Efficient purification of cryoprotective dehydrin protein from the radish (Raphanus sativus) taproot

Title

Efficient purification of cryoprotective dehydrin protein from the radish (*Raphanus sativus*) taproot

Authors

Masakazu Hara[1*], Saki Uchida[1], Takae Murata[1], Hermann Wätzig[2]

[1]Research Institute of Green Science and Technology,
Shizuoka University,
836 Ohya, Shizuoka 422-8529, Japan
[2]Institut für Pharmazeutische Chemie,
Technische Universität Braunschweig,
Beethovenstraße 55, 38106 Braunschweig, Germany

*Name and address for editorial correspondence
Masakazu Hara
Research Institute of Green Science and Technology,
Shizuoka University,
836 Ohya, Shizuoka 422-8529, Japan
Telephone number: +81-54-238-5134
Fax number: +81-54-238-5134
E-mail address: amhara@ipc.shizuoka.ac.jp

Abstract

Dehydrin is a protein that is related to cold stress tolerance in plants. Because dehydrin shows potent cryoprotective activity, it has the potential to be used in food storage applications. In this paper, we presented an efficient purification method for native dehydrin from radishes (*Raphanus sativus*). Immunoblot analysis using an anti-*Arabidopsis* KS type dehydrin antibody revealed that the related dehydrin accumulates in the radish taproot. The radish dehydrin that accumulated in the vascular tissues of the taproot was purified through two simple chromatographic steps: immobilized metal affinity chromatography followed by anion exchange chromatography. The yield was higher than yields previously reported on a fresh weight basis. The cryoprotective activity for malate dehydrogenase shown by purified dehydrin was more potent than that shown by small-molecule cryoprotectants. This suggests that the radish is an appropriate source for the production of native dehydrin.

Key words

Cryoprotective activity; Dehydrin; Protein purification; Radish; *Raphanus sativus*

Introduction

Freezing is an essential process in the preservation and processing of foods and medicinal materials [1]. Since proteins are often damaged during the freezing process, the development of methods for stabilizing proteins under freezing conditions is crucial in the field of food production [2]. Cryoprotectants such as sugars and amino acids are used to maintain the activities of frozen enzymes [3]. However, such cryoprotectants affect taste, such as by adding sweetness, and increase the number of calories in foods [4]. Since proteinaceous cryoprotectants, such as serum albumins and antifreeze proteins, have less of an effect on taste and are low in calories, they are thought to be suitable substitutes for small-molecule cryoprotectants. However, the uses for these proteinaceous cryoprotectants are limited because serum albumins may contain blood-borne pathogens [5] and because the production of antifreeze proteins is still very expensive [6]. Other proteinaceous cryoprotectants are expected to be developed.

Dehydrins are late embryogenesis abundant (LEA) proteins which accumulate in plant seeds at the desiccation stage of seed development. Dehydrins are also produced when plants are exposed to cold and drought stresses [7-12]. It has been documented that dehydrins are related to the development of cold and drought tolerance in plants. Transgenic plants overexpressing dehydrin genes enhanced their tolerance to low temperature [13-16] and osmotic stress [17, 18]. Transgenic seeds of low-dehydrin mutants showed less longevity than the seeds of wild plants [19].

Functional studies have suggested that the cold tolerance enhancement provided by dehydrins may be mainly due to their potent cryoprotective activities (see reviews cited above). The cryoprotection of lactate dehydrogenase provided by the wheat WCS120 protein was a million-fold more efficient than that provided by sucrose on a molar basis [20]. Based on these

data, it has been proposed that dehydrins can be applied to food cryopreservation [20, 21].

Because, in general, consumers prefer food additives from natural sources, it is considered that dehydrins should be prepared from plants. Previous papers have reported that dehydrins could be purified from dry seeds of *Zea mays* [22], *Glycine max* [23, 24], and *Vigna unguiculata* [25], fresh leaves of *Spinacia oleracea* [26], and fresh shoots of *Prunus persica* [27]. The purification yields were noted in some reports. The dehydrin yields from dry seeds were 13 mg kg^{-1} dry weight (*Z. mays*), 27 mg kg^{-1} dry weight (*V. unguiculata*), and 77 mg kg^{-1} dry weight (*G. max*), respectively. The yield from fresh *S. oleracea* leaves was 2.6 mg kg^{-1} fresh weight. Considering that the water content in well-stored seeds is less than 10% [28], the previous yields were less than 8 mg kg^{-1} on a fresh weight basis. A more efficient purification system is needed. In this paper, we report the purification of a KS-type dehydrin from the *Raphanus sativus* taproot. The average yield was 21 mg kg^{-1} fresh weight, which was higher than the previously recorded yields on a fresh weight basis. The cryoprotective activity of the radish dehydrin was determined by the freeze and thaw test using malate dehydrogenase.

Materials and methods

Plant materials

European red radish *(R. sativus* L., cv. 'Comet') seeds purchased from Takii Seed (Kyoto, Japan) were sown on the surface of soil (Peatban, Sakata Seed, Yokohama, Japan) in a plastic pot (100 cm^2 x 10 cm) in a naturally illuminated glasshouse at an uncontrolled temperature at Shizuoka University, Japan. During the cultivation (from October to December in 2010), the plants were watered every week with Hyponex solution (500 times dilution; Hyponex, Tokyo,

Japan). Leaves, taproots, and roots from mature plants were used for immunoblotting. Taproots underwent immunohistochemical analysis. European red radishes and Japanese white radishes (Karami cultivar) purchased at a local market in Shizuoka (October 2011 to April 2012 and October 2012 to April 2013) were used as dehydrin sources for purification.

