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BRIEF REPORT

REVISED An ice-binding protein from an Arctic population of American dunegrass, *Leymus mollis*

[version 2; peer review: 3 approved]

Previously titled: An ice-binding protein from an Arctic grass, *Leymus mollis*

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Abstract

Several cold-hardy grasses have been shown to have ice-binding proteins (IBPs) that protect against freeze-thaw injury. Here, we looked for IBP activity in an Alaskan coastal grass, *Leymus mollis* (Pooidae), that had not previously been examined. Rhizome tissue had strong ice-structuring and ice recrystallization inhibiting (IRI) activities, indicating the probable presence of IBPs. The gene sequence of an IBP was obtained. The sequence encoded a 118-amino acid IRI domain composed of eight repeats and that was 80% identical to the IRI domain of the IBP of perennial ryegrass *Lolium perenne*. The predicted 3D structure of the IRI domain had eight beta-roll coils like those in *L. perenne* IBP

Keywords

Leymus mollis, ice-binding protein, Arctic, Alaska, Pooidae, dune grass, Chukchi Sea

Open Peer Review

Approval Status ✓ ✓ ✓

	1	2	3
version 2 (revision) 17 Aug 2020			
version 1 26 Jun 2020	✓ view	✓ view	✓ view

1. **Peter L. Davies**, Queen's University, Kingston, Canada

2. **Matthew Carlson**, University of Alaska Anchorage, Anchorage, USA
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3. **Hans Ramløv**, Roskilde University, Roskilde, Denmark

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: Sformo TL: Conceptualization, Funding Acquisition, Investigation, Resources, Validation, Writing – Review & Editing; **Raymond JA**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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First published: 26 Jun 2020, **9**:648 https://doi.org/10.12688/f1000research.24328.1

REVISED **Amendments from Version 1**

Many revisions were made in response to reviewer comments.

Leymus mollis is not strictly an Arctic grass so the title was changed to "an Arctic population of American dunegrass, *Leymus mollis*".

The percent identity to *L. perenne* was added to the Abstract.

Details about the growing season on the Arctic coast were added to the Introduction.

We clarified the significance of facets on ice crystals in the Results and discussion section.

We added another control (*Festuca*) to Figure 2 to more clearly show the higher IRI activity of *L. mollis*.

A sentence was added to clarify how IBPs are thought to bind to ice to the Results and discussion section.

We revised the final paragraph to provide some comparative aspects of cold tolerance and adaptation in plants.

We have added a supplementary table (Table S1) as *Extended data*, which gives the frequency of subzero temperatures during the growing season.

Any further responses from the reviewers can be found at the end of the article

Introduction

Within the grass family (Poaceae), the subfamily Pooideae includes many cold-adapted grasses including wheat, barley and forage grasses. These grasses have developed a large family of ice-binding proteins (IBPs) that protect the plants from freezing damage (Sandve *et al.*, 2008). The IBPs, which have negligible effect on the freezing point, are characterized by a C-terminal domain consisting of several repeating units that is associated with strong ice recrystallization inhibition (IRI) activity (Sandve *et al.*, 2008). This region is thus called the IRI domain. Ice recrystallization, in which larger ice grains grow at the expense of smaller grains, occurs mostly at warmer sub-zero temperatures and is thought to cause damage to cell walls. Two of the most studied of these IBPs are from the grasses *Lolium perenne* (Sidebottom *et al.*, 2000) and *Deschampsia antarctica* (John *et al.*, 2009). Their amino acid sequences are very similar in both the N-terminal and IRI domains. The 118-a.a. IRI domain of the *L. perenne* IBP has been crystallized and its structure determined by X-ray crystallography (Middleton *et al.*, 2012). The IRI domain has a beta-roll fold with eight similar coils, one side of which is the predicted ice-binding site. The spacing of the coils is very close to the repeat distance along the *a*-axis of ice. Interestingly, heterologous expression of the *Lolium* IRI domain in tomato was also shown to increase chilling (4°C) tolerance (Balamurugan *et al.*, 2018), although the mechanism remains unclear. Here, we describe a related IBP from a pooid grass from Utqiaġvik (Barrow), Alaska, *Leymus mollis*. This site, at 71.3° N, is the highest latitude at which IBPs have been examined in a grass It is characterized by a short growing season from mid-June to late August with an average air temperature of 4°C (Tieszen, 1972), and frequent subzero air temperatures (Table S1, *Extended data*).

