control, Control imbibition. The hydrated seeds were cooled with −3°C h^{-1} to −20°C and the globulins peptides were extracted. In the control group, the peptides were divided into four types including c-1, c-2, c-3 and c-4. The smallest peptides in 2-Bp (2-Bp-4) and S-h (S-h-4) were also extracted.

showed higher onset temperature and exothermic enthalpy (Figure 8A, B). Compared with the smallest peptide in the control group (c-4), the exothermic enthalpy of the 2-Bp-4 peptide was even significantly higher than that of c-4 (Figure 8A, $^*P < 0.05$).

2-Bp treatment improved the antioxidant activity of seeds

After the slow-cooling treatment, hydrated seeds' superoxide dismutase (SOD) activity was assessed. The SOD activity of the seeds treated with 2-Bp imbibition (2-Bp group; 1689.85 U/g) was higher than those treated with control imbibition (control group; 541.98 U/g) (Figure 9A, $^*P < 0.05$).

Compared with the control group, the hydroxyl radical scavenging ability of the seeds in the 2-Bp group was significantly enhanced by 82%, demonstrating that 2-Bp increased the antioxidant activity in hydrated seeds at freezing temperature (Figure 9B, $^*P < 0.05$).

Discussion

During the slow-cooling process, PPT, a protein involved in the fatty acid synthesis pathway and protein palmitoylation modification, was upregulated, suggesting that fatty acid synthesis and palmitoylation might be involved in the response of hydrated seeds to freezing stress (Figure 1). In this study, the level of unsaturated fatty acid was not affected by the cooling treatment. So we focused on the palmitoylation regulation of frozen seeds.

Palmitoylation is an important reversible protein modification (Xue et al., 2004) and involves two proteins that have opposite functions. One of them is PAT, which is responsible for the palmitoylation of target proteins, enabling them to undergo membrane transport. The second one is PPT which is responsible for the dissociation of palmitic acid from target proteins after the target proteins are transferred to the correct cellular membrane location (Jiang et al., 2018). The palmitoylation was involved in different abiotic stresses (Batistic et al., 2008; Qi et al., 2013).

2-Bp can block the binding of palmitate to protein. Several studies have confirmed that this compound is an inhibitor of protein palmitoylation and mainly inhibits the function of PAT (Zhou et al., 2013; Zhang et al., 2015). In this study, the palmitoylation inhibitor (2-Bp) was applied exogenously in the imbibition medium to explore the effects of palmitoylation on the freezing tolerance of hydrated lettuce seeds. The results showed that the application of 2-Bp could protect the hydrated lettuce seeds from freezing injury based on germination percentage after freezing treatment.

Previous studies (Keefe and Moore, 1981, 1983) indicated that two exothermic peaks were produced in hydrated lettuce seeds during the fast-cooling process. The first was around −15°C, which indicates the freezing in endosperm, and the second was around −18°C, which is the freezing temperature of the embryo. However, during the slow-cooling treatment, only one exothermic peak was observed at approximately −15°C, suggesting that ice

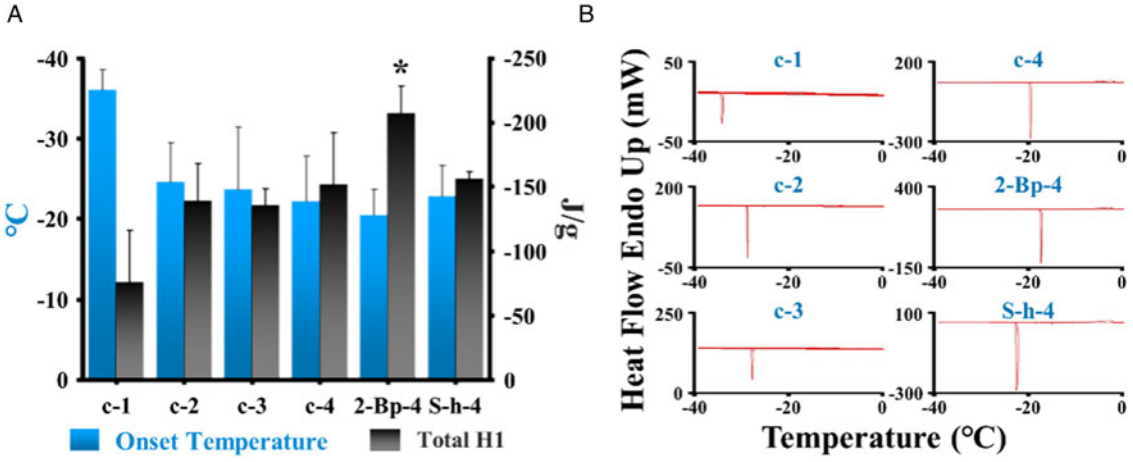

Figure 8. Ice formation of globulin peptides of different molecular weights. (A) The onset and exothermic enthalpy of ice crystal formation in different globulin peptides during the cooling process with a cooling rate of −60°C h^{-1}. Onset: The occurring temperature of the exothermal peak; Total H1: the total enthalpy of heat release during the ice crystal formed. $^*P < 0.05$. (B) The formation of ice crystals of different globulins peptides during the cooling process.