Purification of the radish dehydrin

The radish taproots (100 g fresh weight) were minced by a steel masher. The mashed taproots were further homogenized using a mortar and pestle on ice. The homogenate was centrifuged at 10,000 x *g* for 15 min at 4°C. After the supernatant was removed, the pellet was resuspended with 60 ml of deionized water. The suspension was centrifuged at 10,000 x *g* for 15 min at 4°C, and then the supernatant was discarded. The washed pellet was resuspended with 500 ml of 1 M NaCl and agitated for 1 hr on ice. The suspension was centrifuged at 10,000 x *g* for 15 min at 4°C. The supernatant was passed through a membrane filter (DISMIC, Advantec, Tokyo, Japan) and then applied to a 1-ml HiTrap Chelating HP column (GE Healthcare, Tokyo, Japan) immobilizing Zn^{2+} equilibrated with 25 mM Tris-HCl buffer pH 7.5 containing 500 mM NaCl (1/2EQ buffer). After the column was washed with 1/2EQ buffer, a linear gradient of imidazole (from 0 M to 1 M, 1 ml min^{-1}, 50 ml) based on the 1/2EQ buffer was performed (Econo Gradient Pump, BioRad, Tokyo, Japan). Fractions containing the protein immunologically related to AtHIRD11 (around 100 mM imidazole) were desalted by a NAP-25 column (GE Healthcare) equilibrated with 10 mM Tris-HCl buffer pH 7.5 (QB). The fraction was further purified by anion exchange chromatography (DEAE-Toyopearl 650M, 10 mm x 50 mm, Tosoh, Tokyo, Japan). A linear gradient (50 ml) was performed from 0 M to 1 M NaCl in QB at 1 ml min^{-1}. The fractions containing the purified protein that was identified to be related to

AtHIRD11 by immunoblot analysis were desalted and stored at -80°C until use. The purified protein was designated RsDHN (*R. sativus* dehydrin). The purified RsDHN was freeze-dried and weighed.

During the purification process, the protein amount was determined spectrophotometrically at 595 nm using the Quick Start Bradford Protein Assay (Bio-Rad, Tokyo, Japan), with bovine γ-globulin as a standard protein. Assays were performed according to the manufacturer's instructions. At each purification step, the amount of RsDHN was estimated by semi-quantitative immunoblot. The chemiluminescence intensity of positive signals was compared to that of purified RsDHN, which was weighed as described above. Immunoblot was performed as described below.

The purified RsDHN was identified using a matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) system composed of a 4700 Proteomics Analyzer (Applied Biosystems, Tokyo, Japan). The purified band (approximately 16 kDa in size) in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel stained with colloidal Coomassie blue (Bio-Safe, Bio-Rad) was excised to be digested with trypsin. The peptide fragments formed were analyzed by the above system. The sequence of fragments was matched to the corresponding partial sequence of the predicted RsDHN sequence by considering their molecular weights. The matched sequence was IHGGEGHSSGDHKHDGEK (Fig. 3, underlined).

Immunoblot analysis

The plant organs used were leaves, taproots, and roots of European red radish (Comet cultivar). Fresh organs (2 g fresh weight each) were frozen with liquid N_2 and homogenized with 5

volumes of deionized water at 4°C. The homogenate was centrifuged at 10,000 x *g* for 15 min at 4°C. The supernatant was taken and kept on ice. The pellet was resuspended with deionized water at a volume equal to that of the supernatant. The supernatant and pellet were resolved by SDS-PAGE (17% polyacrylamide gel). Proteins in the gel were blotted onto a polyvinylidene difluoride membrane filter (Immobilon-P, Millipore, Tokyo, Japan) by a Mini Trans-Blot Cell (Bio-Rad). The filter was blocked and incubated with the antibodies by a SNAP i.d. system (Millipore). The primary and secondary antibodies were the anti-AtHIRD11 antibody [29] and horseradish peroxidase conjugated anti-rabbit IgG (GE Healthcare, Tokyo, Japan), respectively. Positive signals were detected using the chemiluminescent Western blotting detection reagent ECL Plus (GE Healthcare). The chemiluminescence was detected by the LAS-4000 imaging system (Fujifilm, Tokyo, Japan).

Immunohistochemical analysis

Immunohistochemistry of tissue sections of European red radish (Comet cultivar) was performed basically according to Takahashi et al. [30]. Radish taproots were fixed with 2% (v/v) glutaraldehyde for 4 hrs at 4°C. Thin sections of the tissue prepared by hand were dehydrated and rehydrated in the ethanol series. The sections were blocked and incubated with antibodies. The primary and secondary antibodies were the anti-AtHIRD11 antibody and alkaline phosphatase-conjugated anti-rabbit IgG (Millipore), respectively. Staining was done with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics, Tokyo, Japan). Preimmune serum was used for the control. Sections were observed under a microscope (SZX7; Olympus, Tokyo, Japan) equipped with a digital photographic camera (C3040Z; Olympus).