L. mollis, also known as American dunegrass, is found in coastal habitats, especially sand dunes across North America and Greenland. It is typically subjected to many stresses, such as low nutrient levels, salt spray, little freshwater, inundation during storms, wind abrasion, and ice storms (Gagné & Houle, 2002). Here we characterize the IBP of *L. mollis* and compare it with the IBP of *L. perenne*, a plant that is not as well adapted to such northern regions (Helgadóttir *et al.*, 2018).

Methods

Grass

A grass sample was collected from a gravel beach at Utqiaġvik (Barrow), Alaska (71.3°N) on 6 October 2019, and stored at -80°C. The grass was shipped frozen to University of Nevada Las Vegas for analysis, but accidentally rose to ~10°C for about one day during shipment.

Activity measurements

To obtain a sample for measuring ice structuring and IRI activities, the brown outer layers of a rhizome from just below the soil surface were peeled off, revealing green tissue underneath. About 70 mg of the green tissue was ground in 1 ml water in a mortar and pestle. The suspension was centrifuged at 14,000 rpm for 5 minutes to yield a slightly cloudy supernatant. Stem tissue from lawn grass (*Festuca* sp.) at the University of Nevada Las Vegas (52 mg) was similarly homogenized and used as a control. Ice-structuring activity was observed by examining the growth of an ice seed crystal with well-defined ice *c*- and *a*-axes submerged in the grass extract supernatant, as described previously (Raymond & Fritsen, 2001). Briefly, the sample was placed in a rectangular tube and the tube was submerged in a controlled temperature bath with front and rear windows. The growth of the crystal was observed at a temperature slightly below the freezing point (~-0.2°C) with a horizontally mounted dissecting microscope. Ice-structuring activity was defined as the appearance of sharply defined facets on the crystal surface. IRI activity was observed as described previously (Raymond & Fritsen, 2001). Briefly, 3 µl drops of supernatant were placed on a slide cover glass. A liquid nitrogen-cooled slide glued to a handle was pressed against the drops to form highly polycrystalline ice between the slide and cover glass. The samples were stored at -3°C in hexane and changes in recrystallization were monitored in the temperature bath described above over 21 hours. Photographs were taken through crossed polarizers.

Sequencing

Green tissue was obtained and homogenized as described above. DNA was extracted with a NucleoSpin Plant II kit (Machery Nagel; catalog number 740770) according to the manufacturer's instructions. To make IBP primers, the nucleotide sequences of IBPs from *L. perenne*, *D. antarctica* and *Triticum aestivum* (GenBank accession numbers EU680848, FJ663044 and KU204387, respectively) were aligned. Regions with high identities at the 5' and 3' ends were selected for primer sequences to obtain an amplicon that covered as much of the gene as possible. Primers for 18S ribosomal RNA were selected from conserved regions in the 18S sequences from *Dupontia fisheri*, *L. perenne* and *D. antarctica* (GenBank accession numbers KP794861, KJ598999 and MH628292, respectively) that flanked a region of high variability. At that time, we

suspected the Utqiaġvik grass sample was *D. fisheri* and did not use the *L. mollis* 18S sequence. PCR was carried out with an Eppendorf Mastercycler Personal thermal cycler, with 3 minutes initial denaturation at 95°C, followed by 35 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 59°C and 40 seconds extension at 72°C, followed by 3 minutes final extension at 72°C using Promega GoTaq polymerase (catalog number M300B). PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and observed and photographed with a UVP transilluminator. IBP and 18S bands of the expected sizes were obtained (see *Underlying data* (Raymond, 2020c)), cleaned up with a Nucleospin gel and PCR clean-up kit (Machery Nagel, catalog number 740609) and sequenced in both directions at the UNLV Genomics Core with an Applied Biosystems 3130 sequencer. The primers that were used for sequencing are shown in Table 1.