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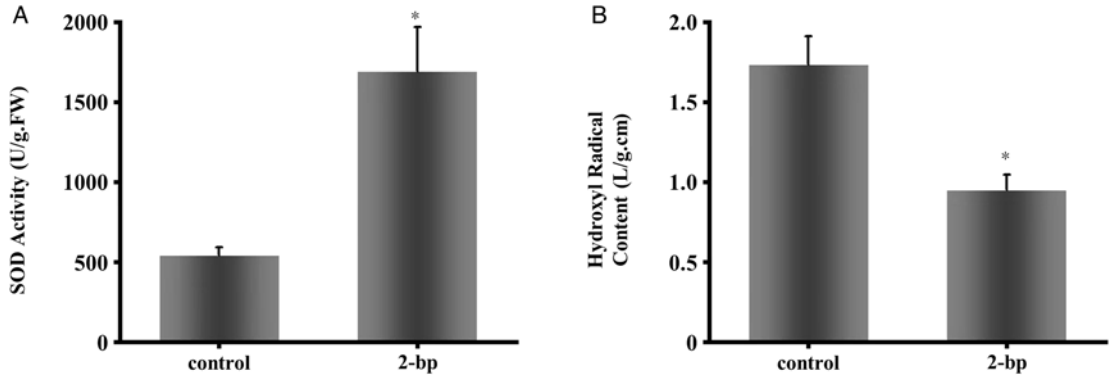

Figure 9. The effects of 2-Bp on the SOD activity (A) and hydroxyl radical scavenging ability (B) of the hydrated lettuce seeds after slow-cooling treatment. *Note*: Control, the control imbibition treatment; 2-Bp, 2-Bp imbibition treatment; Slow cooling: cooled to −20°C with −3°C h^{-1}. * $P < 0.05$.

formation in the outer endosperm made the internal embryo desiccate during slow cooling (Keefe and Moore, 1981). As a result, the exothermic peak of the embryo disappeared, thereby reducing low-temperature damage to the embryo (Keefe and Moore, 1981). In this study, DSC was carried out to detect the formation of ice crystals in 2-Bp treated seeds. The results indicated that the application of 2-Bp (1 mM) led to the disappearance of the exothermic peaks in embryos which might be the reason for the higher viability in 2-Bp treatment (1 mM) after slow cooling.

To further characterize the relationship between survival rate and the ice formation in hydrated seeds, slow cooling was carried out and the sample was taken out after the peak temperature in both control and 2-Bp treatment (detected by DSC), separately. The results in control imbibition indicated that the hydrated seeds died after the first peak, which indicated that embryo was frozen at the same temperature as the endosperm (Supplementary Table S3). In 2-Bp treatment, the seeds kept higher viability after the second heat release peak (Supplementary Table S3). This might be the reason that 2-Bp can promote the survival rate of hydrated seeds in the slow-cooling treatment.

To explore the biological mechanism that 2-Bp exerted on the freezing tolerance, two-D was carried out to detect differentially expressed proteins in 2-Bp treatment. Two-D was an efficient tool to study the proteome of plants (Kosmala et al., 2009). Moreover, it has an advantage in detecting the degradation and hydrolysis of proteins. According to previous studies (Imin et al., 2004; Yan et al., 2006; Kosmala et al., 2009), the protein degradation during low-temperature treatment occurred in the following proteins including Rubisco large subunit, Rubisco activase, sedoheptulose-1,7-bisphosphatase, PSII oxygen-evolving complex protein 2, ATP (adenosine triphosphate) synthase alpha chain and ATP synthase CF1 beta chain. In this study, 2D electrophoresis showed that the addition of 2-Bp accelerated the hydrolysis of 11S and 13S globulins and promoted the accumulation of small molecular peptides. A high-efficiency hydrolysis experiment on globulins with the addition of S-h showed the same effects as the addition of 2-Bp, i.e. globulin peptides with low molecular weight were accumulated and the corresponding survival of hydrated seeds after freezing treatment increased, which implied that hydrolytic peptides might protect the embryo from being damaged.

To check the function of hydrolytic globulin peptides, the ice formation of them during cooling was detected using DSC with the fast-cooling rate (−60°C h^{-1}). The smaller peptides resulted in earlier onset of crystal formation and higher heat release in the process of ice formation. The DSC analysis of 2-Bp treated seeds with the slow-cooling treatment revealed that 2-Bp treatment caused earlier and larger crystal formation, which is consistent with the DSC analysis of hydrolytic peptides. We propose that the accumulation of small hydrolyzed peptides in the endosperm leads to the earlier onset of ice formation in the outer endosperm and more water evaporation from the internal embryo after 2-Bp treatment, resulting in the higher survival rate of hydrated seeds.

Seed globulins are storage proteins, which are correctly formed, assembled and stably accumulated in the process of maturation of the seeds (Kesari et al., 2017). The hydrolysis of globulins is a conventional event in the process of seed germination, which will provide nutrients for the embryo. Previous studies have shown that the proteolysis of seed storage globulin peptides protects seeds from oxidative stress, and peptides with smaller molecular weights exhibit higher antioxidant activity than those with larger molecular weights (Shutov et al., 2003; Galland et al., 2014; Capraro et al., 2021). In this study, the extract of hydrated seeds in the 2-Bp group after slow cooling exhibited higher SOD activity and hydroxyl radical scavenging ability compared with the control group, indicating that small peptides would increase the antioxidant activity of hydrated seeds.

Conclusion

Palmitoylation modification was related to the freezing tolerance of hydrated seeds. De-palmitoylation might result in the hydrolysis of seed globulins, which protect the seeds from freezing injury. The hydrolysis of globulin peptides altered ice crystal formation in seeds during the slow-cooling process. Smaller peptides might promote earlier and greater ice formation in the endosperm, leaving the internal embryo dry and enhancing the seeds' viability. Smaller peptides might also improve the antioxidant activity of seeds. Based on this study, we can deduce some potential methods for protecting recalcitrant seeds from freezing injury, for example, by application of 2-bromopalmitic acid or hydrolytic peptides of seed storage protein in loading solution before cryopreservation of the seeds or embryos from recalcitrant seeds.

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