Cryoprotection assay

The cryoprotective effects of RsDHN, AtHIRD11, bovine serum albumin (BSA), trehalose, proline, glycine betaine, and glycine on the freeze denaturation of malate dehydrogenase (MDH) were determined by the method previously reported [31] with slight modifications. RsDHN was purified from the radish as described above. Recombinant AtHIRD11 was produced according to our previous method [29]. BSA, trehalose, proline, glycinebetaine, and glycine were purchased from Sigma (Tokyo, Japan). The enzyme mixtures contained MDH (Oriental Yeast, Tokyo Japan, 20 U ml^{-1}) and different concentrations of RsDHN (0.8 to 40 mg l^{-1}) in 10 mM Tris-HCl buffer pH 7.5 (QB). For testing the AtHIRD11 and BSA, the concentrations used were 0.8 to 40 mg l^{-1}. In the cases of trehalose, proline, glycinebetaine, and glycine, higher concentrations were used (1 to 100 g l^{-1}). The reaction reagent consisted of 85 mM malate and 2.5 mM NAD^+ in QB (pH was adjusted to 7.5 by the 5 M NaOH solution). The enzyme mixtures (20 μl) were frozen by immersion in liquid nitrogen for 1 min and thawed at room temperature for 3 min. This freeze and thaw cycle was repeated 5 times in total. The solution was mixed with the reaction reagent (980 μl), and then the increase in absorbance at 340 nm was monitored at 25 ^oC to measure the activity. The degree of protection was shown by percentage; 100% protection indicated that the enzyme activity after the freeze and thaw was identical to the control unfrozen sample in the presence of the cryoprotectants. Finally, protective dose 50% (PD_{50}) was used to evaluate the cryoprotective activities.

Amino acid sequence of RsDHN

The expressed sequence tags (ESTs) of *R. sativus* were obtained from the *Arabidopsis* information resource web site (TAIR, http://www.arabidopsis.org/). Sequences were analyzed using GENETYX software (Genetyx, Tokyo).

Results and discussion

Dehydrin accumulation in the radish taproot

Our previous report suggested that among the 10 dehydrin genes of *Arabidopsis thaliana*, one of the most expressed was *AtHIRD11* (gene code: *At1g54410*) [29]. The yield of partially purified AtHIRD11 protein from the mature *Arabidopsis* plants was approximately 10 mg kg^{-1} fresh weight [29]. This suggests that the AtHIRD11 protein may be a good target for the efficient purification of native dehydrin. However, large-scale production of *Arabidopsis* plants is difficult because the mass of the plant is small. In attempting to resolve this problem, we decided to use the radish (*R. sativus*) taproot, which is a large axis tissue, because the AtHIRD11 protein was found to have accumulated in the cambium of axis tissues like the stark and hypocotyl of *Arabidopsis*. Indeed, we found that the antigen for the anti-AtHIRD11 antibody accumulated in the radish taproot (Fig. 1a, TR). In the case of *Arabidopsis*, the antigen for the antibody was detected in a tissue pellet derived from low-speed centrifugation [29]. Similarly, a strong signal was found in the tissue pellet of the radish taproot (Fig. 1a, TR, lane Ppt). The size of the antigen was similar to that of AtHIRD11, i.e. approximately 16 kDa in the SDS-PAGE analysis. Moreover, the antigen was mainly located at the cambial zone in the radish taproot (Fig. 1b, white arrowheads). This location was the same as the AtHIRD11 distribution in *Arabidopsis* [29]. These results suggest that the antigen in the radish taproot was highly related

to AtHIRD11.

Purification of the radish dehydrin

To purify the AtHIRD11-related protein from the radish, we referred to the partial purification process of the AtHIRD11 protein from *Arabidopsis* plants [29]. AtHIRD11 was extracted from the tissue pellet of *Arabidopsis* with 1 M NaCl solution and then partially purified by immobilized metal affinity chromatography (IMAC) [29]. In accordance with this method, we prepared tissue pellets from the mashed taproots of European red radish and Japanese white radish. Although we could purify dehydrins from both radishes, the results for the Japanese white radish are shown below because the yield in the Japanese white radish was better than that in the European red radish. The protein fraction that was obtained from the tissue pellet by the NaCl extraction (Fig. 2a, lane 2) was subjected to IMAC. At this step, a protein whose size was approximately 16 kDa in SDS-PAGE was concentrated (Fig. 2a, lane 3). Further anion exchange chromatography was performed to remove minor impurity proteins. The resulting fractions (Fig. 2a, lane 4) contained the purified protein that was recognized by the anti-AtHIRD11 antibody (Fig. 2b). We designated the AtHIRD11-related protein "RsDHN." The average yield (with standard deviation) of three independent purifications was 20.7 ± 3.4 mg per kg fresh weight. Table 1 was based on one of the three purifications. Typical chromatographies (IMAC and DEAE) are shown in Supplemental Fig. 1.

Identification of RsDHN sequence

The above data suggest that RsDHN is closely related to AtHIRD11. We predicted the amino

acid sequence of RsDHN as follows. By using the radish EST database, which was obtained from the *Arabidopsis* information resource web site (TAIR, http://www.arabidopsis.org/), we found 13 full-length cDNA sequences that were shown as orthologs of AtHIRD11 (At1g54410) by the web system (Supplementary Fig. 2). The amino acid alignment of the 13 sequences revealed that they are highly similar to each other (more than 99% identical). Five amino acid sequences out of the total of 13 were completely identical. The five identical sequences each had a region similar to the ant-AtHIRD11 antibody recognition site (Fig. 3). Moreover, MALDI-TOF/TOF indicated that the purified protein can have IHGGEGHSSGDHKHDGEK, which is a part of the radish sequence (Fig. 3, underlined). These results suggest that the sequence below in Fig. 3 is a strong candidate for RsDHN. RsDHN possesses one K-segment and one S-segment, indicating that RsDHN is a KS-type dehydrin, as is AtHIRD11.

Cryoprotection of enzyme by RsDHN

In general, dehydrins show cryoprotective activity for freezing-sensitive enzymes. Here, we tested whether the purified RsDHN has such cryoprotective activity. The activity of RsDHN was measured and compared to those of AtHIRD11, BSA, trehalose, proline, glycinebetaine, and glycine (Fig. 4). The latter five compounds are known as cryoprotectants [32, 33]. RsDHN showed more potent cryoprotective activity than the small-molecule cryoprotectants (trehalose, proline, glycinebetaine, and glycine), whereas the activity of RsDHN was similar to those of AtHIRD11 and BSA.

