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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/29, 15/82, 15/56, 15/72, 15/81, 9/24, C07K 14/415, C12N 5/10, 1/19, 1/21, A01H 5/00, A01K 67/027, A23L 3/36, A23G 9/00

A2

(11) International Publication Number: **WO 99/06565**

(43) International Publication Date: 11 February 1999 (11.02.99)

(21) International Application Number: PCT/CA98/00745

(22) International Filing Date: 31 July 1998 (31.07.98)

(30) Priority Data:
08/903,872 31 July 1997 (31.07.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 08/903,872 (CIP)
Filed on 31 July 1997 (31.07.97)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ANTIFREEZE PROTEINS, DNA AND EXPRESSION SYSTEMS

(57) Abstract

§he winter rye, upon cold–induction or aclimation, produces a family of antifreeze proteins that are similar to pathogen–related proteins. Two of these proteins, both of which are chitinase–like proteins, are cloned using molecular biology techniques and are expressed in bacterial and yeast (*Pichia*) systems and *Arabidopsis thaliana.* The recombinant proteins showed both chitinase and antifreeze activities. The invention includes the DNA and protein sequences of the chitinase–like antifreeze proteins, any modifications of the said sequences, the expression of these proteins and their appplication in agriculture, food industry and medicine.

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ANTIFREEZE PROTEINS, DNA AND EXPRESSION SYSTEMS

U.K. provisional application 9112774.6 (filed June 13, 1991), U.S. Patent Application Nos. 08/060,425 (filed May 11, 1993), 08/419,061 (filed April 10, 1995), and 08/485,647 (filed June 7, 1995), Canadian Patent Application No. 2,110,510 (filed June 12, 1992), European Patent Application No. 92911435.3 (filed June 12, 1992), PCT application No. PCT/CA92/00255 (filed June 12, 1992) and U.S. application no. 08/903,872 are incorporated by reference herein in their entirety. This application claims priiority from U.S. application no. 08/903,872.

FIELD OF THE INVENTION

The invention relates to plant antifreeze proteins, peptides and polypeptides (also referred to as "AFPs") that bind to ice crystals and inhibit their growth, inhibit ice recrystallization and protect liposomes, cell membranes and proteins, cells and organisms at low temperatures.

The invention also relates to DNA sequences that encode proteins with both chitinase and antifreeze activities and the expression of these DNA sequences in bacteria, yeast, plants and animals for the production of chitinases with antifreeze activity.

BACKGROUND OF THE INVENTION

Low temperature is a major environmental limitation to the production of agricultural crops. For example, late spring frosts delay seed germination, early fall frosts decrease the quality and yield of harvests and low winter temperatures decrease the survival of overwintering crops, such as winter cereals and fruit trees. However, some plants have the ability to withstand prolonged subfreezing temperatures. With the identification and isolation of proteins involved in the development of freezing tolerance in these plants, as well as the corresponding genes, freezing-sensitive crop plants can be transformed into freezing-tolerant crop plants and extend the range of crop production.

Guy et al. (I-5.1) analyzed the total protein content of cold-acclimated spinach tissues compared with tissues from warm-grown plants to find proteins associated with freezing tolerance. Proteins found in cold-acclimated leaf extracts with molecular masses of 110, 82, 66, 55 and 13 kD were not found in warm-grown leaf

extracts. Guy et al. (I-5.2) also examined the total protein content of cold-acclimated spinach leaves and observed the accumulation of high molecular mass proteins (110, 90 and 79 kD). As with Guy et al. (I-5.1), the location and function of these proteins within the plant remain unknown.

5 It was known, however, that many biological organisms survive subzero environments by avoiding ice formation. This strategy requires the synthesis of antifreeze proteins ("AFPs"), also known as thermal hysteresis proteins ("THPs"). AFPs bind to the surfaces of ice crystals to prevent their further growth. The presence of AFPs is determined: 1) by examining the shape of ice crystals as they 10 grow (III-19, III-35, III-22), 2) by measuring thermal hysteresis, which is the difference in temperature at which an ice crystal in solution melts and freezes (III-19), and 3) by measuring the inhibition of the recrystallization of ice (III-16).

Four distinct types of AFPs were identified in fish (II-4) and a number of different THPs were identified in insects (III-12). These previous findings suggest 15 that this adaptive mechanism has arisen independently in different organisms and that this mechanism is responsible for preventing any ice formation within the organism. It was generally understood that AFPs did not exist in plants because most plants that survive prolonged exposure to subzero temperatures actually form ice in their tissues, usually in intercellular spaces and xylem vessels. Kurkela and 20 Franck (I-9.1) reported that a plant gene expressed at low temperature encodes a protein similar in amino acid sequence to the flounder AFP of Davis and Hew (II-4). Kurkela and Franck (I-9.1) did not have sufficient amounts of the encoded protein to determine whether it exhibited antifreeze activity. Cutler et al. (I-1.1) demonstrated that plant tissues infused with the flounder AFP exhibit a slight increase in freezing 25 tolerance. Georges et al. (I-3.1) transformed corn protoplasts with a synthetic gene for the flounder AFP in an attempt to improve plant freezing tolerance. A CAT-AFP fusion protein was produced, but it was not secreted efficiently and no antifreeze activity was detected.

Although AFPs isolated from fish and insects have been used in developing 30 new additives for food products and biological materials stored at low and subzero temperatures and in transforming plants, these proteins have not entered the marketplace because they are perceived by consumers to be inappropriate. For example, the addition of a fish AFP to ice cream is not acceptable whereas the addition of a plant AFP to ice cream is acceptable to consumers. The use of AFPs 35 isolated from plants is expected to be more widely applicable in the marketplace than

AFPs already characterized from animals.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention will be described in relation to the drawings
5 in which:

I. COLD TOLERANCES IN PLANTS

Figure 1 depicts concentrations of extracellular proteins from winter rye leaves grown under various temperature regimes.

Figure 2 depicts SDS-PAGE of extracellular polypeptides isolated from winter rye
10 leaves grown under various temperature regimes. Lane 1, molecular mass markers; lane 2, extracellular polypeptides from rye plants grown at 20/16° C (day/night) with a 16-hour day; lane 3, extracellular polypeptides from rye plants grown at 5/2° C with a 16-hour day; lane 4, extracellular polypeptides from rye plants grown at 5/2 °C with an 8-hour day.

15 **Figure 3** depicts SDS-PAGE of extracellular polypeptides isolated from cold-acclimated winter rye leaves grown with an 8-hour daylength at different stages of development. Lane 1, molecular mass markers; lane 2, extracellular polypeptides from rye plants grown at 20/16° C with a 16-hour day for 7 days; lane 3, extracellular polypeptides from rye plants (20/16 °C, 7 days old) transferred to 5/2° C with an 8-
20 hour day for 28 days; lane 4, extracellular polypeptides from rye plants transferred to 5/2 °C for 43 days; lane 5, extracellular polypeptides from rye plants transferred to 5/2° C for 50 days; lane 6, extracellular polypeptides from rye plants transferred to 5/2 °C for 71 days; lane 7, extracellular polypeptides from rye plants transferred to 5/2 °C for 95 days.

25 **Figure 4** depicts SDS-PAGE of extracellular polypeptides isolated from deacclimated winter rye leaves. Lane 1, molecular mass markers; lane 2, extracellular polypeptides from plants grown at 20/16° C for 7 days and then transferred to 5/2° C with an 8-hour day for 42 days; lanes 3, 4 and 5, extracellular polypeptides from plants grown as described in lane 2 and then transferred to 20/16° C with an 16-hour
30 day for 4, 6 and 8 days, respectively.

Figure 5 depicts the ice nucleation activity of various ultrafiltered extracellular extracts from rye leaves grown under different temperature regimes.

Figure 6 illustrates the antifreeze activity in extracellular extracts of cold- acclimated

winter rye leaves. Antifreeze activity was determined by observing ice crystal morphology using a nanoliter osmometer (Clifton Technical Physics, Hartford, N.Y., U.S.A., II-5). Orientation of the ice crystals in **C, D, E** and **F**: the a-axis represents growth in the basal plane and the c-axis represents growth normal to the basal plane.

5 **Figure 7** illustrates fractionation of extracellular extracts from cold-acclimated rye leaves by column chromatography.

Figure 8 illustrates the ice crystal morphology of partially purified and concentrated antifreeze protein from cold-acclimated winter rye leaves. **A:** Orientation of the crystal as described in Fig. 6. **B, C and D:** Growth sequence of an ice crystal as the temperature
10 was lowered. **B:** Incomplete bipyramid; **C:** bipyramid; **D:** needle-like crystal.

Figure 9 is SDS-PAGE of polypeptides associated with column fractions of each 280 nm peak shown in Fig. 8.

Figure 10 depicts extracellular polypeptides from cold-acclimated winter rye leaves separated by SDS-PAGE using Tris-tricine buffers. The polypeptides were eluted from
15 the gel and assayed for activity. The 161, 93 to 99, 33, 31, 27,23, 15 and 11 kD polypeptides all exhibited antifreeze activity.

Figure 11 depicts extracellular extracts from **A**: nonacclimated and **B**: cold-acclimated winter rye leaves. Polypeptides were solubilized and separated by SDS-PAGE and then probed with anti-chitinase antiserum as the primary antibody. Molecular mass standards
20 (in kD) are shown in lane C.

Figure 12 shows an immunoblot of extracellular extract from cold-acclimated winter rye leaves. Polypeptides were separated by SDS-PAGE, blotted onto nitrocellulose and probed with anti-chitinase antiserum as the primary antibody.

Figure 13 depicts recrystallization inhibition in extracellular extracts obtained from
25 cold-acclimated winter rye plants. At the 1:10,000 dilution, the protein concentration was approximately 28 ug/L. Splats were formed at -20° C and were annealed at -8°C for 6 hours. (*NOTE: Splats were annealed at -8° C for 6 hours to observe recrystallization of ice. Photographed by Dr. Charles Knight, National Center for Atmospheric Research, Boulder, CO, USA.*)

30 **Figure 14** depicts the level of freezing injury, measured as ion leakage, in nonacclimated leaves (σ), cold-acclimated leaves (λ) and cold-acclimated leaves which were extracted before freezing to decrease the levels of extracellular proteins (ν). The leaves were placed in water, cooled to the temperature at which ice

formation occurred and then held at that temperature for 22 min. Leaves were removed from the freezing bath and allowed to thaw slowly on ice. Ion leakage was calculated as the conductivity of the solution after the leaves were frozen divided by the total conductivity of the solution after boiling. The data were corrected for ion
5 leakage of unfrozen samples and are presented as means ± SE, n = 3. Lethal injury occurs at 50% or greater ion leakage from the tissue.

II. ANTIFREEZE ACTIVITY IN COLD-ACCLIMATED HERBACEOUS PLANTS

Figure 15 shows antifreeze activity in leaves of nonacclimated (NA) and cold-
10 acclimated (CA) plants. Antifreeze activity was determined for each leaf extracellular extract by observing the morphology of ice crystals grown in solution. No antifreeze activity was observed in all nonacclimated leaf extracts. Extracts of cold-acclimated kale and winter canola leaves, as well as cold-acclimated winter and spring rye, winter and spring wheat, winter barley and spring oats leaves all exhibited antifreeze
15 activity. All crystals were photographed at the same magnification. The magnification bar represents 17 μm.

Figure 16. Extracellular protein accumulation in leaves during cold acclimation of **A:** winter rye, winter barley and winter wheat; **B:** spring rye, spring wheat, spring oats and maize; **C:** spinach, winter canola and kale; **D:** spring canola and tobacco. Plants
20 were grown under nonacclimating conditions (0 time point) and then transferred to conditions for cold acclimation (5/2°C) for 1 to 7 weeks. Protein concentrations are presented as means ± SE, n = 3.

Figure 17. Accumulation of extracellular polypeptides and immunodetection of antifreeze proteins in winter rye cv Voima. **A:** Equal amounts of polypeptides (5 μg
25 protein per lane) were separated by SDS-PAGE from concentrated extracellular extracts obtained from 3 week-old nonacclimated (NA) rye (lane 1), and from rye cold-acclimated (CA) for 1 week (lane 2), 2 weeks (lane 3), 4 weeks (lane 4), 7 weeks (lane 5) and 9 weeks (lane 6) at 5/2±C (day/night). SDS-polyacrylamide gels loaded with equal amounts (1 μg per lane) of concentrated extracellular extracts
30 were blotted and probed with **B:** anti-glucanase-like protein antiserum (dilution 1:10 000) produced against winter rye 32 kD glucanase-like protein; **C:** anti-chitinase-like protein antiserum (dilution 1:1000) produced against winter rye 35 kD anti-chitinase-like protein; and **D:** anti-thaumatin-like protein antiserum (dilution 1:10 000) produced against winter rye 25 kD thaumatin-like protein. Positive immunodetection of each of

the corresponding polypeptides is indicated on the right. Numbers on the left refer to the Bio-Rad low molecular mass markers. CLP, chitinase-like protein; GLP, glucanase-like protein; TLP, thaumatin-like protein.

Figure 18 depicts the accumulation of extracellular polypeptides in nonacclimated (NA) and fully cold-acclimated (CA) cereals. Equal amounts (1 μg per lane) of concentrated extracellular polypeptides were separated in 15% SDS-polyacrylamide gels. **A:** A gel stained with Bio-Rad silver stain. The gels were blotted and probed with **B:** anti-GLP antiserum (dilution 1:10 000), **C:** anti-CLP antiserum (dilution 1:1000), and **D**: anti-TLP antiserum (dilution 1:10 000). Numbers on the left refer to the Bio-Rad low molecular mass markers.

Figure 19 depicts the accumulation of extracellular polypeptides in nonacclimated (NA) and fully cold-acclimated (CA) dicotyledons. Equal amounts (1 μg per lane) of concentrated extracellular polypeptides were separated in 15% SDS-polyacrylamide gels. **A:** A gel stained with Bio-Rad silver stain. After transfer, immunoblots were probed with **B:** anti-GLP antiserum (dilution 1:10 000), **C:** anti-CLP antiserum (dilution 1:1000), and **D:** anti-TLP antiserum (dilution 1:10 000). Numbers on the left indicate the separation of Bio-Rad low molecular mass markers.

Figure 20 shows the total sugars present in extracellular extracts from nonacclimated (white bars) and cold-acclimated (hatched bars) monocotyledonous (**A**) and dicotyledonous (**B**) plants. Sugar concentration was calculated as mg g^{-1} FW using glucose as a standard and are shown as means ± SE, n = 3.

III. ISOLATION OF DNA AND PROTEIN SEQUENCES FOR CHITINASE-LIKE PROTEINS WITH ANTIFREEZE ACTIVITY

Figure 21 depicts DNA and protein sequences for a chitinase-like protein with antifreeze activity. **a:** ch-9 full length DNA and amino acid sequence; **b:** ch-9 DNA and amino acid sequence (lacking the predicted signal peptide coding region); **c:** ch-9 including a presequence of 20 amino acids that is removed in the plant cell (the final purified protein that has chitinase and antifreeze activity is lacking this presequence); **d:** ch-9 amino acid sequence lacking a presumptive signal sequence of 20 amino acids.

Figure 22 depicts DNA and protein sequences for a chitinase-like protein with antifreeze activity. **a:** ch-46 DNA and amino acid sequence (removed untranslated sequences and the putative presequence); **b:** ch-46 DNA and amino acid sequence

(removed untranslated sequences); **c:** ch-46 (putative signal sequence removed; **d:** ch-46 (untranslated sequence removed).

Figure 23 is a multiple sequence alignment of amino acid sequences of chitinases from different plants. **1** is the amino acid sequence of chitinase-*a* from rye seeds (Protein Information Resource Accession No. JC2071), **rye** is chitinase-*c* from rye seeds (Protein Information Resource Accession No. JN0884, **pCHT9** and **pCHT46** are the predicted amino acid sequences for two winter rye chitinase-like proteins with antifreeze activity.

EXPRESSION OF DNA SEQUENCES ENCODING CHITINASE-LIKE PROTEINS

Figure 24 shows the expression of DNA sequences and secretion of chitinase-like protein in *E.coli.* Total cell extract was prepared from uninduced (lanes 1, 3) and IPTG-induced cells (lanes 2, 4), separated by SDS-PAGE and subjected to immunoblotting. A 32 kD protein recognized by the anti-chitinase-like protein antiserum is evident in the induced cells (lanes 2, 4). Also shown is chitinase-like protein purified from cold-acclimated winter rye (M).

Figure 25a shows the expression and secretion of chitinase-like protein in yeast. Yeast cells were grown in YPD medium for 24 hr (lane 1), 48 hr (lane 2). Cells were centrifuged, the supernatant was mixed with sample buffer, and the polypeptides were separated by SDS-PAGE. A 32 kD polypeptide is present in both lanes.