Structure prediction

A 3D model of the IRI domain of the *Leymus* IBP was predicted with SWISS-MODEL (Waterhouse *et al.*, 2018) using the eight-coil structure of the *Lolium perenne* IRI domain (Protein Data Bank accession no. 3ULT) (Middleton *et al.*, 2012) as template. SWISS MODEL proposed three structures of the *Leymus* IRI domain based on the template. We selected the one with the highest sequence identity (76.5%), which also was the only one that had eight orderly coils like those in the *L. perenne* template. The free energy of this structure was then minimized (from -42.56 to -52.58 MJ mol^{-1}) with the YASARA Energy Minimization Server (Krieger *et al.*, 2009) and displayed with YASARA View. Stereoviews were obtained by rotating the molecule around its vertical axis by 3°. The average distance between coils in the IRI domain was measured with the YASARA distance function as the distance between the alpha carbons of Ser15 and Ser116 divided by seven.

Results and discussion

Species identification

The obtained 18S rRNA sequence (GenBank accession no. MT506010) most closely matched the sequence of *Leymus mollis* (GenBank accession no. EF581964) (98.3% identity). Among the Pooidae, *L. mollis* is a member of the Triticodae, which includes wheat (*Triticum*) and barley (*Hordeum*), while *Lolium* and *Deschampsia* are members of the Poodae. The grass was confirmed as *L. mollis* from photos of the remnant spikes and leaves by Matthew Carlson (University of Alaska Anchorage) and Carolyn Parker, University of Alaska Fairbanks (personal communications to T.S.).

Ice-structuring and recrystallization inhibition activities

An extract from green rhizome tissue strongly affected the growth of an ice seed crystal, causing it to develop sharp facets (Figure 1A). The facets are an indication of the presence of ice-binding proteins. The facets themselves do not have any role in freezing tolerance, nor are they likely to form in frozen tissue; they are only a demonstration that molecules are binding to the ice surface, which is necessary for IRI. In constrast, ice grown in the presence of extract from lawn grass stem tissue (control) is smooth and rounded with no evidence of facet development (Figure 1B). The *Leymus* extract also had strong IRI activity. Polycrystalline ice formed from water and the lawn grass extract recrystallized significantly after 21 hours at -3°C, while the supernatant of the *Leymus* extract showed no recrystallization (Figure 2).

Ice-binding protein

Primers based on the IBPs of other pooid grasses succeeded in amplifying a sequence that encoded the C-terminal part of the IBP gene of *L. mollis*, including the entire IRI domain. The sequence (GenBank accession no. MT506011) encoded 260 a.a., which corresponds to all but the first 25 a.a. of the *L. perenne* IBP sequence. The sequence included the stop codon and contained no introns. Although the sequence was close to the sequence of *L. perenne* (65% in the region of overlap), it most closely matched an IBP-like protein from *Triticum aestivum* (QBE94480) (86% identity, 91% similarity), in agreement with *L. mollis*'s classification as a member of the Triticodae. When only the IRI domains were compared, the *Leymus* IRI domain was also closer to the *Triticum* IRI domain than it was to the *Lolium* IRI domain. The ice-binding activities of *Tritium* spp. IBPs have not yet been reported.

The IRI domain of *L. mollis*, like that of *L. perenne*, has eight repeats, each consisting of 14 or 15 residues (Figure 3A). The consensus sequences of the two IBPs are virtually the same (Figure 3A).

Model of structure

The predicted structure of the *L. mollis* IBP IRI domain, obtained from using the *L. perenne* IBP IRI domain structure as template, resembles the *Lolium* IRI domain in several respects: it has eight repeats corresponding to eight coils in a beta-roll fold (Figure 3B); a flat side (the a side) that is populated with two rows that are rich in Thr and Ser residues (Figure 3B); an irregular side (the b side) that has a bulge in the first three coils (Figure 3C); and an interior that is dominated by Asn/His

Table 1. Primers used in this study.

Primer pair	Fwd	Rev	Size (bp)
IBP	5′-TGCCACCCCGATGACCTCCG-3′	5′-TTAACCTCCTGTCACGACTTTGTTGCTCCC-3′	798
18S	5′-GGAAGGATCATTGTCGTGACCCTGACC-3′	5′- CTGGGGTCGCGGTCGAAGCGTC-3′	623

IBP, ice-binding protein.