Advantage of dehydrin purification from radish

Although small-molecule cryoprotectants, such as sugars and amino acids, are used to stabilize food proteins during freezing storage, these cryoprotectants may add excess tastes and calories to foods. On the other hand, proteinaceous cryoprotectants do not have such problems. BSA is a potent proteinaceous cryoprotectant that can be produced at low cost. However, BSA is considered to have a risk of containing blood-borne pathogens [5]. Since dehydrin is a plant-derived protein, it has little risk of containing such pathogens. Thus, since the cryoprotective activity of dehydrin is similar to that of BSA, there is merit to producing a native dehydrin. Much effort has been put into the preparation of dehydrins. The preparation processes can be divided into two types: the production of recombinant dehydrins by the *Escherichia coli* expression system and the purification of native dehydrins from plants. The recombinant proteins produced by *E. coli* are thought to be unsuitable for food use because they may contain toxic impurities like lipopolysaccharides [34]. As for the derivation of dehydrins from plants, the use of major crop seeds has been preferred to dehydrin purification because dehydrins are known to accumulate in seeds. However, because major crops are primarily used for human and livestock foods, it is better to use plants which are not widely consumed around the world. Here, we used radishes as the dehydrin source. Although radishes can be globally cultivated, the major consumption areas are restricted to East Asian countries. Moreover, the radish taproot has a large biomass. Thus, the radish has potential as a source in dehydrin preparation.

Table 2 summarizes the dehydrin purification from different plant materials. Previous yields were recorded on a fresh weight basis (*S. oleracea*) and a dry weight basis (*V. unguiculata, Z. mays*, and *G. max*). Since the water content in well-stored seeds is less than 10% [28], the yields from dry seeds were calculated as less than 8 mg kg^{-1} on a fresh weight basis. It is apparent that the yield from radishes (21 mg kg^{-1} fresh weight) was higher than those from other plants. Regarding the purification steps, the previous methods required heat treatments and/or more

than three chromatographic steps. Our process has two chromatographic steps without a heat treatment. Taken together, these findings show that the present purification process was more efficient than the previous ones.

Despite this advantage, the large-scale preparation of purified dehydrin is still thought to be costly. Since highly purified dehydrin may be unnecessary for food use, crude dehydrin fractions, for example the desalted fraction obtained after the IMAC step in the present process, may be sufficient. Practical studies should be undertaken to assess the use of radish dehydrin as a food additive.

References

1. Kiani H, Sun DW (2011) Water crystallization and its importance to freezing of foods: A review. Trends Food Sci Technol 22:407-426

2. Bhatnagar BS, Bogner RH, Pikal MJ (2007) Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol 12:505-523

3. Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25:244-255

4. Dey SS, Dora KC (2010) Effect of sodium lactate as cryostabilizer on physico-chemical attributes of croaker (*Johnius gangeticus*) muscle protein. J Food Sci Technol 47:432-436

5. Sun QY, Ding LW, Lomonossoff GP, Sun YB, Luo M, Li CQ, Jiang L, Xu ZF (2011) Improved expression and purification of recombinant human serum albumin from transgenic

tobacco suspension culture. J Biotechnol 155:164-172

6. Christner BC (2010) Bioprospecting for microbial products that affect ice crystal formation and growth. Appl Microbiol Biotechnol 85:481-489

7. Close TJ (1996) Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plant 97:795–803

8. Rorat T (2006) Plant dehydrins--tissue location, structure and function. Cell Mol Biol Lett 11:536-556

9. Tunnacliffe A, Wise MJ (2007) The continuing conundrum of the LEA proteins. Naturwissenschaften 94:791-812

10. Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol 148:6-24

11. Hundertmark M, Hincha DK (2008) LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genom 9:118

12. Hara M (2010) The multifunctionality of dehydrins: An overview. Plant Signal Behav 5:503-508

13. Hara M, Terashima S, Fukaya T, Kuboi T (2003) Enhancement of cold tolerance and

inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. Planta 217:290-298

14. Puhakainen T, Hess MW, Mäkelä P, Svensson J, Heino P, Palva ET (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. Plant Mol Biol 54:743-753

15. Houde M, Dallaire S, N'Dong D, Sarhan F (2004) Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves. Plant Biotechnol J 2:381-387

16. Ochoa-Alfaro AE, Rodríguez-Kessler M, Pérez-Morales MB, Delgado-Sánchez P, Cuevas-Velazquez CL, Gómez-Anduro G, Jiménez-Bremont JF (2012) Functional characterization of an acidic SK(3) dehydrin isolated from an *Opuntia streptacantha* cDNA library. Planta 235:565-578

17. Brini F, Hanin M, Lumbreras V, Amara I, Khoudi H, Hassairi A, Pagès M, Masmoudi K (2007) Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. Plant Cell Rep 26:2017-2026

18. Shekhawat UK, Srinivas L, Ganapathi TR (2011) MusaDHN-1, a novel multiple stress-inducible SK(3)-type dehydrin gene, contributes affirmatively to drought- and salt-stress tolerance in banana. Planta 234:915-932

19. Hundertmark M, Buitink J, Leprince O, Hincha DK (2011) The reduction of seed-specific

dehydrins reduces seed longevity in *Arabidopsis thaliana*. Seed Sci Res 21:165-173

20. Ouellet F (2007) Cold acclimation and freezing tolerance in plants. Encycl Life Sci, John Wiley & Sons, Ltd. published online, doi:10.1002/9780470015902.a0020093

21. Breton G, Danyluk J, Ouellet F, Sarhan F (2000) Biotechnological applications of plant freezing associated proteins. Biotechnol Annu Rev 6:59-101