Figure 25b shows an immunoblot analysis of both supernatant and cell pellet with anti-chitinase-like protein antiserum. Proteins from both the supernatant (lanes 3, 4) and the cell pellet (lanes 1, 2) were solubilized, separated by SDS-PAGE and subjected to immunoblotting. The majority of proteins are found in the supernatant (lanes 3, 4). Also shown is chitinase-like protein purified from cold-acclimated winter rye.

Figure 26 shows heat-stable AFPs separated by SDS-PAGE. Extracellular extracts from cold-acclimated winter rye leaves were heated (**lane A**) to 60°C for 30 min or (**lane B**) to 100°C for 10 min. **Lane C** shows BioRad braod range molecular mass markers (207, 121, 81, 51.2, 33.6, 28.6, 21.1, and 7.5 kD). GLP: glucanase-like protein; TLP: thaumatin-like protein.

SUMMARY OF THE INVENTION

It was the general impression that the mechanism responsible for freezing tolerance in plants resided within the cell so as to protect it from forming ice crystals internally. No one had given any thought to the possibility of the existence of AFPs and ice nucleating proteins in plants. Furthermore, no thought had been given to the possibility that such proteins could be located outside of the cell to effect an entirely different mechanism for protecting the plant from freezing. Quite surprisingly, we have found that a group of polypeptides accumulate extracellularly and exhibit ice nucleation and antifreeze activities that control ice crystal growth in intercellular spaces and xylem. In their native forms, these proteins also exhibit enzymatic activities, such as glucan and chitin hydrolysis, which may modify plant cell walls and/or inhibit the growth of low temperature pathogens. These extracellular polypeptides are located in the extractable portion of the plant apoplast, which includes the outer surface of the plasmalemma, the region between the plasmalemma and the cell wall, the cell wall, the middle lamella, the intercellular spaces and the tracheids and vessels of the xylem. It is understood throughout this specification that the term extracellular has the above meaning.

Winter rye is an overwintering herbaceous annual plant that survives temperatures lower than -20°C. When winter rye leaves freeze, ice grows in intercellular spaces within the mesophyll and in xylem vessels (III-40). The survival of frozen plant tissues depends on preventing cellular damage caused by the growth of intercellular ice crystals. AFPs that accumulate in the apoplast of winter rye leaves during cold acclimation have the ability to modify the growth of ice crystals in frozen tissues (III-19, III-22) and are correlated with greater freezing tolerance of the winter rye leaves (III-35). Moreover, as shown by immunolocalization, the AFPs are associated with the epidermis and with mesophyll cells that surround intercellular spaces of cold-acclimated winter rye leaves (III-2). These locations correspond to the known sites of ice formation on the surface of the leaf and in intercellular spaces within the mesophyll (III-40, III-41) and suggest that AFPs may also prevent inoculative freezing of cells (III-41).

Six AFPs ranging in molecular mass from 16 to 35 kD have been isolated from the apoplast of cold-acclimated winter rye leaves (III-22). They are similar to members of three classes of pathogenesis-related (PR) proteins: two AFPs are chitinase-like proteins (CLPs), two are β -1,3-glucanase-like proteins (GLPs) and two are thaumatin-like proteins (TLPs, III-23). One CLP was purified to homogeneity

from cold-acclimated winter rye leaves and shown to have both antifreeze and chitinase activities (III-23). Because these low temperature rye proteins exhibit two activties, they may play a role in nonspecific disease resistance (chitinase and glucanase activity) as well as in freezing tolerance (antifreeze activity, III-48).

5 If the ability to modify the growth of ice in the apoplast is an important component of freezing tolerance, then it is probably a widespread mechanism among plants. Surveys of overwintering plants have shown that there is antifreeze activity in the sap expressed from the tissues of many plants, including 21 dicotyledonous and 9 monocotyledonous plants (III-19, III-17, III-52, III-11, III-12).
10 An AFP has also been isolated from the expressed sap of *Solanum dulcamara*, an overwintering woody vine known as bittersweet nightshade (III-10). The nightshade AFP is a large (67 kD) glycosylated protein with an usually high glycine content (ca 24%) and a unique N-terminal amino acid sequence (III-10).

AFPs isolated from plant sources are important additives for processes that
15 involve modifying ice formation, reducing disease, decay and spoilage caused by microorganisms at low temperature, and ameliorating the results of exposure to low temperatures, including the production and storage of frozen foods and cryopreservation of biological materials. The genes encoding AFPs are important in expression systems for increased production of AFPs to use as additives and in
20 transgenic organisms to increase AFP synthesis to promote survival and reduce injury and disease in cold environments. According to an aspect of the invention, AFPs common to freezing-tolerant plants are provided. The proteins are located extracellularly to control ice crystal growth in the xylem and intercellular spaces within plant tissues. This modification of ice crystal growth contributes to the plants'
25 ability to withstand freezing. The polypeptides with antifreeze activity are selected from a group of polypeptides having respectively apparent molecular masses of about 5 to 9 kD, about 9 to 11 kD, about 11 to 15 kD, about 21 to 23 kD, about 24 to 27 kD, about 30 to 31 kD, about 31 to 33 kD, about 32 to 36 kD, about 60 and 68 kD, about 89 to 100 kD and about 161 kD.

30 According to another aspect of the invention, polypeptides having the above molecular masses and deriving from extracellular spaces of freezing-tolerant plants are provided. Some of the polypeptides are ice nucleators that initiate ice in extracellular spaces of plant tissues. Some of the polypeptides are AFPs that modify ice crystal growth in extracellular spaces. Some of the polypeptides are enzymes
35 that modify plant cell walls and/or inhibit the growth of pathogens in plant intercellular

spaces.

According to various aspects of the invention, the polypeptides have been characterized by their apparent molecular masses based on their migration in SDS-PAGE gels relative to known molecular mass markers. It is appreciated that the polypeptides of this invention may migrate differently in different types of gels, particularly with different concentrations of acrylamide in the gel, with different reducing conditions and with different running buffers. Hence, the molecular mass characterization of the polypeptides of this invention is intended to cover the equivalent polypeptides as they might have slightly different molecular masses on different gels. Moreover, the extracellular polypeptides are found in extracts containing total soluble proteins from the plant tissues. Hence, the invention is intended to cover these polypeptides whether they are extracted only from the extracellular spaces or from total extracts of the tissues.

According to another aspect of the invention, AFPs from herbaceous monocotyledonous and dicotyledonous plants are provided. Antifreeze activity and total protein accumulation were found in leaf extracellular extracts from eight herbaceous plants grown at low temperature, including both monocotyledons (winter and spring rye, winter and spring wheat *Triticum aestivum* L., winter barley *Hordeum vulgare* L., spring oats *Avena sativa* L.) and dicotyledons (winter canola *Brassica napus* L. and kale *Brassica oleracea* L.). The secretion of extracellular proteins capable of modifying ice crystal growth is one aspect of the mechanism for winter survival in both monocots and dicots. Moreover, protein secretion into the apoplast accompanied by an increase in antifreeze activity is a common response in all plants of the Poaceae family exposed to cold temperatures. Therefore, in another embodiment, the invention relates to an isolated polypeptide having antifreeze activity, selected from the group consisting of members of the Poaceae and Cruciferae families. In a preferred embodiment, the member of the Poaceae is selected from the group consisting of barley, wheat and rye, spring rye, winter rye, spring wheat, winter wheat, winter barley and spring oats, whereas the member of the Cruciferae is selcted from kale and winter canola.

According to another aspect of this invention, certain monocots, upon cold induction or acclimation, produce a family of AFPs that are related to pathogenesis-related proteins as shown by N-terminal sequences of cold-induced proteins and immunoblots. It is provided that related plants from the family Poaceae, including rye, wheat, and barley, accumulate AFPs similar to pathogenesis-related proteins

during cold acclimation. Therefore, the invention also relates to cold-induced pathogenesis-related proteins with antifreeze activity isolated from a monocot. The monocot is preferably from the Poaceae family. In a preferred embodiment, the monocot is selected from the group consisting of barley, wheat and rye, spring rye, winter rye, spring wheat, winter wheat, and winter barley. The invention also includes a mimetic of each polypeptide. In a preferred embodiment, the polypeptide(s) of the invention may be selected from the group consisting of chitinase-like protein, thaumatin-like protein and glucanase-like protein. The polypeptide(s) may be used for producing antifreeze protein, increasing freezing tolerance in plants and microorganisms, inhibition of ice recrystallization in biological matter or food product, reducing disease, decay or spoilage caused by microorganisms active at low temperature in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

According to another aspect of the invention, antibodies to one or more of the aforementioned polypeptides may be developed, such antibodies being optionally adapted for detection in an immunoassay for determining the level of freezing tolerance or for determining if similar AFPs are produced by other plants. Three of the extracellular AFPs from winter rye that correspond to a 32-kD glucanase-like protein, a 35-kD chitinase-like protein and a 25-kD thaumatin-like protein were purified and antisera were raised against each of them.

According to another aspect of the invention, frozen food preparations may include one or more of the above polypeptides and, in particular, ice cream and fruit preparations which include one or more of the polypeptides to provide a superior product having minute crystalline structure. In addition, the polypeptides are useful in the cryopreservation of biological tissues, frozen storage of a variety of tissues and frozen germplasm storage.

According to another aspect of the invention, it is provided that the sugars present in the extracellular extracts of cold-acclimated plants also modify the growth of intercellular ice and inhibit ice recrystallization. These sugars enhance the activity of AFPs.

According to another aspect of the invention, two chitinase-like proteins from winter rye are cloned using molecular biology techniques and are expressed in both bacterial and yeast systems. The expressed proteins showed both chitinase and antifreeze activities. The invention includes the DNA and protein sequence of the

chitinase-like proteins, any modifications of the said sequences, the expression of these sequences and their application in agriculture, food industry and medicine. The invention is a nucleic acid molecule isolated from a monocot, encoding an AFP. In a preferred embodiment, the molecule has the DNA sequence in Figure 21.a,
5 Figure 21.b or Figure 22.a or Figure 22.b. The invention also includes a molecule whose sequence is substantially homologous (homology is discussed below with reference to the amount of sequence identity) to all or part of the DNA sequence in Figure 21.a, Figure 21.b, Figure 22.a or Figure 22.b. The molecule may be selected from a group consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-
10 stranded DNA and double stranded DNA. The invention also relates to a polypeptide encoded by the nucleic acid molecule and a mimetic of the purified and isolated polypeptide. In a preferred embodiment, the polypeptide has the amino acid sequence in Figure 21.a, Figure 21.b, Figure 21.c, Figure 21.d, Figure 22.a, Figure 22.b, Figure 22.c or Figure 22.d. The polypeptide, in another embodiment, can be
15 substantially homologous to all or part of the amino acid sequence in Figure 21.a, Figure 21.b, Figure 21.c, Figure 21.d, Figure 22.a, Figure 22.b, Figure 22.c or Figure 22.d.

The invention also relates to the DNA sequence encoding the signal sequence of the polypeptide that directs its secretion from the cell. The invention
20 includes a molecule whose sequence is substantially homologous to all or part of the DNA sequence in Figure 21.a, Figure 21 .c, Figure 22.b or Figure 22.d. The molecule may be selected from a group consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded DNA and double stranded DNA. The invention also relates to a polypeptide encoded by the nucleic acid molecule and a mimetic of the
25 purified and isolated polypeptide. In a preferred embodiment, the polypeptide has the amino acid sequence in Figure 21.a, Figure 21.c, Figure 22.b, or Figure 22.d. The polypeptide, in another embodiment, can be substantially homologous to all or part of the amino acid sequence in Figure 21.a, Figure 21.c, Figure 22.b, or Figure 22.d. The targetting sequence may be used to direct protein secretion in a
30 transgenic organism or expression system. The expression host can be a plant, plant cell, algal cell, bacterium, yeast, fungus, animal and animal cell.

The invention also includes a system for the expression of a gene encoding a chitinase-like protein, including both an expression vector and cDNA for a chitinase-like protein inserted in the expression vector. In a preferred embodiment, the
35 expression vector is *E. coli* plasmid pET12a. In another embodiment, the system

consists of *Pichia* vector pGAPZαA. In a preferred embodiment, the system includes the chitinase-like DNA sequence in Figure 21.a, Figure 21.b, Figure 22.a or Figure 22.b. The invention also includes a plant, plant cell, animal, animal cell, bacterium, fungus or yeast transformed by the system. Another aspect of the invention includes a method for expressing chitinase-like protein consisting of transforming an expression host with a chitinase-like protein DNA expression vector and culturing the expression host. The expression host can be a plant, plant cell, algal cell, bacterium, yeast, fungus, animal and animal cell. The product of the expression system can be used for producing AFP, increasing freezing tolerance and low temperature disease resistance in plants, animals and microorganisms, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

The invention also relates to DNA sequences encoding cold-induced endochitinases from all members of Monocots and/or the Poaceae for the same uses as the winter rye sequences described in this application.

The invention also relates to organisms (plants, animals and microorganisms) transformed by AFP DNA sequences to promote survival during frozen storage, winter survival and/or freezing tolerance in transgenic organisms. Organisms may be transformed to overproduce the protein, and the AFPs also allow storage of the organisms themselves close to zero Celsius or at a subzero temperature while permitting continued work with them. This is advantageous because many bacterial, animal, plant and yeast cell lines are quite freezing or cold sensitive.

The invention is a nucleic acid molecule isolated from a monocot, encoding an antifreeze protein. In one embodiment, nucleic acid molecule comprises the DNA sequence in Figure 21(a), Figure 21(b), Figure 22(a) or Figure 22(b). The nucleic acid molecule may omit the targeting sequence shown in Figures 21 or 22. The molecule preferably has at least 40% sequence identity to all or part of the DNA sequence Figure 21(a), Figure 21(b), Figure 22(a) or Figure 22(b). The molecule is preferably selected from a group consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded DNA and double-stranded DNA. The invention also includes nucleic acid molecules encoding the same amino acid sequence as the nucleic acid molecules in Figures 21 and 22. The invention also includes a nucleic acid molecule that encodes a chitinase-like antifreeze polypeptide that hybridizes to the nucleic acid molecule of the coding strand from positions 340 to 744 of Figure 22 (a) under a wash stringency of 0.2X SSC to 2X SSC, 0.1% SDS, at 42°C. Another

embodiment of the invention also includes a nucleic acid molecule that encodes chitinase-like antifreeze proteins that hybridizes to the nucleotide sequence of the coding strand from positions 541 to 745 of Figure 21 (a) under a wash stringency of 0.2X SSC to 2X SSC, 0.1% SDS, at 42°C.

5 In another embodiment, the invention includes a polypeptide encoded by a nucleic acid molecule of the invention, such as those described in the preceding paragraph. The invention also includes a mimetic of the purified and isolated polypeptide. The polypeptide preferably includes the amino acid sequence in Figure 21(a), Figure 21(b), Figure 21(c), Figure 21(d), Figure 22(a), Figure 22(b), Figure 22(c) or Figure 10 22(d). In another embodiment, the polypeptide includes at least 40% sequence identity to all or part of the amino acid sequence in Figure 21(a), Figure 21(b), Figure 21(c), Figure 21(d), Figure 22(a), Figure 22(b), Figure 22(c) or Figure 22(d).

A variation of the invention includes a cold-induced antifreeze polypeptide isolated from a monocot or a cold-induced pathogenesis-related protein with 15 antifreeze activity isolated from a monocot. The monocot is preferably from the family Poaceae. The monocot is preferably selected from the group consisting of barley, wheat and rye, spring rye, spring wheat, winter wheat, winter barley, and spring oats. The invention also includes mimetics of these polypeptides.

The invention includes an isolated polypeptide having antifreeze activity, 20 selected from the group consisting of barley, wheat and rye, spring rye, spring wheat, winter wheat and winter barley, winter canola, spring oats and kale. The polypeptide is preferably, selected from the group consisting of chitinase, thaumatin and glucanase. The polypeptide is useful for producing antifreeze protein, increasing freezing tolerance in plants and microorganisms, increasing field survival and yields 25 of plants, animals and microorgansims exposed to subzero temperatures, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

The invention also includes a recombinant DNA comprising a DNA molecule 30 of the invention and a promoter region, operatively linked so that the promoter enhances transcription of said DNA molecule in a host cell. The invention also includes a system for the expression of a chitinase gene, comprising an expression vector and chitinase cDNA inserted in the expression vector. The expression vector preferably includes a plasmid such as an *E. coli* vector (for example, pET12a). The 35 expression vector may also a *Pichia* vector (for example pGAPZαA). In the

expression system, the chitinase DNA preferably comprises all or part of the DNA sequence in Figure 21(a), Figure 21(b), Figure 22(a) or Figure 22(b). The invention also includes a plant, plant cell, animal, animal cell, bacterium or yeast transformed by the system.

5 In another embodiment, the invention is a method for expressing chitinase comprising: transforming an expression host with a chitinase DNA expression vector; and culturing the expression host. In the method, the expression host is preferably selected from the group consisting of a plant, plant cell, bacterium, yeast, fungus, protozoa, algae, animal and animal cell. The product of the expression system is
10 preferably useful for producing antifreeze protein, increasing freezing tolerance in plants, animals and microorganisms, increasing field survival and yields of plants, animals and microorgansims exposed to subzero temperatures, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor
15 cells.