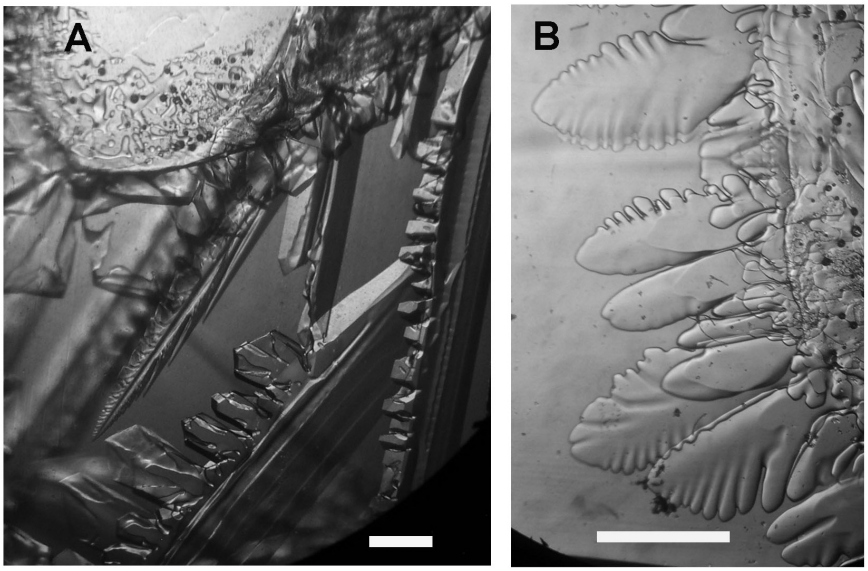


Figure 1. Ice-structuring abilities of grass extracts. (**A**) *Leymus mollis* rhizome tissue. (**B**) Lawn grass (*Festuca* sp.) stem tissue (control). Ice seed crystals were placed in the extracts at slightly below the freezing point. Scale bars, 1 mm.

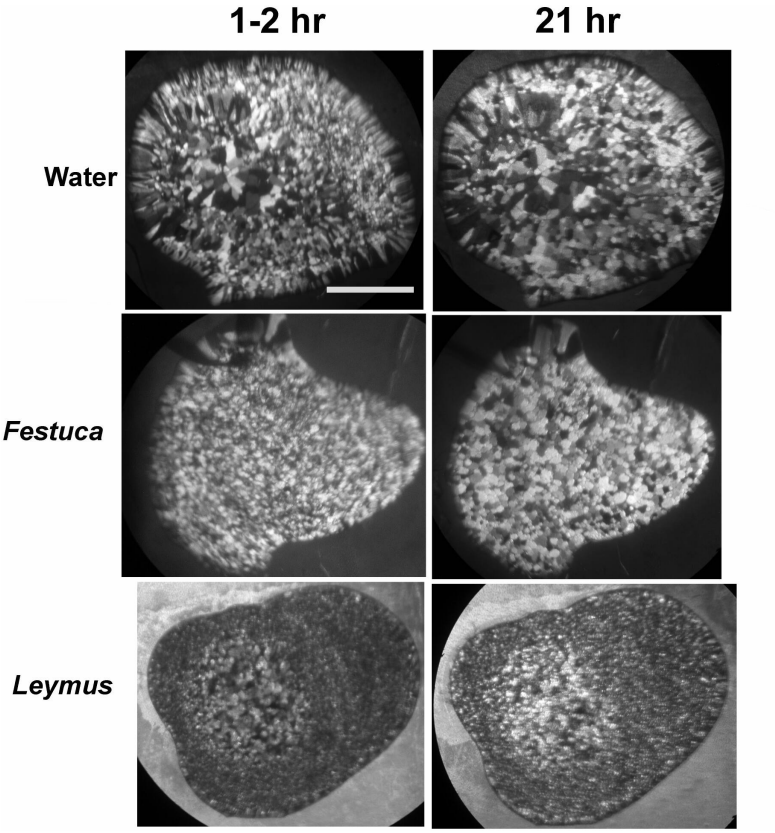


Figure 2. Comparison of ice recrystallization in 3 µl drops of water and extracts from lawn grass (*Festuca*) and *L. mollis* (*Leymus*). Scale bar, 1 mm.