22. Ceccardi TL, Meyer NC, Close TJ (1994) Purification of a maize dehydrin. Protein Expr Purif 5:266-269

23. Momma M, Haraguchi K, Saito M, Chikuni K, Harada K (1997) Purification and characterization of the acid soluble 26-kDa polypeptide from soybean seeds. Biosci Biotechnol Biochem 61:1286-1291

24. Herzer S, Kinealy K, Asbury R, Beckett P, Eriksson K, Moore P (2003) Purification of native dehydrin from *Glycine max* cv., *Pisum sativum*, and *Rosmarinum officinalis* by affinity chromatography. Protein Expr Purif 28:232-240

25. Ismail AM, Hall AE, Close TJ (1999) Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence of cowpea. Plant Physiol 120:237-244

26. Kazuoka T, Oeda K (1994) Purification and characterization of COR85-oligomeric complex from cold-acclimated spinach. Plant Cell Physiol 35:601-611

27. Arora R, Wisniewski ME, Scorza R (1992) Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch): I. Seasonal changes in cold hardiness and polypeptides of bark and xylem tissues. Plant Physiol 99:1562-1568

28. Gambhir PN (1992) Applications of low-resolution pulsed NMR to the determination of oil and moisture in oil seed. Trends Food Sci Technol 3:191-196

29. Hara M, Shinoda Y, Kubo M, Kashima D, Takahashi I, Kato T, Horiike T, Kuboi T (2011) Biochemical characterization of the *Arabidopsis* KS-type dehydrin protein, whose gene expression is constitutively abundant rather than stress dependent. Acta Physiol Plant 33:2103–2116

30. Takahashi I, Kuboi T, Fujiwara T, Hara M (2012) Overexpression of an extraplastidic β-amylase which accumulates in the radish taproot influences the starch content of *Arabidopsis thaliana*. Plant Biotechnol 29:447-455

31. Hara M, Terashima S, Kuboi T (2001) Characterization and cryoprotective activity of cold-responsive dehydrin from *Citrus unshiu*. J Plant Physiol 158:1333-1339

32. Kundu CN, Das K, Majumder GC (2001) Effect of amino acids on goat cauda epididymal sperm cryopreservation using a chemically defined model system. Cryobiology 42:21-27

33. Cleland D, Krader P, McCree C, Tang J, Emerson D (2004) Glycine betaine as a

cryoprotectant for prokaryotes. J Microbiol Meth 58:31-38

34. Warzecha H (2008) Biopharmaceuticals from plants: a multitude of options for posttranslational modifications. Biotechnol Genet Eng Rev 25:315-330

Figure legends

Fig. 1 Accumulation of dehydrin in a radish. **a** Immunoblot detecting dehydrin in the leaf (L), taproot (TR), and root (R) of the radish. Sup and Ppt indicate the supernatant and pellet formed by centrifugation, respectively. A black arrowhead shows the position of the dehydrin. **b** Immunohistochemical analysis of dehydrin in the taproot of a radish. Transverse sections were examined using the anti-AtHIRD11 antibody (**b1**, **b4**) and preimmune antiserum (**b3**). The tissue was stained with toluidine blue O (**b2**). White arrowheads indicate the cambial zone. Bars represent 0.5 mm (**b1**, **b2**, and **b3**) and 0.2 mm (**b4**), respectively.

Fig 2 Purification of radish dehydrin. Proteins in each purification step were analyzed by SDS-PAGE stained with Coomassie blue (**a**) and immunoblot with anti-AtHIRD11 antibody (**b**). **a** Lane 1, taproot total protein (1 μg); lane 2, NaCl extract from tissue pellet (1 μg); lane 3, fraction after HiTrap Chelating HP (2 μg); lane 4, fraction after DEAE (2 μg). **b** The fraction after DEAE (0.1 μg) was subjected to immunoblot. Arrowheads indicate the position of dehydrin.

Fig. 3 Amino acid sequences of dehydrins. Sequences of AtHIRD11 (*Arabidopsis*) and RsDHN (radish) are shown. The red sequence indicates the epitope of anti-AtHIRD11 antibody. A sequence identified by MS is underlined. Asterisks represent amino acids that are identical

between two sequences.

Fig. 4 Cryoprotection of malate dehydrogenase by dehydrins and other compounds. Protective dose 50% (PD_{50}) values are shown. Values and bars represent means ± SD (n=3). *Significant difference ($p<0.05$) in comparison to RsDHN determined by Student's t-test. No PD_{50} value for Gly was determined due to its low level of protective activity.

Table 1 Purification of radish dehydrin.

Steps	Total protein (mg)	RsDHN (mg)	Purity (%)	Purification Factors	Yields (%)
Radish taproot	522.5	ca. 5.9	1.1	1	100
Pellet	117.3	5.3	4.3	4	90
NaCl extraction	14.7	4.5	31.1	28	76
IMAC	2.9	2.3	80.7	73	39
DEAE	2.1	2.0	96.1	87	34

Japanese white radish (100 g fresh weight) was used.