The invention also includes a nucleotide sequence that targets protein secretion in plants consisting of the targeting sequence of the coding strand or its complement thereof shown in Figure 21 (a), positions 1 through 60. The nucleotide sequence that targets protein secretion in plants preferably includes of the coding
20 strand or its complement thereof shown in Figure 22 (a), positions 1 through 66.

The invention includes a method of enhancing antifreeze activity, comprising combining antifreeze polypeptide with sugars to enhance the activity of antifreeze polypeptide to inhibit the recrystallization of ice and modify the normal growth of ice. The invention includes a composition comprising one or more antifreeze proteins
25 combined with one or more sugars. The polypeptides of the invention may be combined with a sugar for a use such as producing antifreeze protein, increasing freezing tolerance in plants and microorganisms, increasing field survival and yields of plants, animals and microorganisms exposed to subzero temperatures, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells,
30 hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

The polypeptides of the invention may be used for inhibition of the initiation or progression of a disease or spoilage caused by a low temperature pathogen in a plant, a frozen food or any cryopreserved biological matter. The invention also
35 includes a method for separating antifreeze proteins from plant materials or

recombinant expression systems comprising heating soluble extracts to temperatures of at least about 60°C and then centrifuging or filtering the heated extracts to remove denatured proteins and insoluble materials.The invention relates to an isolated cDNA encoding an antifreeze polypeptide from a monocot. The monocot is preferably from the family Poaceae or the group Triticum (most preferably winter rye). The cDNA preferably also encodes a signal sequence for translocation of the polypeptide. The invention also relates to nucleic acid molecules encoding cold-induced endochitinases from a monocot, the family Poaceae or the group Triticum. The invention also includes polypeptides produced from the nucleic acid molecules. The invention also includes the use of these nucleic the same uses as are described for winter rye and other antifreeze polypeptides in this application.

The invention includes an isolated DNA molecule comprising a nucleotide sequence selected from the group consisting of: a nucleic acid molecule shown in Figure 21 or 22, or the complement thereof; a nucleotide sequence that hybridizes to said nucleotide sequence of (a) under moderate or high stringency conditions, for example the conditions described in this application, and which encodes an antifreeze polypeptide; an antifreeze nucleic acid molecule having at least 40%, at least 60%, at least 80%, at least 90%, at least 95%, 97%, 98% or 99% sequence identity with the nucleotide sequence of (a) and which encodes an antifreeze polypeptide; a nucleotide sequence encoding the same amino acid sequence as the nucleotide sequence of (a); and a nucleotide sequence encoding the same amino acid sequence as the nucleotide sequence of (b).

The invention also relates to an isolated nucleic acid molecule capable of hybridizing with a nucleotide sequence in monocots under conditions of moderate stringency or high stringency, the sequence encoding a polypeptide having antifreeze activity, and wherein the DNA molecule is capable of hybridizing to a nucleotide sequence of Figure 21 or Figure 22 under conditions of moderate or high stringency.

"Moderate" stringency is used in Examples 25, 25.1, and 25.2.6. A "higher" stringency may also be used, for example by increasing the temperature to 55 C in example 25.2.2 and to 65 C with the gene-specific probe for CHT46 described in example 26.1. The stringency level used most often (0.2 XSSC, 0.1% SDS, 30 min at 42 C) and the one we claimed for our hybridizationprobes is defined as moderate stringency in Short Protocols in Molecular Biology, F. Ausubel et al., 1995, eds, John Wiley and Sons, Chapter 4, p 25.

The invention also relates to a ch9 or ch46 nucleic acid molecule cloned from winter rye and an expression vector comprising the nucleic acid molecule and a promoter controlling expression of the molecule. The invention also includes the expression product of the nucleic acid molecule. The invention also relates to an
5 antifreeze nucleic acid molecule or gene cloned from a monocot from the family Poaceae, preferably winter rye.

The invention also relates to a method of delecting the presence of ch46, ch9 or a nucleic acid molecule encoding an antifreeze protein in a sample by a) selecting a probe from the nucleic acid sequences in Figure 21 or Figure 22, b) hybridizing the
10 probe with the biological sample, and c) detecting the presence of a hybridization complex formed by the hybridization of the probe with the ch46, ch9 or antifreeze nucleic acid molecule in the sample, wherein the presence of the complex is indicative of the presence of ch46, ch9 or antifreeze nucleic acid molecule in the sample. The nucleic acid can be DNA or RNA. The probe is preferably at least 75%
15 complementary to a nucleotide sequence of 10 contiguous molecules present in a probe disclosed in this application. Sequences complementary to the probes described in this application are also useful.

The invention also relates to a cold tolerant plant having inserted into its genome a recombinant, double-stranded DNA molecule comprising in sequence:

20
- a) a promoter region which functions in plants to cause the production of an RNA sequence, operatively linked to;
- b) a DNA sequence that causes the production of an RNA sequence which encodes an antifreeze polypeptide having the sequence in Figure 21 or 22, a variant or biologically functional equivalent polypeptide.

25 In alternate embodiments of the invention, the DNA sequence is operatively linked to at least one of a signal sequence and an untranslated sequence. The promoter is preferably heterologous with respect to the DNA sequence and causes sufficient expression of the antifreeze polypeptide in plant cells or tissue to enhance the cold tolerance of a plant transformed with the gene.

30 The nucleic acid molecules of the invention may be used in a method to transform a host cell capable of producing antifreeze polypeptide by transforming a host cell with an expression vector comprising the nucleic acid molecule. The invention also relates to a method for altering the level of antifreeze polypeptide in a cell (overexpressing antifreeze polypeptide) by transforming a host cell with an

expression vector comprising a nucleic acid molecule of the invention and expressing the polypeptide encoded by the nucleic acid molecule. The expression vector preferably includes an antifreeze nucleic acid molecule and a promoter region, operatively linked such that the promoter enhances transcription of said DNA molecule in a host cell. The invention also includes a method of producing a genetically transformed plant which expresses antifreeze polypeptide including the steps of: inserting into the genome of a host plant cell a recombinant, double-stranded DNA molecule comprising: a promoter which functions in plant cells to enhance transcription of an adjacent DNA coding sequence; an antifreeze DNA molecule of the invention operatively linked to the promoter; and regenerating a genetically transformed plant from said host plant cell. The DNA molecule preferably comprises an isolated DNA molecule that is ch9 or ch46 or that hybridizes to a DNA molecule having the ch9 or ch46 nucleotide sequence of Figure 21 or 22, a variant or biologically functional equivalent molecule under conditions of moderate or high stringency. The invention also includes plant produced by the method.

The invention also relates to a method for altering the level of antifreeze polypeptide in a plant comprising:

- transferring DNA to a plant cell from which the plant is regenerated, wherein the DNA comprises an isolated DNA molecule that is ch9 or ch46 or that hybridizes to a DNA molecule having the ch9 or ch46 nucleotide sequence of Figure 21 or 22, a variant or biologically functional equivalent molecule under conditions of moderate or high stringency, wherein said DNA encodes an antifreeze polypeptide;
- and regenerating the plant from the plant cell, such that said plant expresses the DNA.

In a preferred embodiment of the method, the DNA is a nucleic acid molecule is from a monocot from the family Poaceae, preferably winter rye. The molecule may also be from a dicot. The preferably DNA changes the level of endogenous antifreeze polypeptide such that the cold tolerance of the plant is altered relative to the norm for the plant species.

The invention also includes a method of providing a cell or plant with low temperature disease resistance (preferably antifungal disease resistance) by transforming a cell or plant with the nucleic acid molecules of the invention and expressing the gene. The method also includes applying a composition including polypeptides of the invention and an agriculturally acceptable carrier. Chitinase,

glucanase and thaumatins are all antifungal proteins. The polypeptides of the invention exhibit chitinase, glucanase and thaumatin activity. This activity is retained even when the polypeptides acquire antifreeze activity. There are many diseases caused by microorganisms that grow well at low temperature. Freezing retards but
5 does not prevent microbial growth and any microbes that are in the food or biological material can grow rapidly during thawing.

The invention also relates to a composition for providing cold tolerance to plants, comprising an antifreeze polypeptide and an agriculturally acceptable carrier. The invention also includes a composition for addition to food products comprising
10 an antifreeze polypeptide and a carrier suitable for human consumption.

DETAILED DESCRIPTION OF THE INVENTION

Antifreeze proteins have many interesting properties. These include the binding and inhibition of ice crystal growth, the inhibition of ice recrystallization and
15 the protection of liposomes, proteins, cell membranes and cells (for a more detailed description, see III-21, III-17). As result, these proteins will find wide application in the food industry to inhibit ice recrystallization to improve the texture, quality and shelf life and reduce losses due to microbial spoilage of the foods, vegetables, fruits, meats and many other food products that are either eaten while frozen or stored in a
20 frozen state. In addition, these proteins can be used in the cryopreservation of cells, embryos, oocytes, tissues (III-4), and hypothermic protection of cells and tissues (III-44). Some of the more recent applications are the use of AFPs in the cold protection of human platelets (III-49). At high concentrations, AFPs also modify the growth of ice in a way that can be used in other biomedical applications to kill certain cells such
25 as tumor cells. In transgenic plants, the AFPs can be used to improve freezing tolerance and increase resistance to low temperature pathogens in the plants expressing these proteins.

COLD TOLERANCES IN PLANTS

30 The novel proteins which we discovered and which are associated with plant freezing tolerance are of 3 categories:

i) ice nucleation proteins,

ii) antifreeze proteins (AFPs), and

iii) polypeptides for enzymatic modification of plant and fungal cell walls.

In the process of freezing the water in a plant, tissue water migrates through the cell walls into the intercellular spaces where the water is allowed to freeze under controlled conditions, resulting in the formation of intercellular ice crystals. The role 5 of the ice nucleation proteins is to initiate ice crystal formation outside the cells in the intercellular spaces in plant tissues. The AFPs are also located extracellularly to bind to and modify ice crystal growth in the intercellular spaces so as to prevent rupture of the cell membranes. The enzymes present in extracellular spaces function to modify cell wall material, which may increase the flexibility of the cell wall 10 material and increase cell viability upon freezing and thawing of the plant tissue. These enzymes may also degrade the cell wall material of low temperature pathogens and provide some resistance to low temperature pathogens.

It is believed that the proteins associated with freezing tolerance are made endogenously by the plant cells and are secreted through the plasma membrane into 15 the intercellular spaces to effect and modify ice crystal formation during exposure to freezing temperatures. It is understood that the make up of the freezing-tolerant proteins may comprise one or more of the identified polypeptides. More than one of the identified polypeptides may combine to provide a protein structure which provides one or more of the noted frost tolerant properties of ice nucleation, 20 antifreeze or enzymatic action.

We have found that the polypeptides associated with freezing tolerance are produced to a lesser extent by plant cells at warmer temperatures such as 20°C. The production of the polypeptides associated with frost tolerance is dramatically increased as plants are subjected to conditions which resemble early spring or late 25 fall when frost can set in. To our knowledge this is the first finding of frost tolerance-inducing polypeptides being located in plant tissue. In view of our having located the subject polypeptides in intercellular spaces we extracted the polypeptides from those spaces (I-16 and II-12). Generally, the process is two-step and includes:

i) severed or cut leaves are vacuum infiltrated with an extraction buffer 30 preferably containing 20 mM calcium chloride and 10 mM ascorbate, and

ii) the infiltrate is extracted from the plant tissue while the cells remain unbroken.

The recovered extract exhibits ice nucleation activity, glucanase activity and

chitinase activity as well as antifreeze activity. Preferably ice nucleation activity can be measured by the droplet freezing assay. The ice nucleation activity decreases upon addition of sulfhydryl reducing agents such as dithiothreitol and mercaptoethanol in the manner to be discussed with respect to the examples. The
5 antifreeze activity of the extract was determined by observing ice crystal growth on a freezing stage mounted on a light microscope. In the presence of the extract, the ice crystals form bipyrimidal and hexagonal structures that indicate modification of crystalline growth. Such structures are similar to those formed in the presence of other types of antifreeze proteins isolated from other sources such as the sea raven
10 fish (II-4). It has been found that the addition of protease to the extract eliminates the antifreeze activity, thus confirming the presence of AFP(s). The glucanase activity is measured as the enzymatic release of glucose equivalents from soluble laminaran (poly-β-1, 3-glucose). The glucanase is more active in the presence of calcium. Chitinase activity is measured as the enzymatic release of glucosamine
15 from colloidal chitin (exochitinase) and from chitin oligomers (endochitinase) [I-10.1].

It is understood that various separation techniques may be employed which remove the infiltrate from the intercellular spaces without rupturing the plant cells. Such techniques include vertically orienting the leaves in a funnel or cone placed inside a centrifuge tube to avoid bending the leaves. The leaves are then
20 centrifuged to recover the infiltrate without rupturing the cells. Other techniques are available for polypeptide extraction. For example, leaves may also be extracted by perfusion with appropriate extraction solutions. The extracellular polypeptides are water-soluble and are found in the total soluble fraction when plant tissues are homogenized.

25 The frost tolerance-inducing polypeptides are beneficial to any type of plant where ice formation occurs. Any plant tissue can, in a variety of ways, be adapted to provide or include these polypeptides so that they can modify ice crystal growth. Plants containing these proteins are more likely to withstand exposure to lower cold or freezing temperatures for longer time periods, survive harsher climates, and have
30 longer growing seasons because they are less affected by spring and fall frosts.

In addition, the use of the individual proteins provides alternative survival mechanisms for plants exposed to subzero temperatures. For example, some plants avoid freezing damage by completely preventing ice formation. This strategy of supercooling requires the absence of ice nucleators or the presence of substances
35 that can inactivate ice nucleators. AFPs have been shown to inhibit ice nucleation

(II-13). Thus, any plant tissue can, in a variety of ways, be adapted or provided with AFPs to prevent ice nucleation and promote supercooling of the plant liquids

As can be appreciated, the proteins involved in freezing tolerance as provided by this invention have a variety of uses. A significant use is in the detection of characteristics involved in freezing tolerance in plants. Antibodies have been produced for several of the polypeptides. Other plants can be tested for the presence of similar polypeptides by way of an immunoassay to determine their ability to produce cold-induced AFPs, ice nucleators and enzymes, and to assay their capacity for freezing tolerance. It is also understood that plants could be transformed with genetic information that encodes the subject polypeptides to improve or provide frost tolerance in other types of plants. The polypeptides of this invention may also be applied to plant matter. Suitable carriers for the polypeptides may be used which expedite absorption of the polypeptides into plants, fruit, foods or other biological materials. The AFPs will have to be in an aqueous solution to affect ice formation. To enhance absorption, we use a physical method such as vacuum infiltration, perfusion, mixing, blending, and soaking with and without abrasion. The polypeptides can be applied by spraying techniques to avoid frost damage to vegetable crops, fruit crops and the like when ambient temperatures drop suddenly below freezing temperature. Furthermore, the polypeptides would be useful in the cryopreservation of biological tissues. Polypeptides also have a broad application in improving the quality of all frozen foods and, in particular, ice cream and other foods that are eaten while frozen. The use of the polypeptides produces a minute crystalline structure in the ice and decreases recrystallization to produce a higher quality product. In principle, the polypeptides of this invention are useful whenever it is desired to inhibit recrystallization of ice particles. Such inhibition of recrystallization maintains small ice crystal size, preventing damage to cell walls and membranes and maintaining viability of stored cells and tissues. Further aspects of the invention will be understood based on the following specific discussion and exemplification of the invention.

Example 1: Extraction and quantification of extracellular proteins

Winter rye seeds (*Secale cereale* L. cv. Musketeer) were sown in 15 cm plastic pots containing coarse vermiculite and germinated for one week at 20:16 °C (day:night) with a 16 hour daylength. Plants transferred to a at 5:2 °C (day:night) and a light regime of 16:8 h (day:night) are referred to as cold-acclimated rye (RH). Plants grown at 5:2 °C (day:night), but with a light regime of 8:16 h (day:night) are

referred to as cold acclimated rye - short day (RH-SD). The pots that remained in the growth chamber at 20 °C for another three weeks are control or nonacclimated rye plants (RNH). Rye plants that were grown at 5:2 °C (day:night) (8 h day:16 h night) for exactly seven weeks and then were shifted to the growth chamber at 20° C for
5 four days are referred as deacclimated (Deacc). All plants were watered with modified Hoagland nutrient solution as described by Huner and Macdowall (I-6).

Extracellular proteins were extracted from the leaves of RNH, RH, RH-SD and Deacc plants. In each instance, the extracellular extracts were prepared by vacuum infiltration of the leaves with 5 mM EDTA, 10 mM ascorbic acid, 10 mM
10 mercaptoethanol, 1 mM phenylmethyl sulfonylfluoride, 2 mM caproic acid and 2 mM benzamidine. The vacuum infiltration is in accordance with the process described in Mauch and Staehelin (I-16). The treated leaves were packed vertically in a funnel placed in a centrifuge tube to avoid bending the leaves. With the leaves packed in the funnel, the material was centrifuged to remove the extracellular infiltrate that is
15 captured in the centrifuge tube as an extract. The extract is obtained without rupturing the cells of the leaves.