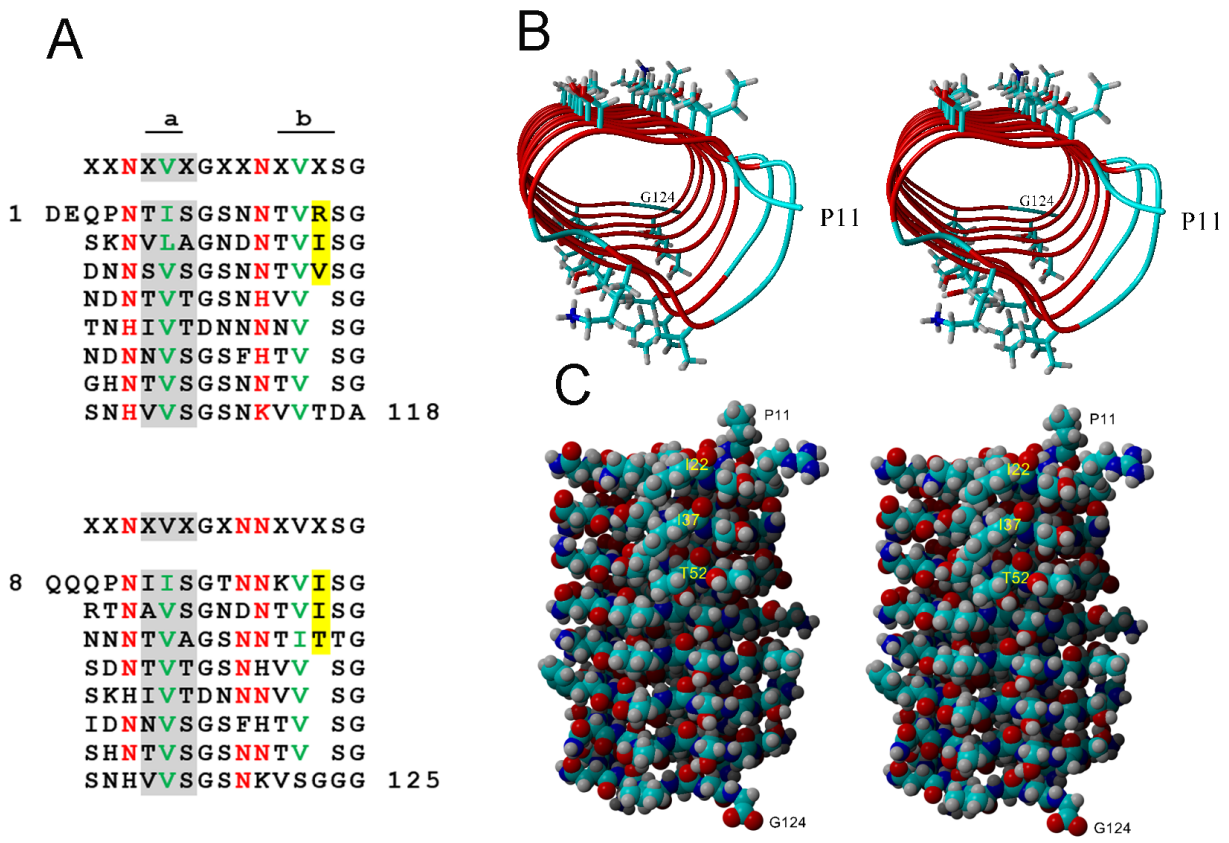


Figure 3. Structure of the IRI domain of *Leymus mollis* IBP. (**A**) Comparison of the Leymus repeats (bottom) with those in the IRI domain of *Lolium perenne* (top). The *Lolium* data and the color scheme have been reproduced with permission from Middleton *et al.* (2012). Both domains have eight repeats. Consensus sequences are shown on top. Numbers indicate a.a. residue numbers. In the *Lolium* sequence, the gray background indicates the ice-binding site called the a side and the yellow background indicates a bulge on the b side. Similar features are found in the *Leymus* sequence. (**B**, **C**) Stereoviews predicted by SWISS MODEL using the IRI domain of *L. perenne* as template. SWISS MODEL was able to model the *Leymus* structure from Pro11 to Gly124, which corresponds to Asp1 to Ala118 in *L. perenne*. (**B**) View through the center of the coils, in which the ice-binding site (the a side) is on top. Amino acid side chains are shown only for the a and b sides. Color code of the ribbon: red, beta strand; cyan, coil. Color code of amino acid side chain: Cyan, C; blue, N, red, O; gray, H. (**C**) View of the b side of a space-filling model. A bulge is created by two residues on each of the first three coils. The first of the two residues in each coil is labeled. Colors are the same as those for the side chains in **A**.