Table 2 Summary of dehydrin purifications

Plant species	Parts	Yields	Purification steps	References	DHN types
Fresh vegetative tissues					
Raphanus sativus	Taproots	20.7 mg/kg FW	EX - 2 CRGs	This study	KnS
Spinacia oleracea	Leaves	2.55 mg/kg FW	EX - HT - 2 CRGs	[26]	Kn
Dry seeds					
Vigna unguiculata	Dry seeds	26.8 mg/kg DW	EX - HT - 3 CRGs	[25]	YSKn
Zea mays	Maize kernels	13.1 mg/kg DW	EX - HT - 3 CRGs	[22]	YSKn
Glycine max	Dry seeds	77.3 mg/kg DW	Defatting - EX - Acid filtration - 3 CRGs	[24]	unknown

CRG, chromatography; DW, dry weight; EX, extraction; FW, fresh weight; HT, heat treatment

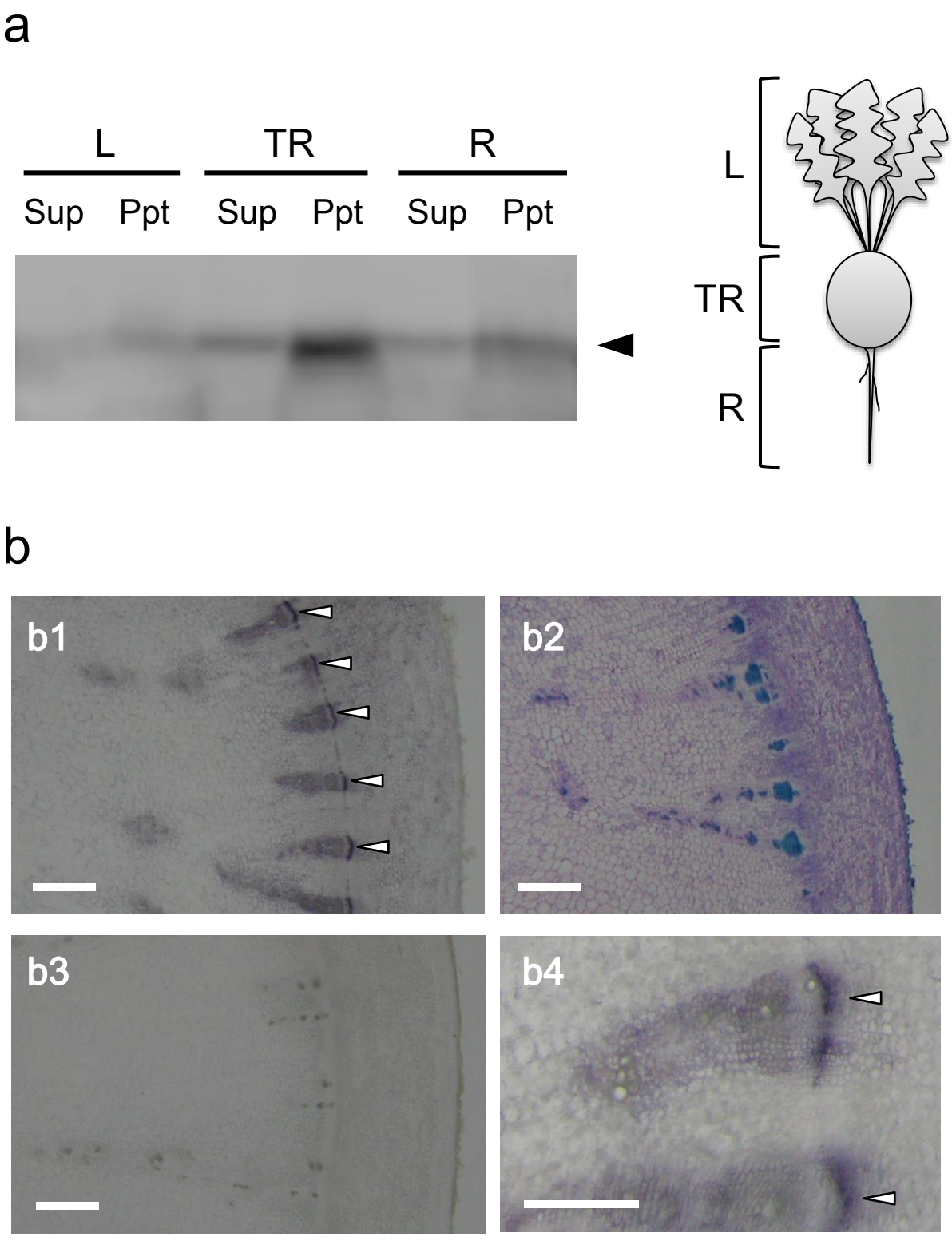


Fig. 1 Hara et al.

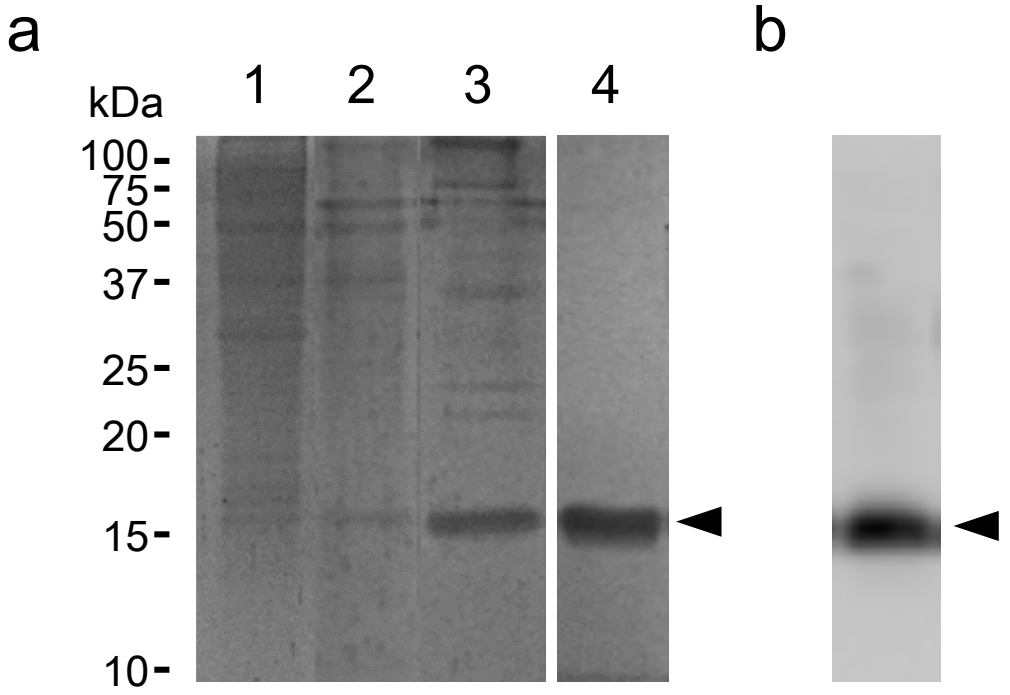


Fig. 2 Hara et al.