Extracellular proteins were extracted from RNH, RH, RH-SD and Deacc rye leaves, as outlined above in "Protein Extraction". Protein concentrations in the extracts were determined by the Bio-Rad method with BSA as the standard, using at
20 least four independent replicates. The amount of proteins extracted from the extracellular space varies with the growth conditions of the plants. Nonacclimated leaves have an average extracellular protein content of 0.034 mg protein/g fresh weight. Leaves grown at 5° C with a 16-hour or 8-hour daylength have an average extracellular protein content of 0.149 mg/g fresh weight, or 0.307 mg/g fresh weight,
25 respectively. Thus there is a nine-fold increase in extracellular protein contents in rye plants grown at low temperature with a short daylength. These protein levels decreased when the leaves were shifted back to 20°C to acclimate (Figure 1).

Example 2: Electrophoresis of extracellular proteins

For the results presented in Figures 2, 3 and 4, the extracellular proteins
30 were precipitated from extracellular extracts for purposes of electrophoresis by the addition of 1.5 volumes of 1% acetic acid in methanol and incubating overnight at -20°C. The protein pellet was washed with 100% ethanol and 70% ethanol at 5°C and then dried in a desiccator. The protein was resuspended in Laemmli (I-10) sample buffer [60 mM Tris-HC1, pH 6.8; 10% glycerol; 2% sodium dodecyl sulfate
35 (SDS); 5% mercaptoethanol] and separated by electrophoresis, along with Biorad

unstained standards, on 10% acrylamide gels using 90 V for the stacking gel and 110 V for the separating gel (I-10). The gels were stained with Coomassie blue. For the results presented in Figure 9, proteins present in column fractions of the extracellular extracts were solubilized directly in Laemmli sample buffer (I-10) and separated by electrophoresis, along with Biorad prestained standards, on 13.5% acrylamide gels at 200 V. The gels stained with ammoniacal silver.

The protein profile of extracellular extracts shows remarkable changes between the different types of leaves. SDS-PAGE (10% acrylamide gels) revealed the presence of at least 12 polypeptides in the extracellular extracts. Two of these extracellular polypeptides with molecular masses of 77 and 73 kD, which stained red with Coomassie blue, were observed in extracellular fluids from cold acclimated leaves only (Figure 2; lanes 3 and 4). Increases of eight polypeptides with molecular masses of 36, 33, 30, 25, 21, 15, 14 and 13 kD were observed in extracellular fluids from acclimated leaves (Figure 2). Increases in two polypeptides with molecular masses of 23 and 20 kD were observed only in leaves cold-acclimated with a short day.

Example 3: Accumulation of polypeptides during cold acclimation

To further characterize the polypeptides of the intercellular spaces, a time course study was carried out with the aim of correlating the appearance of these polypeptides with the development of the freezing tolerance. Most of the polypeptides of the intercellular spaces were detected at very low levels in nonacclimated leaves (Figure 3, lane 2). The polypeptides accumulated steadily during cold acclimation for 35, 50, 57 and 78 days (Figures 3, lanes 3, 4, 5 and 6). At 78 days, rye leaves cold-acclimated with a short day are maximally freezing-tolerant (LT50 = -30°C), exhibit the highest levels of all extracellular polypeptides and exhibit a new polypeptide at 109 kD. After cold acclimation for 102 days, the 109, 77 and 73 kD polypeptides were no longer present and the leaves are less freezing-tolerant, presumably because the plants are vernalized (Figure 3, lane 7). This finding shows that the appearance of most of these 13 extracellular polypeptides is correlated with changes in freezing tolerance and vernalization. The extracellular protein profile was also monitored during deacclimation by transferring cold-acclimated plants at their hardiest stage to 20°C for different lengths of time. As shown in Figure 4, the levels of most of the 12 extracellular polypeptides (lane 2, maximally cold-acclimated) were greatly reduced following 4 days of deacclimation (lane 3) and continued to decline steadily after deacclimation for 6 and 8 days (lanes

4 and 5). The molecular mass markers are in the left hand column, which are understood to approximate the molecular masses of the polypeptides in lane 1. The column of molecular mass markers between lane 1 and lane 2 is believed to be more accurate.

5 **Example 4: Ice nucleation activity in extracellular extracts**

Leaf extracellular proteins were concentrated ten-fold by ultrafiltration through an Amicon minicon (I-13). Concentrated extracts were used in the droplet freezing technique to determine the spectrum of active ice nuclei within a given temperature range. During freezing (Figure 5), extracellular extracts from cold-acclimated rye
10 leaves grown under a short photoperiod nucleate ice at -9°C, whereas extracts from cold-acclimated rye leaves grown under a long photoperiod nucleate ice at –10°C. The extracts from nonacclimated and deacclimated leaves initiate ice formation at the lowest temperature (-13° C). The difference in ice nucleation activity of the extracellular extracts between nonacclimated and cold-acclimated leaves (Figure 5)
15 may be attributed to the fact that acclimated leaves maintain higher levels of proteins in the extracellular spaces (Figure 1). The effect of protein concentration was examined by using ultrafiltration to obtain nonacclimated and cold-acclimated extracellular extracts that were equal in protein content. When ice nucleation activity was assayed using frozen droplets and calculated for the two extracts, a statistically
20 significant ($p = 0.01$) increase in the cumulative number of ice nuclei per gram fresh weight was found in cold acclimated leaves. The number of ice nuclei per gram fresh weight (mean standard deviation, $n = 4$) at –15° C was 2269 ± 292 in extracellular extracts from nonacclimated leaves and 7048 ± 917 in extracts from cold-acclimated rye leaves grown under a long daylength. The low threshold
25 temperature for nucleation shows that the ice nucleators present in the extracts are not intact ice nucleation sites.

Example 5: Antifreeze activity of extracellular extracts

Extracellular extracts prepared in accordance with the above technique were evaluated with respect to antifreeze activity. The antifreeze activity was assayed by
30 observing the morphology of ice crystals formed in the extracts using a nanoliter osmometer (Clifton Technical Physics, Hartford, N.Y., U.S.A., II-3, II-5). In pure water, an ice crystal normally grows parallel to the basal plane (a-axes) of the crystal lattice with little growth perpendicular to the basal plane (the c-axis), so that the ice crystals appear flat and round (II-5) (Fig. 6A). In contrast, low (nM) concentrations of
35 AFPs preferentially inhibit the a-axis growth of ice so that the hexagonal prism faces

of the crystal are expressed (II-6) (Fig. 6G). At higher concentrations (μM) of AFPs, the crystals grow predominantly along the c-axis to form hexagonal bipyramids (II-6)(Fig. 8C).

In this experiment, extracellular extracts of nonacclimated rye leaves froze like distilled water; i.e., only thin, round ice crystals were observed (Fig. 6B). In contrast, all crude extracts of the extracellular space of cold-acclimated winter rye leaves formed hexagonal ice crystals upon freezing (Figs. 6C to 6G). As the temperature was lowered, the crystals expanded first along the c-axis to form incomplete hexagonal bipyramids (Fig. 6C) and then along the a-axis to form both hexagonal columns (Fig. 6D) and larger hexagonal plates of ice (Figs. 6E to 6G). The formation of hexagonal ice and growth of the ice crystals along the c-axis indicate that antifreeze activity is present in these crude extracts of winter rye (II-3, 5). Furthermore, the fact that these effects on ice crystal morphology were lost when extracellular extracts from cold-acclimated rye leaves were incubated with 5% (w/v) *Streptomyces griseus* protease (Sigma Chemical Co., St. Louis, MO, U.S.A.) at 22° C for one hour shows that the antifreeze activity is associated with a protein.

Example 6: Recrystallization inhibition by extracellular proteins

One role of AFPs in freezing-tolerant plants and organisms is to prevent the recrystallization of ice (II-10). Although ice may initially form as small crystals, these crystals can amalgamate into larger ice crystals over time and cause mechanical damage to the tissue in the absence of AFPs. Recrystallization was assayed by the "splat assay" where a small volume of an extracellular extract was dropped onto a surface at -20°C to form a thin layer of small ice crystals. The splat was then annealed at -8°C for 6 hours. The size of the ice crystals in extracellular extracts of this invention and in distilled water were compared after annealing using a light microscope and polarized light to determine whether the extracts were able to inhibit the recrystallization observed in water (Figure 13). The crystals present in all dilutions of the extracellular extracts were still significantly smaller than the crystals observed in water after annealing. Thus the extracellular extracts exhibited significant recrystallization inhibition at a dilution of 1: 10,000, which represents a concentration of approximately 28 μg of protein per litre.

Example 7: Fractionation of proteins in extracellular extracts

Extracellular extracts were concentrated five-fold, exchanged into 50 mM NH_4HCO_3 by ultrafiltration (Centriprep-10, Amicon Canada Ltd., Oakville, ON,

Canada) and applied to a Sephacryl 200 (Pharmacia LKB Biotechnology, Uppsala, Sweden) column (0.5 x 32 cm) in 50 mM NH_4HCO_3. The eluate was monitored for UV absorbance at 280 nm (O -- O) and 230 nm (ν --ν). Protein standards were eluted separately to estimate protein size. Ferritin, 440 kD, eluted at 9.5 ml;
5 aldolase, 158 kD, eluted at 11.5 ml: bovine serum albumin, 67 kD, eluted at 13.5 ml: and trypsinogen, 24 kD, eluted at 16.5 ml. As shown in Figure 7, four peaks were observed with apparent molecular masses of 305 kD (peak 1), 5 kD (peak 2), 2 kD (peak 3) and < 1 kD (peak 4). Only fractions associated with peak 2 formed hexagonal and bipyramidal ice crystals upon testing fraction antifreeze properties.

10 The polypeptides associated with column fractions of each peak at 280 nm as shown in Figure 7 were evaluated by SDS-PAGE (13.5% acrylamide, silver-stained gel shown in Figure 9). Lane 1 contains prestained molecular mass standards; lane 2 contains crude extracellular extract; lane 3 contains polypeptides eluted at eluted at 8 ml (peak 1); lane 4 contains polypeptides eluted 18 ml (shoulder of peak 2); lane
15 5 contains polypeptides eluted at 22 ml (peak 2); lane 6 contains polypeptides eluted at 26 ml (shoulder of peak 2); lane 7 contains polypeptides eluted at 31 ml (peak 3); and lane 8 contains polypeptides eluted at 35 ml (peak 4). Column fractions obtained from peak 2 (Figure 7) which exhibited antifreeze activity contain several major polypeptides ranging in size from 5 to 36 kD (Fig. 9, lane 4, and Tables I and
20 II).

Example 8: Ice nucleation activity of fractionated extracellular polypeptides

When proteins are eluted off the Sephacryl column, low ice nucleation activity is detected in peak 1 and peak 4, of Figure 7, with higher levels of activity observed in peak 3. SDS-PAGE separated out two polypeptides at molecular weights of about
25 60 and 68 kD. The ice nucleating protein can be one or a combination of these two polypeptides. These two polypeptides are distinct from the 77 and 73 kD polypeptides shown in Figures 2, 3 and 4 because the 60 and 68 kD polypeptides stain blue with Coomassie blue.

Example 9: Thermal hysteresis of fractionated extracellular polypeptides

30 AFPs lower the freezing temperature of a solution noncolligatively by binding to ice crystals and inhibiting crystal growth, but the proteins alter the melting temperature of the solution only by colligative effects (II-5). This thermal hysteresis (the difference between freezing and melting temperatures) is determined by observing the effect of temperature on the growth of a single ice crystal. Melting

occurs when faces of the ice crystal become round; freezing occurs when the ice crystal elongates along its c-axis (II-5).

In order to demonstrate thermal hysteresis, we pooled column fractions exhibiting both absorbance at 280 nm and antifreeze activity (peak 2), which were lyophilized and resolubilized in distilled water, for the determination of thermal hysteresis. At this higher protein concentration, ice crystal growth was inhibited along the a-axis (Figs. 8B to 8D). Furthermore, the ice crystals spiked along the c-axis (Fig. 8D) at an average freezing temperature of -1.10°C for five ice crystals. The average melting temperature was -0.78°C, and so the thermal hysteresis was calculated to be 0.33 ∀ 0.06°C (mean ∀ S.D., n = 5). Thus, winter rye leaves produce AFP(s) that has(have) the ability to modify the normal growth pattern of ice and to depress the freezing temperature of a solution noncolligatively. The thermal hysteresis exhibited by the winter rye AFP(s) is smaller than that observed for other AFPs found in polar fish (approximately 0.6°C, II-7) or in insects (5°C, II-7, II-8). This may be due to the fact that the AFPs from winter rye are not completely purified or to a difference in structure and function.

Example 10: Antifreeze activity of at least 11 of the extracellular polypeptides

Extracellular polypeptides were extracted from winter rye leaves using 20 mM $CaCl_2$ and 10 mM ascorbate and were separated by SDS-polyacrylamide gel electrophoresis using a Tris-tricine buffer system with no reducing agent (no dithiothreitol) and large (16 x 18 x 0. 15 cm) 12 % acrylamide gels. The polypeptides were visualized in the gels after a 10 min incubation in ice-cold 0.25 M KCl. After washing the gel in distilled H_20, the bands were cut and eluted from the gel in 0.1% SDS and 50 mM Tris-HCl. The polypeptides were precipitated from the elution buffer in 80% acetone at -20°C, pelleted and air-dried. The polypeptides were then redissolved in 0.1 M NH_4HCO_3 and assayed individually for antifreeze activity by observing changes in ice crystal morphology. As shown in Figure 10, 8 polypeptides, ranging from 111 to 161 kD in molecular mass, altered the normal pattern of ice crystal growth so that hexagonal ice crystals were formed. The 93 kD polypeptide actually represents a group of polypeptides that exhibit antifreeze activity and are in the size range of 93 to 99 kD in the Tris-tricine gel system. These polypeptides are distinguished by the fact that they stain a reddish-purple color with Coomassie brilliant blue. In the earlier experiment, polypeptides were separated using a Tris-glycine buffer system and either 12.5% gels or gradient gels, and so the sizes of the polypeptides are somewhat different in this system compared with those of the

earlier gels.

Example 11: Amino acid analyses of AFPs

Amino acid analysis of the polypeptides with antifreeze activity shows that they are relatively enriched in glycine, asparagine or aspartate, alanine, glutamine or glutamate and serine (see Table III for all of the polypeptides) but do not contain hexosamines (within the limits of detection by amino acid analysis after 4 h hydrolysis of 15 picomoles of each polypeptide). None of the polypeptides exhibits the high alanine content characteristic of antifreeze glycoproteins and type I AFPs (II-10). Instead, the rye polypeptides exhibit high hydrophilic amino acid contents, as observed in sea raven and ocean pout (II-4), and also contain the high glycine content observed in some insect AFPs (II-17,Table III).

Example 12: Identification of chitinase-like proteins in extracellular extracts by immunoblotting

Extracellular polypeptides were separated by SDS-PAGE and electroblotted onto nitrocellulose. The blots were probed with primary antibodies to chitinase obtained from Dr. Michel Legrand, Laboratoire de Virologie, Institut de Biologie Moleculaire et Cellulaire de la Recherche Scientifique, 15, rue Descartes, 67000 Strasbourg, France (I-10.1). The blots were probed with a secondary antibody (anti-rabbit IgG conjugated with alkaline phosphatase) for visualization. The results show that two extracellular polypeptides, 27 and 32 kD, have an epitope similar to that of chitinase (Figure 11). The 27 kD polypeptide is expressed at higher levels in cold-acclimated leaves than in nonacclimated leaves, whereas the 32 kD polypeptide is induced by low temperature. A second immunoblot is presented as Figure 12. The lanes in this blot represent extracellular polypeptides from winter rye plants grown at 5°C for 2, 5, 6, 7, 8, and 9 weeks, which were probed with the antibody to chitinase. The 27 kD chitinase is not apparent in leaves of 2-week-old plants, but accumulates during the entire 9 week period. The 32 kD chitinase only becomes apparent after the plants have grown at 5 C for 7 weeks. At 9 weeks, both chitinase polypeptides appear as doublets (32 and 31 kD, 27 and 26 kD).

Example 13: Role of extracellular polypeptides in freezing tolerance in rye.