ladders (red residues in Figure 3A). The a side has been identified as the ice-binding site of *L. perenne* IBP (Middleton *et al.*, 2012). In *Lolium*, the average spacing between the alpha carbons of the row of amino acids on the ice-binding site is 4.5 Å, which is very similar to the spacing along the *a*-axis of ice (4.51 Å). In *Leymus*, the average spacing was calculated as 4.77 Å. The regular array of hydrophilic residues is thought to order water molecules that act as a glue between the IBP and the ice (Middleton *et al.*, 2012).

In summary, we describe an ice-binding protein from the grass *Leymus mollis* with ice-structuring and ice recrystallization inhibition (IRI) activities. The collection site of the grass, a gravelly beach on the Chukchi Sea, is an extreme habitat subject to numerous environmental stresses. *L. mollis* appears to be better adapted to high latitudes than *L. perenne*, which does not grow well north of 60°N (Helgadóttir *et al.*, 2018). It seems unlikely that the greater cold hardiness of *Leymus* can be attributed to its IBP as the IBPs of the two grasses are very similar. However, many proteins contribute to the cold hardiness of forage grasses, including transcription factors, cell membrane proteins, and proteins involved in cell signalling, cellular transport and photosynthesis (Sandve *et al.*, 2011). It is thus likely that many of these factors are responsible for *L. mollis*'s survival in the high Arctic. One approach to identifying them would be a comparison of transcriptomes of the Arctic population with a California population that never experiences freezing conditions.

Data availability

Underlying data

Leymus mollis 18S ribosomal sequence on GenBank, Accession number MT506010

Leymus mollis IBP sequence on GenBank, Accession number MT506011

Figshare: Ice structuring by ice-binding protein of Leymus mollis DSCN5945.JPG. https://doi.org/10.6084/m9.figshare.12401966.v1 (Raymond, 2020a)

Figshare: Leymus mollis ice recrsytallization inhibition. https://doi.org/10.6084/m9.figshare.12401954.v1 (Raymond, 2020b)

Figshare: PCR amplification of IBP and 18S genes of Leymus mollis. https://doi.org/10.6084/m9.figshare.12401957.v1 (Raymond, 2020c)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Extended data

Figshare: Table S1. Number of days with subzero air temperatures during the growing season at Utqiaġvik, AK. https://doi.org/10.6084/m9.figshare.12765983.v1 (Raymond & Sformo, 2020)

This project contains the following extended data:

- Table S1.docx - Number of days with subzero air temperatures during the growing season at Utqiaġvik, AK. Data provided by the National Climatic Data Center.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

JR thanks the School of Life Sciences, University of Nevada Las Vegas, for providing facilities for carrying out this study.

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PubMed Abstract | Publisher Full Text | Free Full Text

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PubMed Abstract | Publisher Full Text

Raymond J: **Ice structuring by ice-binding protein of Leymus mollis DSCN5945.JPG.** *figshare.* Figure. 2020a.
http://www.doi.org/10.6084/m9.figshare.12401966.v1

Raymond J: **Leymus mollis ice recrsytallization inhibition.** *figshare.* Figure. 2020b.
http://www.doi.org/10.6084/m9.figshare.12401954.v1

Raymond J: **PCR amplification of IBP and 18S genes of Leymus mollis.** *figshare.* Figure. 2020c.
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PubMed Abstract | Publisher Full Text

Raymond J, Sformo TL: **Table S1. Number of days with subzero air temperatures during the growing season at Utqiaġvik, AK.** *figshare.* Dataset. 2020.
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Publisher Full Text

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PubMed Abstract | Publisher Full Text | Free Full Text

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Current Peer Review Status: ✔ ✔ ✔

Version 1

Reviewer Report 27 July 2020

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✔ **Hans Ramløv**
[1] Department of Science and Environment, Roskilde University, Roskilde, Denmark
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- In the present paper Sformo and Raymond present data on an ice binding and recrystallisation inhibiting protein from the Alaskan grass Leymus mollis, which lives in areas where it is exposed to several kinds of stresses including low temperatures.