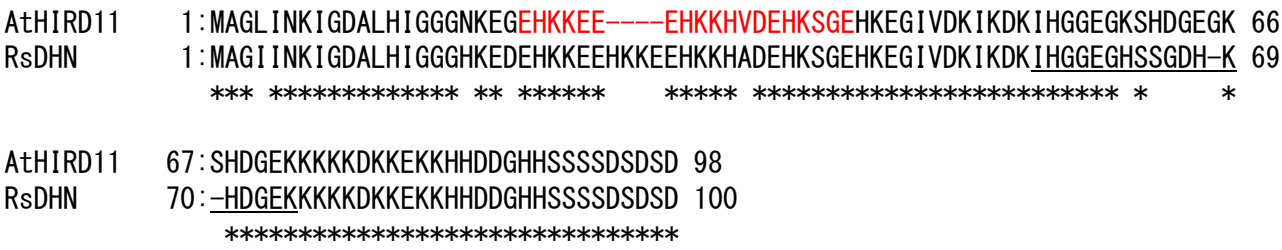


Fig. 3 Hara et al.

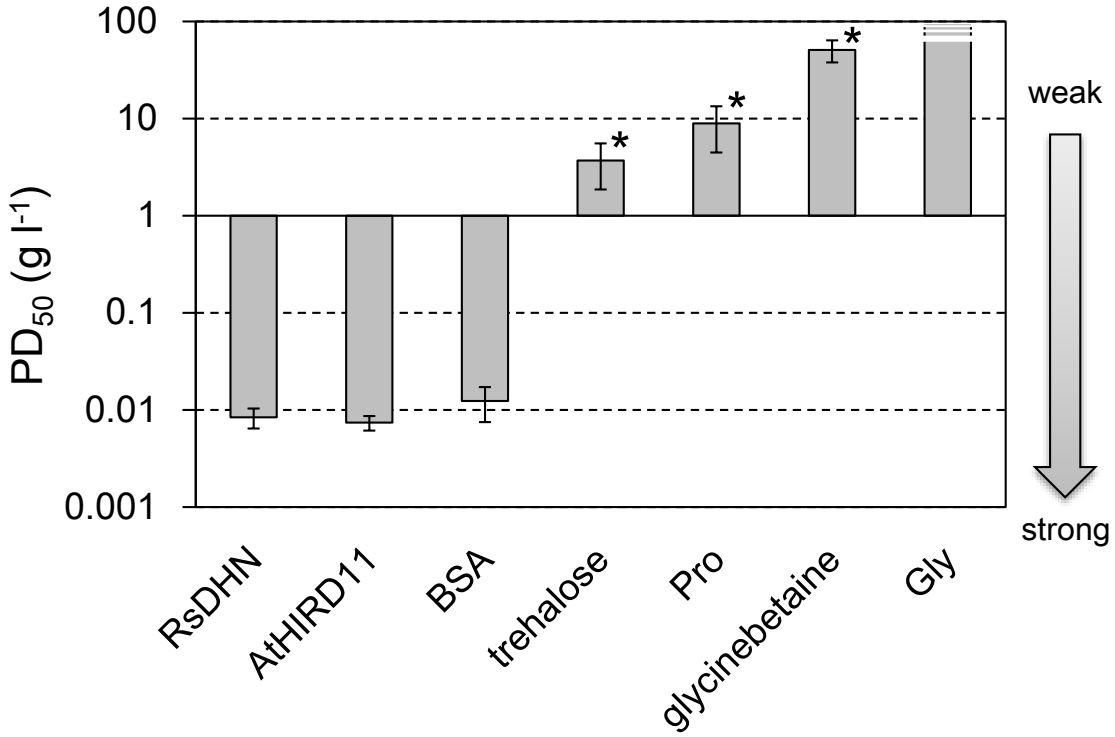
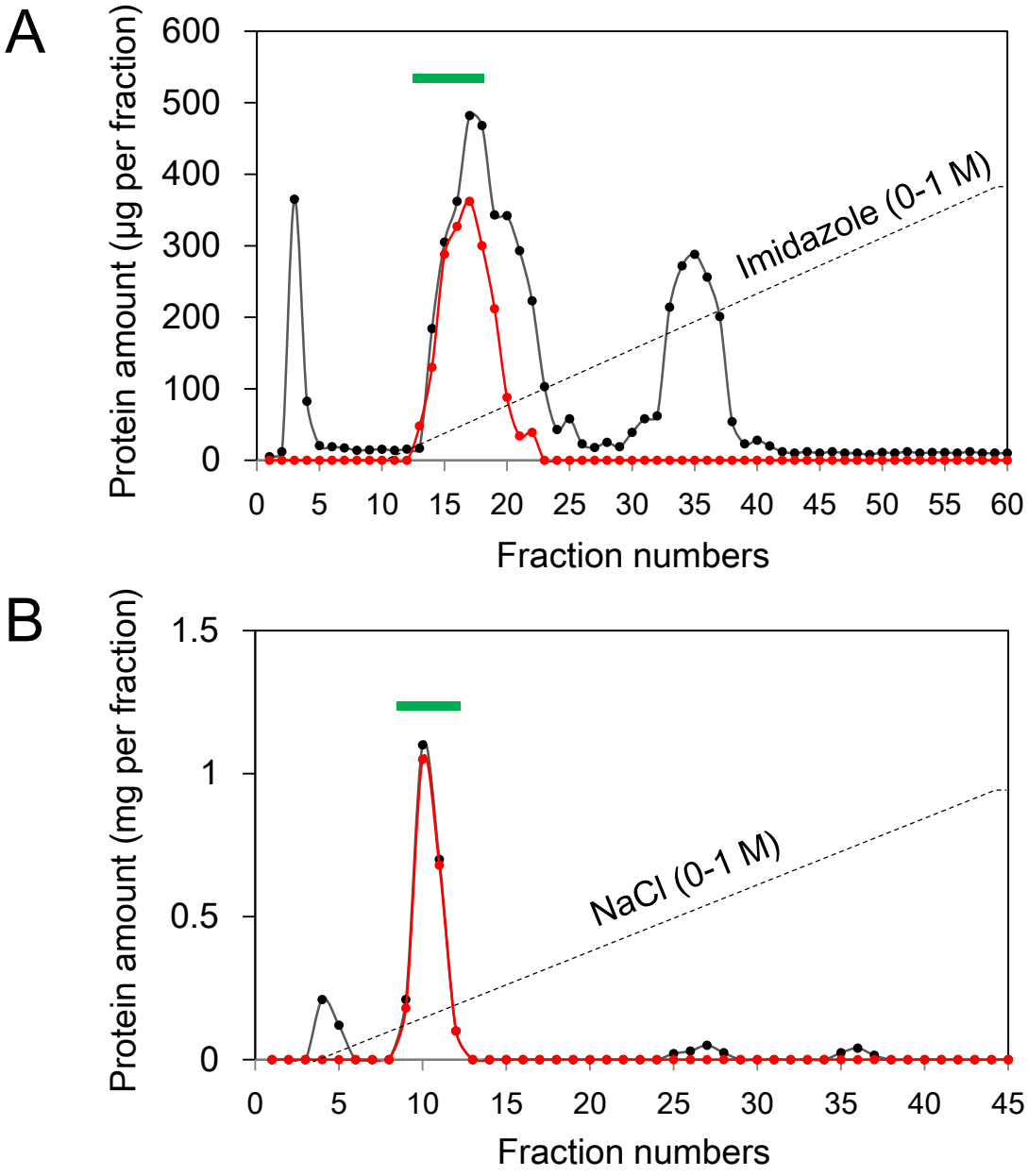