Winter rye leaves that had been cold-acclimated at 5°C with an 8 hour daylength were extracted with 20 mM $CaCl_2$ and 10 mM ascorbic acid to reduce the protein concentration present in the apoplast. Nonacclimated leaves, cold-acclimated leaves and cold-acclimated leaves that had been extracted were cut into

2.5 cm lengths and rinsed well with distilled water. For each of the three treatments, leaf pieces were placed in each of 50 tubes containing 4 mLs HPLC-grade water. The tubes were positioned in a freezing bath, and the temperature was lowered at 1°C intervals every 22 min. At each temperature, the samples that had frozen were removed and placed on ice to thaw slowly. The samples were then brought to room temperature and the conductivity of the samples was measured. The samples were boiled to release all internal ions, cooled to room temperature, and the conductivity of the solution was measured again. The results are shown in Figure 14. Extraction of the extracellular proteins caused lethal freezing injury to occur at -11 C in cold-acclimated leaves. If not extracted, cold-acclimated leaves normally survive temperatures as low as -30°C when ice is nucleated at -1° C. Even when the unextracted cold-acclimated leaves are allowed to freeze spontaneously, they are not killed at temperatures above –13°C. Thus the presence of extracellular proteins does decrease the level of injury caused by freezing.

Example 14: N-Terminal amino acid sequence analysis of AFPs, Identification of AFPs as similar to pathogenesis-related proteins

Partial amino acid sequences for three of the seven major polypeptides shown in lane 4 of Figure 9 were determined. The first 20 amino acids of the N-terminus of the 9 kD polypeptide that exhibits antifreeze activity have been sequenced:

NH_2-ALA-ILE-PHE-CYS-GLY-GLN-VAL-ASN-PRO-ALA-LEU-GLY-PRO-PRO-ILE-TYR-PRO-ALA-PHE-GLY-.

The first 16 amino acids of the 11 kD polypeptide are:

NH_2-ARG-SER-PHE-SER-ILE-THR-ASN-ARG-CYS-TRP-SER-PHE-THR-VAL-PRO-GLY-

The first 11 amino acids exhibit 55% homology with a kinase-related transforming protein (listed in the Protein Information Resource under the file names MUSHCK and TVMSHC).

The first 30 residues of the N-terminal sequence for the 30 kD protein are as follows:

NH_2-ILE-GLY-VAL-CYS-TYR-GLY-VAL-ILE-GLY-ASN-ASN-LEU-PRO-SER-ARG-SER-ASP-VAL-VAL-GLN-LEU-TYR-ARG-SER-GLY-X-ILE-ASN-X-MET- wherein X indicates an unknown amino acid residue.

This sequence was checked for homology with protein sequences listed in

the National Cancer Institute's Supercomputer databanks. This sequence has 63% homology with the glucan endo-1,3-beta-glucosidase (EC 3.2.1.39) previously purified from barley. The 30 kD band sometimes appears as a 31 to 33 kD band thought to be an endoglucanase precursor. These results show that one of the
5 processes induced by cold acclimation is the ability to modify cell walls. Increased cell wall flexibility may be important when cells shrink and then swell during a freeze-thaw cycle. The glucanase activity may also inhibit the growth of fungal hyphae and provide resistance to low-temperature diseases.

Additional amino acid sequences were obtained for six extracellular
10 polypeptides that exhibit antifreeze activity. The polypeptides were separated by SDS-PAGE using Tris-tricine buffers, eluted from the gels, assayed for antifreeze activity, as shown in Figure 10, and then used for sequence analysis. The N-terminal sequences are as follows:

11 kD polypeptide:

15 NH_2 - ALA - ILE - SER - X - GLY - GLU - GLN - VAL - ASN -

SER - ALA - LEU - [GLY]- PRO - X - ILE - [SER] - TYR -

ALA - [ARG] - [GLY].

A FASTA search of the Protein Information Resource revealed that this sequence has 80% identity in the 20 amino acid overlap area with a lipid transfer
20 protein from barley with a molecular mass of 9 kD (II-14.2). This 11 kD polypeptide from Figure 10 corresponds to the 9 kD polypeptide in Figure 9 that was sequenced above. As noted above, this divergence in molecular mass is due to the variability in the gel electrophoresis.

15 kD polypeptide:

25 NH_2 - ARG - SER - PHE - SER - ILE - THR - ASN - ARG - X -

ALA - PHE - THR - VAL - X - PRO - ALA - ALA - THR - PRO -

VAL - GLY - GLY - GLY - GLY - GLN

A FASTA search of the Protein Information Resource of the National Biomedical Research Foundation revealed that this sequence has 75% identity in a
30 24 amino acid overlap with the reported sequence for a thaumatin-like protein from *Oryza sativa*. This 15 kD polypeptide from Figure 10 may correspond to the 11 kD polypeptide from Figure 9 sequenced above.

25 kD polypeptide:

NH_2 - ALA - THR - ILE - THR - VAL - VAL - ASN - (LYS] -

PHE - SER - TYR - THR - VAL -X - PRO - GLY - ALA - LEU -

PRO - PHE - GLY - GLY - VAL - GLY - LEU - GLY - PRO - GLY

5 - GLN -

A FASTA search revealed that this sequence has 79% identity in a 29 amino acid overlap with thaumatin homolog protein 1 from barley. It also has 77% homology in a 22 amino acid overlap with avematin isolated from oat and 82% identity in a 22 amino acid overlap with trimatin isolated from wheat. Thaumatin-like 10 proteins have been shown to exhibit a number of activities, including alpha-amylase, protease and membrane permeabilizing activities (II - 8.2).

31 kD polypeptide in Figure 10 which corresponds to the 30 kD polypeptide in Figure 9 for which a longer amino acid sequence is described above:

NH_2 - ILE - GLY - VAL - X - TYR - GLY - VAL - ILE

15 32 kD polypeptide:

NH_2 - ILE - GLY - VAL - X - TYR - GLY - VAL - ILE - GLY -

ASN - ASN - LEU - PRO - [SER] - ARG - [SER] - ASP - VAL -

VAL - GLU

33 kD polypeptide:

20 NH_2 - ILE - GLY - VAL - X - TYR - GLY - VAL - ILE - GLY -

ASN - ASN - LEU - PRO - SER

All of the three sequences listed above exhibit significant homology to glucan endo-1,3- -glucosidase (EC 3.2.1.39).

33 kD polypeptide:

25 NH_2 - GLU - GLN - X - GLY - SER - GLN - ALA - GLY - GLY -

ALA - THR - X - PRO ASN - ASN - LEU - LEU -

A FASTA search revealed that this sequence has 81 % identity in a 16 amino acid overlap with hevein from the para rubber tree. The sequence also has 88% identity in a 16 amino acid overlap with endochitinase (EC 3.2.1.14) isolated from rice and 30 tobacco, as well as 92% identity in a 14 amino acid overlap with agglutinin from

wheat also known as isolectin 11.

Proteins such as thaumatin-like proteins (TLPs), glucanases and chitinases are known as pathogenesis-related proteins because they are accumulated by plants that have been infested with a pathogen. We have now shown that thaumatin-like
5 proteins (TLPs), glucanase-like proteins (GLPs) and chitinase-like proteins (CLPs) accumulate during cold-acclimation of freezing-tolerant plants and exhibit antifreeze activity. Other than our own work, there are no reports in the scientific literature showing that pathogenesis-related proteins can be induced by low temperature, no reports indicating that cold-induced pathogenesis-related proteins exhibit antifreeze
10 activity, and no reports indicating that cold-induced proteins or products of cold-induced genes exhibit antifreeze activity.

Example 15: Glucanase and chitinase activities in extracellular extracts

Extracellular extracts of winter rye leaves were obtained using an extraction solution of 20 mM calcium chloride and 10 mM ascorbic acid, pH 3.5. Beta-1, 3-
15 glucanase activity was measured in extracellular extracts using the dinitrosalicylic reagent to assay the release of glucose equivalents from laminarin (II-1.0). In our experiments, the glucanase assay was optimal under the following conditions: pH 3.5, 1% laminarin, presence of $CaCl_2$ (as opposed to $MgCl_2$ or $MnCl_2$), 5°C, and measuring absorbance of 470 nm. In crude extracellular extracts, the beta-1,3-
20 glucanase activity was approximately 312 mg glucose equivalents per mg total protein per hour. The extract did not exhibit beta-1,4-glucanase activity when carboxymethylcellulose was used as a substrate.

In crude extracellular extracts, the endochitinase activity (I-10.1) was 115 nmol glucosamine released per gram fresh weight per hour, and the exochitinase activity
25 was 9 nmol glucosamine released per gram fresh weight per hour in winter rye plants grown at 5°C with a short day. Rye plants grown at 20°C exhibited an endochitinase activity of 34 nmol glucosamine released per gram fresh weight per hour and an exochitinase activity of less than 3 nmol glucosamine released per gram fresh weight per hour.

30 **Example 16: Characterization of ice nucleation activity in cell suspensions**

Suspensions of single mesophyll cells were obtained from 20°C and 5°C winter rye leaves by pectolytic degradation of the leaf tissue and purification using density gradients. In order to quantify the number of ice nucleators present in winter rye, dilution series of the single cell suspensions were assayed for ice nucleation activity

using the droplet technique. The mean threshold ice nucleation temperature for mesophyll cells isolated from 20°C and from 5°C leaves was not significantly different and averaged -7.3°C (Tables IV and V).

The composition of the ice nucleators from leaves of plants grown under different conditions was determined by incubating single cells in the presence of compounds and enzymes known to affect proteins, sulfhydryl groups and disulfide bonds associated with proteins, carbohydrates and phospholipids (Table IV). Treating cells with 3 M urea and heating to 90°C denatures proteins and dramatically decreased ice nucleation activity. Nonspecific proteases (Pronase E and Proteinase K) also decreased ice nucleation activity. Thus the ice nucleators associated with winter rye mesophyll cells have a proteinaceous component. Reduction of disulfide bonds with dithiothreitol and reaction of free sulfhydryl groups with N-ethylmaleimide also decreased ice nucleation activity, which shows that the structure of the protein is important in producing ice nucleation activity. Boric acid and periodic acid both react with carbohydrates and both compounds reduced ice nucleation activity, thus demonstrating that the ice nucleators also contain a carbohydrate component. Finally, treatment with phospholipase C, which releases the phosphate and head group of phospholipids, also decreased ice nucleation activity. Taken, together, these results show that ice nucleators associated with winter rye mesophyll cells have protein, carbohydrate and phospholipid components. While the ice nucleation activity of the isolated polypeptides occurs in the intercellular spaces of the plant tissue, it appears from the cell suspension experiments that the ice nucleation proteins may be bound to the cell wall and released by reagents that reduce disulfide bonds.

Example 17: Antibodies to the polypeptides with antifreeze activity

Polyclonal and monoclonal antibodies were prepared for polypeptides present in extracellular extracts from cold-hardened winter rye leaves. Individual polypeptides separated by SDS-PAGE and electroeluted from gels (using Bio-Rad Prep Cell) were used as antigens. The antibodies are purified and used for immunopurification of the polypeptides in order to determine the ice nucleation, antifreeze and glucanase or chitinase activities of each. Furthermore, the antibodies are used for immunoassays of the polypeptides. The procedures for antibody production and purification, immunopurification of antigens, and immunoassays are described in detail by Harlow and Lane (II-8.1). The detection and quantification of these polypeptides can be used in selection programs designed to decrease plant

injury and yield losses caused by freezing temperatures. Previous selection programs have relied on winter survival to increase frost tolerance in overwintering crops and have been unsuccessful. Because of their sensitivity, the immunoassays provide a nondestructive means for selecting plants that have high concentrations of polypeptides associated with either freezing avoidance (presence of AFP and absence of ice nucleators) or freezing tolerance (presence of ice nucleation, antifreeze and glucanase proteins). Antibodies are also used to identify and isolate antifreeze proteins from other plants. Furthermore, these antibodies may be bound to the AFP(s) to enhance their antifreeze activity.

Example 18: Antifreeze activity in crude extracts and different plant organs

Cold-acclimated winter rye plants were separated into leaves, crowns and roots. These parts of the plants were placed in plastic bags, frozen in liquid nitrogen and allowed to thaw at room temperature. The plant tissues were then squeezed, filtered and centrifuged treated to obtain soluble fractions. These fractions were assayed for their ability to modify ice crystal growth. The soluble fractions of leaves, crowns and roots all exhibited the formation of hexagonal bipyrimids in the assay for antifreeze activity. These results demonstrate that antifreeze activity can be found in crude plant extracts and that the activity is present in all parts of the vegetative plant.

Example 19: Different species and cultivars having antifreeze activity

Sixteen different species or cultivars including both monocots and dicots (see Table IV) were grown at 5/2°C (day/night temperature) with a 16 hour daylength. Leaves from all sixteen plants were extracted by vacuum infiltration followed by centrifugation using a solution of 20 mM $MgCl_2$ and 10 mM ascorbic acid, pH 3.5. All sixteen intercellular extracts exhibited the ability to modify the normal pattern of ice crystal growth, although this ability varied between cultivars and species. Of the plants tested, winter rye (*Secale cereale* cv. Musketeer), periwinkle *(Vinca minor),* winter wheat *(Triticum aestivum* cv. Karat and Ruby) and winter barley *(Hordeum* vulgare cv. Acton) extracts exhibited the greatest effect on ice crystal growth with the formation of hexagonal bipyrimids, whereas winter canola *(Brassica napus* cv. Ceres) extract exhibited the least effect with only the formation of hexagonal discs.

As demonstrated by the further characterization of the polypeptides, at least 11 polypeptides are synthesized at low non-freezing temperatures, namely those of lane 4 of Figure 9 and Figure 10 ranging in molecular weight from 5 kD to 36 kD and the

additional polypeptides of 60, 68 kD, 93 to 99 kD and 16 kD. We have determined that six of the polypeptides, two glucanases, two chitinases and two thaumatin-like proteins, have an unexpected antifreeze property. Also these polypeptides along with the other polypeptides of this invention exhibit recrystallization inhibition activity at very low concentrations.

It is important to note the results of Figure 3 where a time course examines changes in intercellular proteins of rye leaves during cold acclimation. A correlation exists between the degree of frost hardiness and the increased appearance of the extracellular polypeptides. The intensity of the extracellular polypeptides reaches a maximum at 78 days, which corresponds to the hardiest stage of rye plants cold acclimated with a daylength of 8 hours. Most of the extracellular polypeptides decrease in intensity while others were no longer detected at 102 days after germination. This result shows the loss of freezing tolerance when plants are vernalized. Ice nucleation activity is also indicated in Table V at levels as high as –7°C. This is believed to be the first report of ice nuclei of proteinaous nature in higher plants. It is also likely that several ice nucleating molecules are required in the assembly of a template upon which an ice crystal can grow. It is understood that the ice nucleating proteins are important in the extracellular spaces for the development of freezing tolerance in cold-acclimated leaves.

The polypeptides as isolated and characterized in accordance with this invention establish that plants withstand frost by the combined efforts of ice nucleation and ice crystal modification by virtue of antifreeze mechanism. These same extracellular proteins have antifungal activities and provide nonspecific resistance against low temperature pathogens such as fungi and bacteria. It has been demonstrated that cold-acclimated winter rye leaves are not injured by ice formation even when the leaves are first undercooled to temperatures as low as -12°C, whereas nonacclimated winter rye leaves exhibit injury whenever ice forms. In accordance with this invention, ice formation in the extracellular spaces indicates that it is not the presence of AFPs alone that determines the lowest limit of cell survival at freezing temperatures. As temperatures decrease intracellular water is lost to the growing extracellular ice masses and the cells themselves become dehydrated. The lowest temperature which frost tolerant plants survive is correlated with desiccation tolerance of the cells (II-16, II-18).

Conventional breeding programs have failed to improve freezing resistance in crop plants because specific physiological markers are not yet available (II-2). The

discovery of ice nucleation and AFPs intrinsically produced by a freezing-tolerant plant as demonstrated by this invention represents an important breakthrough in agriculture for two reasons. First of all, the ice nucleation and AFPs are the first polypeptides demonstrated to be directly involved in influencing the process of ice
5 formation in plants. Antifreeze and ice nucleation polypeptides may prove useful as selection markers for increasing survival in overwintering crops. Secondly, further isolation and characterization of the ice nucleation and AFPs will be useful for producing transgenic plants with increased survival and productivity. In the future, it will be possible to raise crops successfully in regions or in seasons where crop
10 production is now limited by freezing temperatures.

As already indicated the polypeptides are useful in production of frozen foods and cryogenic storage of biological tissues. Treatment of frozen foods with the polypeptides can ensure superior food quality upon thawing of the product. Also, with the manufacture of products such as ice cream as well as in the cryopreservation of
15 biological tissues it is desirable to have a minute crystalline structure. The use of the AFPs in limiting crystalline size and in preventing recrystallization are very useful in providing a superior product and reducing storage costs. The amount of polypeptides used in these biological matter and food compositions is minimal, as demonstrated in the examples. For example, an effective amount of the polypeptide
20 may be as little as 25 μg of the polypeptide per litre of water contained in the biological matter or food product.