- The authors have observed ice structuring, recrystallisation inhibition activity in aqueous homogenates of the rhizome tissue. They have sequenced DNA from similar homogenates using primers based upon the conserved sequences from other IBP containing grasses. The obtained DNA sequences were translated into the amino acid sequences presumably involved in the ice recrystallisation inhibition and ice structuring. The amino acid sequence of the IRI domain of *L. mollis* was closely matched to the *Triticum aestivum* IRI domain.

- The 3D structure of the ice binding domain was predicted using the obtained sequence and the structure of the IRI domain in *Lolium perenne*. It appears that the tree dimensional structure of the ice binding domain of *L. mollis* IBP quite resembles that of *L. perenne*.

- The paper is an interesting comparative account of the ice related properties of a grass found at high latitudes - again indicating that ice structuring and recrystallisation inhibition is very important in plants adapted to cold climates.

- I miss an extension of the discussion of the comparative aspects of cold tolerance and adaptive aspects of the findings in the paper.

- Figure 1 could be explained a bit better as some readers may not understand the differences between the A and the B panel very well.

- Figure 2 is not very clear and also this figure would benefit from a bit more explanation in the text.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cold tolerance of ectothermic animals, antifreeze proteins, cryptobiosis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 30 Jul 2020

James Raymond

- I miss an extension of the discussion of the comparative aspects of cold tolerance and adaptive aspects of the findings in the paper.

We revised the last paragraph to give some perspective to the cold tolerance of L. mollis and how it probably differs from that of L. perenne.

- Figure 1 could be explained a bit better as some readers may not understand the differences between the A and the B panel very well.

We added some sentences to better explain the difference between Leymus and Festuca.

- Figure 2 is not very clear and also this figure would benefit from a bit more explanation in the text.

We added new photos to show RI of Festuca. It more clearly shows that Leymus has much stronger RI than Festuca.

Competing Interests: The authors declare no competing interest.

Reviewer Report 23 July 2020

https://doi.org/10.5256/f1000research.26842.r65704

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Matthew Carlson
[1] Alaska Center for Conservation Science, University of Alaska Anchorage, Anchorage, AK, USA
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This brief report outlines a description of the sequence and structure of ice-binding protein in Leymus mollis from the Beaufort Coast in Arctic Alaska, and compares the findings to IBPs of other more temperate grass species.

The paper seems pretty straightforward, however, I do not have the expertise to comment on the amino acid sequence, protein modeling, and comparative approach to other species. The paper was well-written, the figures were informative, and I believe it warrants indexing.

While this is a brief note, I do think that additional interpretation of results and providing some brief explanations related to mechanisms of freeze tolerance in light of their findings would make the manuscript substantially more valuable. I was not able to gauge if the similarities and differences between Leymus and Lolium/Festuca are likely to have physiological/ecological bearing on their respective environmental contexts. (As an aside, since Leymus grows all down the Pacific Coast to San Diego it would be interesting to know if the IBP structure or concentrations/ability to upregulate are different along that substantial environmental gradient.).

I provide additional comments and edits embedded as notes in a PDF - please see the file here.

I appreciate the opportunity to read this paper and I sincerely hope that my comments are useful in generating a more robust paper.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant ecology and evolution - emphasis on Arctic and Boreal systems

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 27 Jul 2020

James Raymond

Thank you for many good comments. Revisions have been made to address all of them.

Competing Interests: the authors declare no competing interests.

Author Response 05 Aug 2020

James Raymond

Thank you for many helpful comments. We revised the manuscript to address each of them. Our response to your comments can be viewed in the PDF file here.

Competing Interests: The authors declare no competing interests.

Reviewer Report 20 July 2020

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Sformo and Raymond have prepared aqueous extracts of a hardy coastal grass (*Leymus mollis*) collected in Alaska and have observed the presence of an ice-binding protein (IBP) based on ice crystal faceting and the inhibition of ice recrystallization. From another extract they prepared DNA and used primers based on conserved regions in other grass IBP transcripts to amplify the *L. mollis* gene, which fortunately lacks introns. From the sequence of this DNA they were able to provide the amino acid sequence of the IBP domain involved in ice recrystallization. They used this to model the three-dimensional structure of the domain, which looks very much like that of *Lollium perenne*. Another set of primers was used to amplify a diagnostic section of the 18S ribosomal RNA gene whose sequence identified the species.