Fig. 4 Hara et al.



Supplemental Fig. 1
Typical chromatography patterns. **a** HiTrap Chelating HP. An imidazole gradient (0-1 M) was produced. **b** DEAE-Toyopearl. A NaCl gradient (0-1 M) was performed. Black and red lines show the total protein amounts and the RsDHN amounts, respectively. To make the chromatographic charts, the protein amount in each fraction was determined by measuring the intensities of the protein bands separated by the SDS-PAGE gel stained with Coomassie brilliant blue. Band intensities were analyzed using NIH image software (http://rsb.info.nih.gov/nih-image/). Green bars indicate the fractions collected.

Title: Efficient purification of cryoprotective dehydrin protein from the radish (*Raphanus sativus*) taproot
Authors: Hara M, Uchida S, Murata T, Wätzig H
Journal: Eur Food Res Technol

```
AA_RS1A356JQ   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGERKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS1AW77TF   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS1CA51JQ   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEERKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS1FS64TF   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDYKH 70
AA_RS1FZ84JQ   1:MAGIINKIGDALHIGVGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS1FZ84TF   1:MAGIINKIGDALHIGVGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS2F763JQ   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS2F763TF   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS2FS53JQ   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS2FS53TF   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS2FV08TF   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDLKH 70
AA_RS3FZ43JQ   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
AA_RS3FZ43TF   1:MAGIINKIGDALHIGGGHKEDEHKKEEHKKEEHKKHADEHKSGEHKEGIVDKIKDKIHGGEGHSSGDHKH 70
                 ***************.***************.***********.**********************.**

AA_RS1A356JQ  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS1AW77TF  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS1CA51JQ  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS1FS64TF  71:DGEKKKKKDKKEKKHHDDGRHSSSSDSDSD                                      100
AA_RS1FZ84JQ  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS1FZ84TF  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS2F763JQ  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS2F763TF  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS2FS53JQ  71:DGEKKKKKDKKEKKHHDDGHHSGSSDSDSD                                      100
AA_RS2FS53TF  71:DGEKKKKKDKKEKKHHDDGHHSGSSDSDSD                                      100
AA_RS2FV08TF  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS3FZ43JQ  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
AA_RS3FZ43TF  71:DGEKKKKKDKKEKKHHDDGHHSSSSDSDSD                                      100
                 ********************.**.*******
```

Supplemental Fig. 2

Thirteen full-length cDNA sequences from a radish, shown as orthologs of *Arabidopsis* dehydrin AtHIRD11 (At1g54410) on the Arabidopsis information resource web site (TAIR, http://www.arabidopsis.org/). Alignment was done using GENETYX software (Genetyx, Tokyo, Japan). Letters starting from AA_RS represent the names of the corresponding cDNAs. Asterisks represent amino acids that are identical among the 13 sequences. Sequences of the 5 clones shown in red are totally identical.

Title: Efficient purification of cryoprotective dehydrin protein from the radish *(Raphanus sativus*) taproot

Authors: Hara M, Uchida S, Murata T, Wätzig H

Journal: Eur Food Res Technol