ANTIFREEZE ACTIVITY IN COLD-ACCLIMATED HERBACEOUS PLANTS

Example 20: Antifreeze activity in various plants

25 Winter rye (*Secale cereale* L. cv. Voima), spring rye (*S. cereale* L. cv. Jo 02), winter wheat (*Triticum aestivum* L. cv. Ruby), spring wheat (*T. aestivum* L. cv. Katepwa), and winter barley (*Hordeum vulgare* L. cv. Huron) were planted in coarse vermiculite, grown at 20/16°C (day/night) with a 16-hour daylength, and watered weekly with modified Hoagland solution (III-34). After 7 days, plants were harvested to provide
30 nonacclimated tissue or transferred to low temperature (5/2°C) with an 8-hour daylength for 7 weeks of cold acclimation. Spring oats (*Avena sativa* L. cv. Ogle), winter canola (*Brassica napus* cv. Ceres), kale (*B. oleracea* var. *acephala* cv. Dwarf Blue Curled) and tobacco (*Nicotiana tabacum* L.) were planted in Pro-Mix and grown at 23°C with a daylength of 16 hours until they were large enough to provide

sufficient leaf material for the experiments. Plants were then either harvested to provide nonacclimated tissue or transferred for cold acclimation as described above for 7 weeks.

Extracellular extracts from leaves of winter barley, winter and spring wheat, winter and spring rye, spring oats, spinach, winter and spring canola, and kale were assayed for antifreeze activity (Fig. 15). Antifreeze activity was not detected in any of the nonacclimated plants as only flat, circular ice crystals formed in the extracellular extracts (Fig. 15). After cold acclimation, the leaves of all spring and winter cereals tolerated freezing temperatures in the range of -10 to -27°C, and all exhibited antifreeze activity. Antifreeze activity was detected in cold-acclimated winter rye, winter wheat, winter barley, spring rye, spring wheat and spring oats, winter canola and kale. Thus, antifreeze activity is present in extracellular extracts of the leaves of all freezing-tolerant monocotyledons examined in this experiment, which included all the members of the Poaceae family (winter rye, winter wheat, winter barley, spring rye, spring wheat) that were examined (Fig. 15, Tab. 1).

Low levels of antifreeze activity were also detected in extracellular extracts of cold-acclimated dicotyledonous winter canola and kale (Fig. 15). Duman and Olsen (III-11) also observed a high level of thermal hysteresis in the total expressed sap of kale leaves, whereas we examined only extracellular extracts. It is possible that some of the antifreeze activity in kale could be intracellular. The first intracellular AFP was identified in the skin of winter flounder (III-15), where it is thought to prevent inoculation of epidermal cells by external ice. Another explanation for the low antifreeze activity we observed in kale may be related to the developmental stage or to environmental conditions. Urrutia et al. (III-52) observed thermal hysteresis activity in other members of the Cruciferae, including *Brassica oleracea* subspecies such as cabbage and Brussel's sprout, only after 4 months of cold acclimation at 5°C. Moreover, it has been shown that *Brassica napus* achieves greater freezing tolerance only after exposure to mild freezing events (III-27), and so it is possible that exposure to light frosts may be required for the production of AFPs in overwintering dicotyledons. No antifreeze activity was detected in tobacco, even after cold acclimation.

Table. 1. Freezing tolerance (LT50) of 12 herbaceous plants following cold acclimation at 5/2°C with an 8-h daylength. LT50 values (°C) were determined by conductivity and are the means ± SE of leaves from plants obtained from at least

three separate cold acclimation treatments.

Monocotyledons	LT50 (°C)	Dicotyledons	LT50 (°C)
Winter rye	-27.0 ∀1.0	Spinach	-17.3 ∀1.3
Winter wheat	-19.5 ∀1.7	Winter canola	-16.0 ∀1.2
Spring wheat	-15.0 ∀0.6	Kale	-14.7 ∀0.7
Winter barley	-14.7 ∀1.3	Spring canola	-14.0 ∀0.0
Spring rye	-14.0 ∀1.6	Tobacco	>1
Spring oats	-10.3 ∀0.5		
Maize	>-2		

∀ = ±

Example 21: Accumulation of extracellular proteins among various plants

We quantified the accumulation of extracellular proteins in various plants at low temperature. Cereal leaves were cut into 3-cm sections for extracellular protein extraction, which was performed according to Hon et al. (III-22). Broad leaves were cut into pieces of equal size (3 cm x 1 cm) and treated in a similar manner as those of cereals. Extracellular proteins were extracted in 20 mM ascorbic acid and 20 mM calcium chloride (pH 3) for 30 min by vacuum infiltration followed by centrifugation. Protein concentrations were determined in three independent replicates using the Bradford (1976) protein assay, as modified by Bio-Rad Laboratories Ltd., Mississauga, ON, Canada, with BSA as standard. The results of these experiments showed that the accumulation of protein in the apoplast at low temperatures occurs commonly in monocots (Fig.16). Small amounts of extracellular protein also accumulated in freezing-tolerant dicotyledonous plants (Fig. 16).

Example 22: Immunodetection of AFPs in various cold-acclimated plants

The extracellular proteins that accumulated during cold acclimation were concentrated and examined by SDS-PAGE. For immunodetection of AFPs, antisera that were previously raised against three classes of Musketeer rye AFPs (III-2) were used to probe blots of extracellular polypeptides. These three antisera have been shown to react specifically with the 35 and 32 kD GLPs, the 35 and 28 kD CLPs, and

the 25 and 16 kD TLPs, respectively. These polypeptides all exhibit antifreeze activity in extracellular extracts from cold-acclimated Musketeer rye leaves (III-2).

Equal amounts of extracted extracellular proteins were separated in 15% SDS polyacrylamide gels according to Laemmli (1970). Dilute extracts were
5 concentrated by ultrafiltration and the polypeptides were electrophoresed and stained either with Coomassie brilliant blue or silver stain (Bio-Rad). For immunoblotting, proteins were transferred onto 0.45- m nitrocellulose membranes (Bio-Rad) using the Mini Trans-Blot cell (Bio-Rad) according to manufacturer's instructions. The blots were blocked in a buffer of 25 mM Tris-HCl (pH 7.6), 140 mM
10 NaCl, 0.01% (v/v) Tween-20 and 5% (w/v) skim milk powder. Antisera against the winter rye GLP and TLP were used in a 1:10 000 dilution, and antiserum against the winter rye CLP was diluted 1:1000 for overnight incubation as described by Antikainen et al. (1996). The immunoreaction was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St Louis, MO,
15 USA) with 5-bromo-4-chloro-3-indolylphosphate-toluidine salt (BCIP; Sigma) and nitro blue tetrazolium (NBT; Sigma) as substrates. Extracellular proteins from winter rye cv. Musketeer were separated and transferred as positive controls on all immunoblots. The blots were allowed to develop as long as the positive controls remained visible against the background.

20 All three classes of AFPs (GLPs, CLPs, and TLPs) were detected immunologically in extracellular extracts from Musketeer winter rye, Voima winter rye, spring rye, winter wheat, spring wheat and winter barley, but not from oats (Figs. 17, 18). These AFPs are found in all members of the Triticum group of the Poaceae that have been examined (III-6). In dicotyledons, the anti-TLP antiserum was the
25 only one that detected an extracellular polypeptide (molecular mass of 25 kD) in cold-acclimated spinach (Fig. 19D). There is a possibility that antisera raised against winter rye polypeptides would not cross-react with dicotyledon polypeptides.

The distribution of the cold-specific wheat protein WCS120 is similar but not identical to the distribution of cold-induced AFPs (III-24). WCS120 is found in winter
30 wheat, winter rye and winter barley, but it was not detected in oats or in freezing-sensitive maize and rice or in freezing-tolerant dicotyledons such as winter canola (III-24).

Example 23: Role of extracellular sugars in modifying the growth of intercellular ice

35 Plants may also use secreted sugars for modifying the growth of intercellular

ice. We assayed sugar concentrations in extracellular extracts of all nonacclimated and fully cold-acclimated plants (Fig. 20) using the anthrone assay. The extracellular extracts were subjected to hydrolysis in concentrated sulfuric acid, so that the assay probably measured both monosaccharides present in the extracts and monosaccharides released by hydrolysis from polysaccharides and glycosylated proteins. By using a t-test at the 5% level of significance, we found that the extracellular sugars increased significantly after cold acclimation in winter rye, spring oats, winter barley and winter wheat (Fig. 20A). Smaller changes in extracellular sugars were observed in spring wheat or spring rye. In maize, the cold-induced increase in the extracellular sugar level was not statistically significant. Our results demonstrate that sugars generally accumulate in the apoplast of the same plants that also accumulate AFPs (Figs 16, 17, 18 and 20). These results show that extracellular sugar accumulation does not substitute for AFPs in modifying ice formation. Instead, it is more likely that these sugars enhance antifreeze activity in cold-acclimated plant tissues. The accumulation of extracellular sugars during cold acclimation in winter rye has been observed previously (III-37, III-38, III-39), but the sugars in the earlier studies were larger polysaccharides identified as arabinoxylans (III-29).

It is useful to add sugar to antifreeze protein in order to decrease the amount of antifreeze protein needed to be effective or to enhance or complement the activity of antifreeze proteins. A composition including one or more types of sugar and one or more types of antifreeze proteins is useful for the same purposes as one or more antifreeze proteins alone. Suitable sugars include fructose, glucose, sucrose or fructans (fructose polymers). For example, fructose and other sugars may be added to ice cream before, at the same, or after one or more types of antifreeze proteins are added to the ice cream in order to increase antifreeze protein activity.

Example 24: Purification of winter rye antifreeze proteins by heating

The heat stability of winter rye AFPs was determined to simplify purification procedures. Winter rye and winter wheat leaves were grown at cold temperatures as described in Example 1. Whole leaf extracts were obtained by homogenizing leaf and/or crown tissues in an aqueous buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$, or in a buffer containing 20 mM ammonium bicarbonate, and extracellular extracts were obtained as described in Example 1. Whole leaf and extracellular extracts were heated to either 60°C for 30 min or to 100°C for 10 min and the denatured proteins and particulate matter were removed by centrifugation.

Antifreeze activity, measured as ice crystal modification, was present in the supernatant of whole leaf and extracellular extracts ranging in pH from 3 to 8 of winter wheat and winter rye both before and after heating. The heated extracts were examined by SDS-PAGE, which revealed that the glucanase-like and thaumatin-like
5 proteins were still soluble after heating (Fig. 26). This technique is useful with recombinant proteins as well to simplify purification. This technique is also useful with the other polypeptides or fragments of polypeptides described in this application.

The invention includes a method for separating antifreeze proteins from plant materials or recombinant expression systems comprising heating soluble extracts to
10 temperatures of at least about 60°C and then centrifuging or filtering the heated extracts to remove denatured proteins and insoluble materials.

.ISOLATION OF DNA AND PROTEIN SEQUENCES FOR CHITINASE-LIKE AFPS (CH-9 AND CH-46)

EXAMPLE 25: Isolation of cDNAs for Chitinase-like AFPs

15 The goal of this project was to isolate chitinase cDNAs and determine which ones encoded the chitinase-like AFPs. Those skilled in the art believed that cloning chitinases with antifreeze activity would be difficult because plant genomes contain families of genes encoding chitinases. It was also thought that only a minor modification of a protein is required in some cases to confer antifreeze activity, so
20 that it would be difficult to distinguish a chitinase without antifreeze activity from a chitinase with antifreeze activity. For example, KV Ewart et al (1998, Biochemistry 37:4080-4085) showed that changing Glu-Pro-Asn to Glu-Pro-Asp in a C-type lectin from fish confers antifreeze activity. We were able to clone the chitinases with antifreeze activity by isolating mRNA from rye plants grown under conditions when
25 only the chitinases with antifreeze activity would be expressed, i.e. at low temperatures in the absence of pathogens or other stresses. The cold-induced chitinase cDNAs were isolated and then sorted to determine which cDNAs encoded proteins with ice-binding ability. Those skilled in the art will recognize that there are additional methods that can be used to clone the genes encoding rye AFPs.

30 **25 Southern analysis of genomic DNA of winter rye**

Southern blot analysis of genomic DNA was performed in order to estimate the total number of chitinase genes present in the rye genome and thus provide an idea of the number of possible clones that would be isolated. Genomic DNA was extracted as described with modifications (III-42). Leaves and crown tissue were

collected from 8 day-old rye plants, frozen in liquid nitrogen and ground to a powder in a mortar and pestle. The tissue was added to 5 mL of 60°C extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2%(w/v) CTAB, 0.3% (v/v) 2-mercaptoethanol added before use) and 50 mg PVP were added. The solution was incubated at 60°C with shaking for 1 hr, cooled to room temperature, and extracted with 6 mL chloroform:octanol (24:1). After centrifugation at 3000 rpm for 20 min, the top layer was extracted again with 6 mL chloroform:octanol. The DNA was precipitated by adding 0.5 vol 5 M NaCl and 2 volumes of 95% EtOH and incubating for 30 min at 4°C and 10 min at -20°C. After centrifugation, the pellet was washed twice with 70% EtOH, dried and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). DNA was treated with RNase A and proteinase K, then extracted with twice with phenol:chloroform:IAA (25:24:1), and precipitated with 0.1 volume 3 M NaOAc, pH 5.2 and 2 volumes of 100% EtOH at -20°C. After centrifugation, the pellet was washed twice with 70% EtOH, dried and resuspended in 200 μL TE.

Total genomic DNA from 8-day-old nonacclimated winter rye leaves and crowns was digested with Bam HI, Eco RI, Hind III, Xba I or Xho I restriction enzymes. The fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane. A basic barley chitinase cDNA, pHvcht2a, which is known to be pathogen-inducible, was used to identify chitinase genes. The membrane was hybridized to pHvcht2a at two different conditions of stringency : 30 or 50% formamide with 6 X SSC at 42°C, and washed to 0.2 X SSC and 0.1% SDS at 42°C, after which it was exposed to autoradiograph film (Fig. 3B). At least three genes were present under high stringency (50% formamide) as three DNA fragments in each of the Eco RI, Hind III and Xba I lanes hybridized with the pHvcht2a probe. There were two bands in the Bam HI lane under high stringency. Under lower stringency hybridization, two more genes were apparent, as indicated in the Eco RI lanes with five bands present. Five DNA fragments hybridized to the probe, with three being very faint. Similarly, there appeared to be at least two extra bands in the Xba I and Hind III lanes.

25.1 Screening of cDNA library from cold-acclimated winter rye leaves

A basic barley chitinase cDNA, pHvcht2a, which is known to be pathogen-inducible, was used to screen the cold-acclimated rye leaf cDNA library. The sequence for pHvcht2a is a 1028 bp insert in the Eco RI site of pBluescript SK+. Eco RI was used to isolate the fragment for use as a probe. The size of the fragment used as a probe is 915 bp because the insert has an Eco RI restriction site

near the 3' end of the cDNA. pHvcht2a encodes a chitinase that has the highly conserved chitinase catalytic domain but lacks the chitin-binding domain. Because barley is also a monocot and is very closely related to rye (III-6), it is conceivable that barley and rye chitinases may have very similar nucleotide sequences.

5 Before screening the library, northern analysis was performed to determine whether pHvcht2a was suitable to screen the library and detect all of the rye chitinases. Total RNA was isolated from nonacclimated and cold-acclimated rye leaves were separated by gel electrophoresis in a denaturing formaldehyde agarose gel and transferred to a nylon membrane. The membrane was hybridized with
10 pHvcht2a under high stringency conditions (50% formamide, 6 X SSC, 42°C) and washed to 0.2 X SSC and 0.1% SDS at 42°C. The blot was exposed to autoradiograph film, which revealed mRNA that hybridized to the probe. Two different chitinase mRNAs were identified with sizes of 1.25 and 1.00 kb. Another transcript, about 3.7 kb in size, also hybridized to pHvcht2a but may be due to
15 unspecific binding to the probe. The 1.25 and 1.00 kb transcripts corresponded to the predicted transcript sizes for the 35 and 26 kD chitinase-like AFPs, respectively. The probe pHvcht2a was used to screen the λ Zap-cDNA library made from poly A + RNA isolated from cold-acclimated rye leaves to identify full-length chitinase cDNAs as described below.

20 Total RNA was extracted from tissues as previously described (III-33) with modifications. Plant tissue was ground in a mortar and pestle while frozen by liquid nitrogen. Z6 buffer (8 M guanidine hydrochloride, 20 mM MES pH 7.0, 20 mM EDTA) and 200 μL 2-mercaptoethanol were added to the tissue, which was further homogenized while frozen. The slurry was mixed with 1 vol of phenol: chloroform:
25 isoamyl alcohol (25:24:1) to extract the proteins. After centrifugation at 8000 xg for 15 min, the top aqueous layer was extracted again with 1 vol phenol: chloroform: isoamyl alcohol, mixed with 1 vol of isopropanol and 0.1 vol 3 M NaOAc pH 5.2, and incubated at -20°C for 2 hrs to precipitate the RNA. The RNA was pelleted by centrifuging the solution at 8000 xg for 45 min. The pellet was washed 2 times with
30 70% EtOH, dried and resuspended in DEPC-treated H_2O. The RNA was precipitated using 8 M LiCl at -20°C. The RNA was pelleted by centrifuging at 8000 rpm for 45 min and washed two times with 70% EtOH. The pellet was dried and resuspended in DEPC-treated H_2O. Poly A+ mRNA was isolated from total RNA using the PolyATtract mRNA Isolation system (Promega, Madison, WI, USA).