There is an element of comparative science here, and one would not need to see a repeat of this detailed analysis on other grasses/cereals. The presence of sequence homologs in transcriptomes as a way of seeing the distribution and variation of this IBP type should suffice from here on. Nevertheless, it is interesting to see this grass from a high latitude in Alaska (Beaufort Sea coast) extracted and analyzed. This report is also a good illustration of the use of molecular modeling to avoid solving a structure that would not differ much from the template. Indeed, the main features of *the Lolium perenne* IBP fold are retained in the *L. mollis* model, including the outward bulge of the beta-solenoid at one end on the non-ice-binding side.

Suggestions for improvement:

- The Abstract should include the percent identity of the IRI domain between the two grass species. This type of information should also be provided in the Introduction where the sequences of the IBPs of *L. perenne* and *D. antarctica* are compared. It is not helpful to say their sequences “are very similar”. In the last paragraph of the Introduction the *L. mollis* IBP sequence is again said to be “very similar” to those of the other two grasses. Please provide % identity and % similarity.

- Figure 1A shows ice structuring. However, since panel B also shows ice crystals it is important to point out to the average reader that the distinction lies in the faceting seen in A where flat, angular surfaces are the result of IBP binding to ice. The ice in B is smoother and rounded.

- Figure 2 is a rather poor presentation of ice recrystallization. One must look closely at the ice control images to see the grains are larger after 21 h. The methods say the ice recrystallization was done in water. From our experience the inclusion of 150 mM NaCl makes a huge difference to the visibility of the ice grains. The other suggestion is to take the starting picture at zero time with the ice held colder than – 3 °C. We see substantial recrystallization happening in the first hour, even at -6 °C, and will typically hold the ice wafer at -10 °C until the starting picture is captured, and then raise the temperature for more rapid recrystallization. I recommend that this analysis be repeated and improved.

- In Methods, under ‘Structure prediction’, it is stated that three structures were proposed by SWISS MODEL operating with *L. perenne* IBP as a template. Then, the one with the ‘highest sequence identity’ was selected. Surely the sequence identity is the same for all models

because they are based on the same template. We would typically compare models by root mean square deviation (RMSD) – how the atoms deviate from the model on average. Is this what is meant here?

- Although the sequence of the *L. mollis* open reading frame was incomplete, it would be useful to report the sequence upstream of the ice-binding domain. Presumably this is a series of leucine-rich repeats similar to those seen in other grass IBPs?

- In reference to ice crystal axes the a- and c- should be italicized.

- Replace the contraction "didn't" with "did not".

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Protein biochemistry, structural biology, ice-binding proteins

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 27 Jul 2020

James Raymond

Percent identities added to Abstract. Identities were removed from Introduction to make a revision requested by reviewer 2.

Fig. 1A: The sentence was revised to point out that the ice is smooth and rounded with no evidence of facet development.

Fig. 2: The experiment requires much preparation and is difficult to repeat now that one of the authors is back in Alaska. However, we think there is a clear difference in recrystallization between the two samples when the figure is viewed at full scale.

Swiss-Model: Swiss Model tries different regions of the the sequence to model, e.g., residues 131-247 in one model and residues 144-228 in another. Each gives a slightly different model. We selected the one that had the highest a.a. sequence identity.

N-terminal region of IBP: For this, we would need a consensus sequence of the 5' UTR in other grasses (for use as a forward primer) but we were unable to find such a sequence.

Competing Interests: the authors declare no competing interests

Author Response 28 Jul 2020

James Raymond

Percent identity was added to Abstract and the Results. Identities were removed from Introduction to make a revision requested by reviewer 2.

Fig. 1A: The sentence was revised to point out that the ice is smooth and rounded with no evidence of facet development.

Fig. 2: We added a new control, Festuca (lawn grass) extract, which shows more clearly the greater RI activity of Leymus.

Swiss-Model: Swiss Model tries different regions of the sequence to model, e.g., residues 131-247 in one model and residues 144-228 in another. Each gives a slightly different model. We selected the one that had the highest a.a. sequence identity.

N-terminal region of IBP: For this, we would need a consensus sequence of the 5' UTR in other grasses (for use as a forward primer) but we were unable to find such a sequence.

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