35 The poly A+ RNA was made into a cDNA library using the λ ZAP-cDNA

Gigapack Cloning Kit (Stratagene, La Jolla, CA, USA). The membranes were prehybridized in 50% formamide solution containing 6 X SSC, 0.5% (w/v) SDS, 50% (v/v) formamide, 5 X Denhardt's solution (1 X Denhardt's solution is 0.02% (w/v) Ficoll, 0.02% (w/v) PVP, 0.02% (w/v) BSA) and 100 mg mL^{-1} sheared denatured salmon sperm DNA at 42°C for at least 3 hr. The blots were hybridized at 42°C overnight with the barley chitinase cDNA probe (pHvcht2a,1028 bp) provided by Dr. Tomas Bryngelsson (The Swedish University of Agricultural Sciences, Svalöv, Sweden) that was labelled with a ^{32}P-dCTP by random priming (III-13) to a specific activity of approximately 1 X 10^8 to 2 X 10^8 cpm μg^{-1} DNA. The hybridization solution was the same as that for the prehybrization except that salmon sperm was omitted from the solution and the freshly denatured labelled probe was added to a final concentration of 1 X 10^6 cpm mL^{-1}. Following incubation at 42°C overnight, the membranes were washed to various stringencies from 1 X SSC and 0.1% (v/v) SDS to 0.2 X SSC and 0.1 % SDS at 42°C for 30 min. Approximately 92,700 colonies were screened and 89 positive areas were identified. The phage from positive areas were recovered and pooled into 17 groups for a second round of screening. *E. coli* cells were reinfected with the pooled phage, replated, transferred onto nitrocellulose membranes, and rescreened using pHvcht2a. Forty-eight putative clones were identified and reconstituted into pBluescript SK- plasmid from the phage by *in vivo* excision. These clones were denoted recombinant plasmids pCHT-1 to pCHT-48.

25.2 Separating the putative clones into groups

25.2.1 Pvu II restriction enzyme digestion and G-tracking analysis

Pvu II restriction enzyme digests were performed on each of the reconstituted pBluescript plasmids to release the inserts and to determine the size of the insert. The pBluescript plasmid has two Pvu II cutting sites, 445 bp apart, which flank the multi-cloning site. Digestion with this enzyme releases the insert and adds 445 bp to the actual size of the insert. The plasmid size appears to be 2500 bp on an agarose gel with the insert appearing as the other band. If the insert itself contained any Pvu II restriction sites, more than two bands would appear in total, with the 2500 bp representing the plasmid and the other bands totalling the insert size. Agarose gel electrophoresis of the digested clones revealed clones with different insert sizes ranging from 305 bp to 2655 bp. Some of the clones appeared to have the same pattern of bands. These clones were grouped together initially based on Pvu II restriction enzyme cutting pattern. There were 17 groups of clones that had similar-sized bands after digestion. Notably 10 groups composed of 31 clones had inserts

that were larger than 950 bp while only 7 groups or 17 clones had inserts less than 950 bp. The minimum size required to have a full-length transcript as predicted for the 36 and 26 kD chitinase-like AFPs and the northern blot data using pHvcht2a, was 950 bp.

5 G-tracking analysis was performed on the clones to determine which clones were the same and thus redundant. The G reaction of the Sanger sequencing reaction was performed for each clone using T3 and T7 sequencing primers and separated by polyacrylamide gel electrophoresis. The banding pattern of each clone was compared with one another within a primer group. G-tracking allows some 10 sequence data to be obtained and indicate which clones are the same. There were eight groups of clones with nine clones having no match with any other clone.

25.2.2 Hybridization with pHvcht2a

After separation of the digests by gel electrophoresis, the DNA was 15 transferred to a nylon membrane, probed with pHvcht2a at high stringency (6 X SSC, 50% formamide, 42°C) and washed at high stringency (0.2 X SSC, 0.1% SDS, 55°C) to determine which clones were false positives (Fig. 6B). There were 9 clones that did not appear to rehybridize with pHvcht2a. These clones were pCHT-10, -13, -17, -18, -19, -28, -32, -38, and -39 and may not be chitinase cDNAs.

20
25.2.3 Sequence analysis of the putative chitinase cDNAs

Preliminary sequencing analysis was performed on some of the clones that were predicted to be full-length based on the Pvu II restriction data. Representative clones of groups that had inserts bigger than 950 bp were chosen. The clones were sequenced initially using the T3 and T7 universal primers. From the data, sequence 25 strategies were made to obtain further sequence data. Some of the sequences revealed restriction sites that would allow a part of the insert to be deleted from the plasmid. After the deletion was made, the plasmid was religated and sequenced using the T3 or T7 primer to get more sequence data. In some cases this was not possible and primers were designed from the sequence data to continue sequencing.

30

The pCHT9 insert was determined to have the same predicted N-terminal amino acid sequence as the one determined for the 35 kD chitinase-like AFP determined by N-terminal sequencing (III-23). The pCHT46 insert was also determined to be full-length. The translated gene product has the same size as the

26 kD chitinase-like AFP and appears to have the longest 5' untranslated region when compared to other members of its group. The predicted N-terminal amino acid sequence of the translated gene product is very similar to the pHvcht2a predicted amino acid sequence. Both clones have an ATG start codon and contain open reading frames (ORF) that follow amino acid sequences consistent with known chitinases.

25.2.4 Characterization of CHT9

The sequence of CHT9 was determined in both directions using the ALFexpress DNA sequencer at the Sick Children's Hospital Biotechnology Service Centre, (University of Toronto, Toronto, ON, Canada) with various plasmid constructs and primers designed against the initial and growing sequence data. The cDNA was determined to be 1193 bp in length with a poly A tail (Fig. 21). The ORF contained 318 amino acids that began with the methionine codon 48 bp downstream from the 5' terminus of the insert and ended with the stop codon 955 bp from the ATG start codon. The predicted first amino acid in the gene product is glutamate at position 21 of the ORF as the N-terminal amino acid sequence data for the 35 kD chitinase-like AFP is known. The predicted gene product consists of 301 amino acids with a molecular weight of 31647 Da and a pI of 6.96. There were 190 bp of noncoding region at the 3' terminus. PSORT analysis was performed on this cDNA and predicted that there was a 20 amino acid signal sequence that targets the protein to the outside of the cell (Fig. 21(c). The invention also includes variants of this signal sequence which are biologically functional equivalent peptides, polypeptides and proteins of the signal sequence (or the encoding nucleic acid molecule) that exhibit the same or similar signal targeting activity this signal sequence. The discussion of variants with respect to antifreeze polypeptides, below, also applies to the signal sequence by itself. The signal sequence is easily attached to other polypeptides besides antifreeze polypeptides for targeting them outside of the cell. The invention also includes a method for translocating a polypeptide, peptide or protein outside of a cell by expressing recombinant DNA, using protein synthesis or other techniques known in the art to produce a polypeptide, peptide or protein fixed to the signal sequence (or biologically functional equivalent) such that the polypeptide, peptide or protein is translocated of of cell.

25.2.5 Characterization of CHT46

There were many possible clone candidates that could encode the 26 kD chitinase-like AFP. Possible candidates were sequenced using the Pharmacia T7 kit

to determine which clones were full-length and encoded for the 26 kD chitinase-like AFP. After aligning the sequence data generated using the T3 and T7 primers, CHT46 was determined to encode for a full-length chitinase that could encode the 26 kD chitinase-like AFP and was sequenced completely (Fig. 22). CHT46 was shown to have a 998 bp insert that had 78 bp upstream from the first ATG that initiated the ORF of 252 amino acids. The predicted gene product has a molecular mass of 26835 Da and a pl of 8.25. The stop codon is located 757 bp from the ATG start codon with 163 of 3' untranslated region. PSORT also predicted that this protein has a 22 amino acid sequence (Fig. 22(d) and is targeted to the outside of the cell.

25.2.6 Sequence comparison of CHT9 and CHT46

Sequence comparison, using the GAP program of the GCG sequence analysis software package, between pCHT9 and pCHT46 (Fig. 23) revealed 62.1% identity at the nucleotide level, and 58.6% identity and 63.3% similarity at the amino acid level. As well, CHT46 is more similar to pHvcht2a at the nucleotide and amino acid levels than pCHT9 (greater than 90% for both levels). A BLAST search performed on each clone revealed similarities to other chitinases from closely related species. CHT9 has the highest homology (over 90% similarity at the nucleotide and amino acids levels) with Chinese spring wheat chitinase (GenBank accession no. X76041). Comparisons were performed between CHT9 and CHT46 and two of the chitinase proteins found in rye seeds (Fig. 23). CHT9 is 82.2% identical to the rye seed chitinase with 86.6% similarity in amino acids. CHT46 is only 57.4% identical to the rye seed chitinase with 62.2% similarity in amino acids. Multiple sequence alignment of the predicted amino acid sequence for CHT9, CHT46, Hvcht2a, a tobacco chitinase (X16939), a rice chitinase (D16223) and a spring wheat chitinase (X76041) with both of the rye seed chitinases showed that the chitinases from different species are very conserved. Our previous results showed that tobacco does not accumulate extracellular AFPs or chitinases in response to cold temperature (Figs. 16 and 19). Although tobacco and rye chitinase sequences are highly conserved, the fact remains that the rye proteins are cold-induced and exhibit antifreeze activity whereas the tobacco proteins are not cold-induced and do not exhibit antifreeze activity.

From the sequences in Figures 21 and 22, cDNA probes were designed to identify chitinase-like AFPs. One 404 bp probe (ch46hp) consists of a sequence complementary to the sequence of CHT46 beginning at nucleotide 340 and ending at nucleotide 744 (Fig. 22(b). A second 404 bp probe (ch9hp) is a sequence that

complements the homologous region in the CHT9 sequence beginning at nucleotide 541 and ending at nucleotide 945 (Fig. 21(a). Variants of the nucleotide sequence of the probes (biologically funtionally equivalent probes) are also useful. These probes are useful in the following method to identify similar DNA, cDNA and/or RNA sequences. The DNA or RNA in question is transferred to a membrane. Preferably, following prehybridization at 42°C using 50% formamide, 6X SSC, 0.5% SDS, 5X Denhardt solution (a 1X solution is 0.02% w/v Ficoll, 0.02% w/v PVP, 0.02% w/v BSA) and100 $\mu g\ mL^{-1}$ sheared denatured salmon sperm DNA, the DNA or RNA in question is allowed to hybridize under the same conditions using either labelled, denatured ch46hp or labelled, denatured ch9hp in place of the salmon sperm DNA. The membranes are preferably washed with 0.2X SSC and 0.1% SPS at 42°C for 30 min.

As will be discussed in more detail below, the present invention includes not only the rye chitinase-like AFP encoded by sequences presented in Figures 21 and 22, but also biologically functional equivalent peptides, polypeptides and proteins that exhibit the same or similar antifreeze activity as rye AFPs. The invention also includes nucleotide sequences that are biologically functionally equivalent to CHT9, CHT46, PCHT9 or PCHT46.

Example 26: Expression analysis of chitinase CHT9 and CHT46

26.1 Expression in cold- and nonacclimated leaves

Total RNA was isolated from nonacclimated and cold-acclimated rye leaves. Equal amounts of RNA were separated by formaldehyde denaturing agarose gel electrophoresis (Fig. 11A) as seen by the ribosomal RNA bands. The RNA was transferred to a nylon membrane and allowed to hybridize to gene-specific probes for CHT9 and CHT46. Both gene-specific probes are designed from their respective 3' untranslated regions. The gene-specific probe for CHT9 is a 30-mer oligonucleotide: CGAATAATGGTGCAATCCATCGCAAGATGC. The gene-specific probe for CHT46 is a cDNA fragment 292 bp in length, which includes the poly A tail of the transcript (TCGAGTGCGGCATGGGCCGGAACGACGCCAACGTCGACCGCATCGGCTACTA CACACGCTACTGCGGCATGCTTGGCACGGCCACCGGGGGCAACCTCGACTGC TACACCCAGCGAAACTTCGCTAGCTAGACAGTGTATGCACGTGTTATAAATAAAT GGCAATGCATATGCCATCCCCGAATAAATAATTCAACATGTGACAGTTGATTTGT ATGGTAATACGAGTAAGTTGTTGCAACAAATTATGAATATTGAATAAAATCAAATT TTATCAAAAAAAAAAAAAAAAAA). The gene-specific probe for CHT9 hybridized to a 1.25 kb transcript whereas the gene-specific probe for CHT46 hybridized to a 1.0 kb

transcript. For the CHT46 gene-specific probe, a higher stringency wash was added (0.2 X SSC, 0.1% SDS at 65°C) to the protocol to reduce the occurrence of unspecific binding due to the poly A tail of the probe. The relative intensities of the bands between nonacclimated and cold-acclimated RNA were determined by densitometry. There was a 2.5-fold increase in signal intensity in cold-acclimated leaves for CHT9 and a 1.3-fold increase in signal intensity in cold-acclimated leaves for CHT46 when compared with nonacclimated leaves.

26.2 Expression in different rye tissues during cold-acclimation

Rye tissues were sampled during cold-acclimation. Crown (meristem or dividing tissue), new leaf, old leaf, and root samples were taken after 1 week, 3 weeks, 5 weeks, and 7 weeks at cold-acclimating conditions. Tissues from plants grown for 4 weeks at nonacclimated conditions were used as a control. New leaves appeared only after the plants were transferred to the cold-acclimating temperature. Old leaves were leaves that expanded under nonacclimated conditions before the plant was transferred.

Total RNA was extracted from these tissues and analysed by denaturing formaldehyde gel electrophoresis (Fig 13A) and transferred to nylon membranes. The blots were analysed by northern blotting using CHT9 and CHT46 gene-specific probes as described above. CHT9 hybridized to a 1.25 kb transcript that appeared in all of the tissues except the roots. The signal for this transcript has a lower intensity for all of the tissues after 1 week cold-acclimation and progressively increases during the cold acclimation. The signal has the strongest intensity in the crown tissue after 7 weeks of cold-acclimation. The CHT46 gene-specific probe hybridized to a 1.0 kb transcript. The transcript appeared in all of the tissues with low intensity in tissues after 1 week cold-acclimation and gradually increasing intensities over time during cold-acclimation. The CHT46 transcript appears to be present in all of the tissues including the roots.

26.3 Expression in leaves from nonacclimated, cold-acclimated and deacclimated conditions

In order to demonstrate that cold-induction was reversible, rye plants were grown for one week in nonacclimating conditions, transferred to cold-acclimating conditions for a period of 7 weeks, then transferred back to nonacclimating conditions. Tissue samples were taken from plants that were grown in nonacclimated conditions for 2 weeks as a control. Tissues were sampled after 6,

12 and 36 hrs and after 1, 3, 5 and 7 weeks of being in the cold-acclimating conditions. After 7 weeks, tissue samples were taken after 6, 12, and 30 hrs and 9 days after being transferred back to nonacclimating conditions. Total RNA was extracted from these tissues and analysed by denaturing formaldehyde agarose gel
5 electrophoresisand hybridization with gene-specific probes (see Example 26.1). For CHT9, a 1.25 kb transcript was present in the 7 week cold-acclimation sample and in the samples taken 6 and 12 hr after being transferred from cold to nonacclimating conditions, after which the signal decreases. There does not appear to be any signal in the other samples. The same blot was hybridized to pCHT46 gene-specific probe
10 at low stringency. A 1 kb transcript hybridized in all of the samples. The intensity of the transcript signal increased over time during cold-acclimation. The message disappeared slowly after plants were transferred to nonacclimating conditions. These results confirm that expression of CHT9 and CHT46 is upregulated by cold temperature.

15 **26.4 Expression in leaves treated with salicylic acid**

Salicylic acid induces expression of genes encoding antifungal chitinases. Rye plants grown at nonacclimated conditions for 2 weeks were sprayed once a day with 20 ppm salicylic acid in 0.5% (v/v) Tween for 8 days. Unsprayed plants were used as controls. Leaf tissue was harvested daily from these plants 1 hr after the
20 salicylic acid treatment and used to isolate total RNA. Expression was analysed by northern blotting using the CHT46 gene-specific probe by using low stringency wash conditions. One kb and 3.7 kb transcripts were apparent in all the samples whether treated or not treated with salicylic acid. The 3.7 kb transcript may result from unspecific binding due to the low stringency washes. Initially, the signals for both
25 control and treated samples were similar in intensity. After 5 and 6 days of treatment, the signal was stronger in the control plants than in the treated plant samples. The CHT9 gene-specific probe when used did not produce any positive results. These results show that CHT46 and CHT9 expression is not induced by the same signals as antifungal chitinase genes.

30 **Example 27: Overexpression of CHT46 and CHT9 in *Arabidopsis thaliana***

CHT9 and CHT46 were cloned into the pKYLX7.1-35S^2 *Agrobacterium tumefaciens*-compatible vector. These vectors were transformed into a disarmed *A. tumefaciens* strain (C58 pGv3850) by electroporation. These strains were introduced into *Arabidopsis thaliana* by vacuum infiltration and seeds were harvested
35 from the plants. These seeds were screened for kanamycin resistance and the T1

plants were analyzed for the number of inserts. Plants with one insert of either CHT9 or CHT46 were advanced to homozygosity in the T3 generation. Expression of CHT46 and CHT9 in T3 plants is confirmed by Northern blotting using gene-specific probes described in Example 25. Accumulation of chitinase-like AFPs is confirmed by immunoblotting as described in Example 22 and assays of antifreeze activity. Antifreeze activity can be assayed in either crude extracts as described in Example 18 or in extracellular extracts as described in Example 5.

OVEREXPRESSION OF DNA ENCODING CHITINASE-LIKE AFPs

The term "encoding DNA" refers to chromosomal DNA, plasmid DNA, cDNA or synthetic DNA that encodes proteins similar to winter rye AFPs or other AFPs of the invention, preferably the chitinase-like AFPs. Encoding DNAs of the present invention may be introduced into microbial, plant or animal host. Microbial hosts include algae, bacteria, fungi and protozoa. Encoding DNAs introduced into a host may be incorporated into plasmids or into chromosomes located in microbes, nuclei and organelles. Overexpression refers to the accumulation of a recombinant protein that is not normally present or is present at a higher level than normally seen in expression of the endogenous gene.

Example 28: Expression of CHT9 and secretion of chitinase-like AFP in *E. coli*

28.1 Cloning of CHT9 into a bacterial expression vector

To insert CHT9 into the BamHI site of the *E.coli* expression vector pET12a (Novagen,Madison,WI), the CHT9 was PCR (polymerase chain reaction) amplified with the synthetic oligonucleotides

5'-TTAAGGATCCGGAGCAGTGCGGCTCGCAGGC and

5'-GGTTGGATCCTGCGAACGGCCTCTGGTTGTA as primers to generate BamHI sites at the start and end of the gene. The ca. 800-bp PCR-amplified fragment was digested with BamHI and ligated into the unique BamHI site of pET12a. The ligation mixture was transformed into DH5α, plated onto LB-ampicillin (150 μg/ml) plates, and incubated at 37°C. Plasmids of individual colonies were analyzed to find clones having the BamHI fragment inserted in the correct orientation, *i.e.* CHT9 reads in the same direction as the secretion signal provided by the vector. One clone was identified and named cht9/12a. To confirm the DNA sequence of the PCR-amplified chitinase cDNA, double strand DNA sequencing was performed by dideoxynucleotide chain termination using the T7 DNA sequencing kit according to

the manufacturer's instructions (Pharmacia, Montreal, Quebec, Canada).

28.2 Expression of ch9/12a recombinant plasmid

The plasmid DNA of ch9/12a was purified from DH5α cells by using a QIAGEN DNA purification kit (QIAGEN, Germany) and transformed into the
5 expression host BL-21(DE3) cells from Novagen (Madison, WI) in which a chromosomally inserted T7 polymerase was provided to dictate the expression of the plasmid encoded gene. To express the chitinase-like AFP, a single colony was picked from a freshly streaked LB-plate and was used to inoculate a 2 ml LB/ampicillin culture. The culture was incubated with shaking at 30°C for overnight.
10 The following morning, a 50 ml LB/ampicillin culture was inoculated with 1 ml of the overnight culture in a 250 ml flask and incubated at 30°C with shaking until the OD_{600} reached 0.4. The IPTG (Sigma) was added to a final concentration of 0.4 mM and the incubation was continued for an additional 3hr. The cells were harvested by centrifugation at 5000 x g for 5 min at 4°C.

15 **28.3 Purification and analysis of the expressed proteins**

For purification of periplasmic proteins, the bacteria cell pellet was resuspended in ice-cold 20% sucrose, 2.5 mM EDTA, 20 mM Tris-HCl pH8.0 to a concentration of 5 OD_{550} units/ml and incubated on ice for 10 min. The suspension was centrifuged at 15,000 x g for 5 min and the pellet was resuspended in the same
20 volume of ice-cold 2.5 mM EDTA, 20 mM Tris-HCl pH 8.0, incubated on ice for 10 min, and centrifuged as above for 10 min. The supernatant is the periplasmic fraction and was further analyzed by SDS-PAGE and immunoblotting with antiserum raised against the rye chitinase-like AFP (see Example 17).

The bacteria expression vector pET12a contains the leader sequence of the
25 ompT (outer membrane protein T) protein, and in some cases, the target proteins may be directed to the periplasmic space. The leader sequence is necessary, but not sufficient for export into the periplasm. Translocation also can depend on the mature domain of the target protein. The osmotic shock protocol is a simple method of preparing the periplasmic fraction from BL-21 cells. Figure 24 shows the
30 expression of the chitinase in cells transformed with pET12a/ch9. Protein extracts from total cells showed a major band of ca. 32 kDa (lanes 2,4) which is absent in the extract of control cells (lanes 1, 3). Although the periplasmic fraction contained the expressed protein, the majority of the expressed chitinase-like AFP remained intracellular (data not shown). To evaluate whether the chitinase-like AFP in the

periplasmic fraction had antifreeze activity, this fraction was concentrated 1000-fold and the antifreeze activity was monitored by observing changes in morphology of ice crystals formed during freezing. Antifreeze activity was present in this fraction because the ice crystals grew in the shape of hexagonal bipyramids with straight faces. The recombinant protein also exhibited chitinase activity, which was assayed as described in Example 15.

Example 29: Expression and secretion of chitinase-like AFP in *Pichia pastoris*

29.1 Cloning of a CHT9 construct with a c-terminal His/Myc tag

The *Pichia* expression vector was purchased from Invitrogen (San Diego, CA). To insert the CHT9 cDNA into the EcoRI and NotI site of the expression vector pGAPZαA, CHT9 was PCR-amplified with the synthetic oligonucleotides

5'-ATTGAATTCGAGCAGTGCGGCTCGCAGGCC and

5'-AATTGCGGCCGCTGCGAACGGCCTCTGGTTGTA as primers to generate EcoRI and NotI sites at the 5' and 3' ends of the cDNA, respectively. The amplified fragment was digested with EcoRI and NotI and ligated into the pGAPZαA restricted with the same enzymes. The ligation mixture was transformed into DH5α, plated onto low salt LB-Zeocin (25 μg/ml) plates, and incubated at 37°C. The recombinant plasmids were analyzed, and clones having the EcoRI/NotI fragment inserted in-frame with the yeast α-factor signal sequence were identified and named pGAPZαA/cht9. The DNA sequence of the recombinant plasmid was confirmed by DNA sequencing as described above. The His/Myc tag strategy is useful for producing active chitinases and other antifreeze proteins. Therefore the invention also includes a method of purifying chitinases and other antifreeze proteins. Tags similar to the His/Myc tag may also be used for purification.

29.2 Cloning of a CHT9 construct without the c-terminal tag

A new oligonucleotide 5'-TCTGGAGACTATGCGAACGGCCTCTTGGTT-3' was synthesized, and together with the 5'-oligo described above, were used in a PCR reaction to generate EcoRI and XbaI as the 5' and 3' ends of CHT9, respectively. The amplified fragment was digested with EcoRI and XbaI and ligated into the pGAPzαA restricted with the same enzymes. Such a strategy ensures that the sequences of the His/Myc tag encoded by the vector are not attached to the C-terminus of CHT9. The selection and amplification of the recombinant plasmid were carried out as described above. The positive clone was named pGAPzαA/cht9(-tag)

and is useful for increasing antifreeze activity in the recombinant protein.

29.3 Transformation of pGAPZαA/cht9 and pGAPZαA/cht9(-tag) into *Pichia* strain X-33

Plasmid DNA of pGAPZαA/cht9 and pGAPZαA/cht9(-tag) was purified by using a large scale DNA purification kit (QIAGEN, Germany). Approximately 10 μg of the plasmid DNA was transformed into the yeast cells X-33 (Invitrogen) by electroporation (Bio-Rad GenePulser) according to the parameters recommended by the manufacturer's protocols. The transformed cells were incubated at 30°C on YPDS plates (1% yeast extract, 2% peptone, 2% D-glucose, 1 M sorbitol, 2% agar) containing 100 μg/ml Zeocin for 3 days. The colonies were further purified by streaking for single colonies on fresh YPDS/Zeocin plates. Eight of the Zeocin-resistant *Pichia* transformants for each clone were analyzed for the presence of the CHT9 cDNA. The genomic DNA was isolated from individual clones and a PCR protocol was carried out to detect the presence of the insert according to the *Pichia Expression Manual* (Invitrogen). The clones containing the chromosomally inserted CHT9 cDNA were chosen for expression analysis.

29.4 Expression of the recombinant CHT in *Pichia* and analysis of the chitinase-like AFPs

A single colony from each recombinant clone was used to inoculate 10 ml of YPD medium (1% yeast extract, 2% peptone, 2% glucose) and the culture was incubated with shaking at 28-30°C overnight. Approximately 0.1 ml of the overnight culture was used to inoculate 50 ml of YPD in a 250 ml flask and the incubation was continued for 2-3 days. At the end of each day, 1 ml of culture was collected, centrifuged, the supernatant and the cell pellet were analyzed by SDS-PAGE and immunoblotting.

The pGAPZαA (2.9 kb) vector uses the GAP promoter to constitutively express recombinant proteins in *Pichia pastoris*. GAP is the promoter of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, which is constitutively expressed in many organisms including *Pichia pastoris*. In addition, this vector has an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal, which, in some cases, targets the foreign proteins to the extracellular space (supernatant). Figure 25a shows overexpression and secretion of the recombinant chitinase from the yeast cells. Protein samples prepared from the supernatant of the yeast culture showed a major band in SDS-PAGE of ca. 32 kDa (lanes 1, 2). Figure

25b shows the immunoblot analysis of the same samples. Moreover, unlike the bacterial expression system described above, the majority of the expressed chitinase-like AFP in yeast was present in the supernatant (compare lanes 1, 2 and 3,4), indicating that the secretion was nearly complete. The level of expression and secretion was calculated to be in the range of 50 to 100 mg per liter of culture medium. *Pichia pastoris* secretes little of its own protein, so the successful secretion of the recombinant chitinase-like AFP ensures easy purification and characterization.

Example 30: Expression and secretion of pGAPZαA/cht9(-tag)

In order to obtain a protein best resembling its native form, an alternative clone was constructed in such a way as to delete the His/Myc tag attached to the c-terminus of the native chitinase. The expression and secretion levels of the recombinant protein are comparable to that of the pGAPZαA/cht9. SDS-PAGE and immunoblotting of the expressed proteins from both clones show that the recombinant chitinase-like AFPs have antifreeze activity because hexagonally-shaped ice crystals are formed in the AFP solutions. The recombinant chitinase-like AFPs also exhibit chitinase activity, which was assayed as described in Example 15.

Example 31: Expression of CHT46 in *E. coli*

CHT46 was also expressed in *E. coli* as a fusion protein with a leader sequence containing six histidines using the vectors described in Example 27. The recombinant protein was purified from cell lysate, prepared as described in Example 27, using a nickel chelation column. After refolding and cleavage of the His tag, the mature recombinant protein exhibited definite antifreeze activity as hexagonal ice crystals were formed in solutions of the recombinant AFP.

Example 32: Enhancement of antifreeze activity

The antifreeze activity of the chitinase-like AFP is further increased by carrying out selective site-directed mutagenesis. An example is the increase in AFP activity from the fish Ocean Pout using this approach (III-32). Using protein modelling and other prediction methods, we identify the ice binding domain and other critical amino acid residues in the chitinase-like AFPs that are candidates for mutation, insertion and/or deletion. A DNA plasmid or expression vector containing the chitinase-like AFPs gene is used for these studies using the U.S.E. (Unique site elimination) mutagenesis kit from Pharmacia Biotech or other similar mutagenesis kits that are commercially available. Once the mutation is carried out, and confirmed by DNA sequence analysis, the mutant protein is expressed using any of the

expression systems and its antifreeze activity monitored. This approach is useful not only to enhance antifreeze activity, but also to engineer some functional domains for other properties useful in the purification or application of the proteins or the addition of other biological functions. It is also possible to synthesize a DNA fragment based
5 on the sequence of the chitinase-like AFPs that encodes smaller proteins that retain antifreeze activity and are easier to express. It is also possible to modify the expression of the cDNA so that it is induced under environmental conditions other than cold temperatures or in response to different chemical inducers or hormones. It is also possible to modify the DNA sequence so that the protein is targeted to a
10 different location. All these modifications of the DNA sequences presented in Figures 21 and 22 and the proteins produced by the modified sequences are encompassed by the present invention.

Example 33: Inheritance of antifreeze proteins in winter and spring wheat.

Cheyenne winter wheat (*Triticum aestivum* L. cv Cheyenne), Chinese Spring
15 wheat (*Triticum aestivum* L. cv Chinese Spring), and 21 chromosome substitution lines were grown as described in Example 1. In each of the substitution lines, one pair of chromosomes in Chinese Spring wheat was replaced by the corresponding homologues from the variety Cheyenne, which has a much higher degree of freezing tolerance. Apoplastic extracts were obtained from leaves of Chinese Spring wheat,
20 Cheyenne winter wheat, and the 21 chromosome substitution lines as described in Examples 10 and 21. Each extract was assayed for antifreeze activity as described in Example 5. The proteins present in the extracts were quantified as described in Example 21, then separated by SDS-PAGE and examined by immunoblotting using antisera produced against winter rye AFPs with similarity to glucanases, chitinases
25 and thaumatins as described in Example 22.

After cold acclimation, apoplastic extracts from the leaves of Chinese Spring, Cheyenne and the 21 chromosome substitution lines all exhibited antifreeze activity and all contained glucanase-like, chitinase-like and thaumatin-like AFPs (Chun et al, 1998, Euphytica 102:219-226). The amount of apoplastic potein per leaf and the
30 level of antifreeze activity were positively and significantly correlated with the winter survival of all 23 lines. Chromosomes 5B and 5D carry the major genes for both the greater accumulation of apoplastic proteins and higher antifreeze activity.

Example 34: Production of AFPs in Eukaryotic and Prokaryotic Cells

The DNA sequences (also referred to as nucleic acid molecules in this
35 application) of the invention may be obtained from a cDNA library, for example by

using the methods of Example 25 (genomic DNA libraries). The nucleotide molecules can also be obtained from other sources known in the art such as expressed sequence tag analysis or *in vitro* synthesis. The AFP-encoding DNA described in this application (including variants that are biologically functional equivalents) can be introduced into and expressed in a variety of eukaryotic and prokaryotic host cells. A recombinant nucleic acid molecule for the AFPs contains suitable operatively linked transcriptional or translational regulatory elements. Suitable regulatory elements are derived from a variety of sources, and they may be readily selected by one with ordinary skill in the art (Sambrook, J, Fritsch, E.E. & Maniatis, T. (1989). Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press. New York; Ausubel et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). For example, if one were to upregulate the expression of the gene, one could insert the sense sequence and the appropriate promoter into the vector. Plant promoters can be inducible or constitutive, environmentally - or developmentally-regulated, or cell - or tissue-specific. Transcription is enhanced with promoters known in the art such as the "Super-promoter" [Ni et al., (1995) Plant Journal 7:661-676] or the 35S promoter of cauliflower mosaic virus [Shah et al., (1986) Science 233:478-481.].

Inducible promoters include: a) drought- and ABA-inducible promoters which may include ABA-responsive elements [Ono et al., (1996) Plant Physiol 112:483-491; Abe et al., (1997) Plant Cell 9:1859-1868.]; b) heat shock-inducible promoters which may contain HSEs (heat shock elements) as well as CCAAT box sequences [Rieping M and Schoffl F (1992) Mol Gen Genet 231:226-232]; c) salt-inducible promoters which may include AT and PR elements [Raghothama et al., (1997) Plant Mol Biol 34:393-402]; d) Copper-inducible promoter that includes ACE1 binding sites [Mett et al., (1996) Transgenic Res 5:105-113.]; e) steroid-inducible promoter that includes the glucocorticoid response element along with an expression vector coding for a mammalian steroid receptor [Schena et al., (1991) PNAS 88:10421-10425.].

In addition, tissue specific expression is achieved with the use of tissue-specific promoters such as, the Fd (Ferredoxin) promoter that mediates high levels of expression in green leaves [Vorst et al. (1990) Plant Mol Biol 14:491-499.] and peroxidase promoter for root-specific expression [Wanapu & Shinmyo (1996) Ann. NY Acad. Sci. 782:107-114.]. Pollen-, flower-, fruit- and seed-specific promoters can also be used. These promoters vary in their transcription initiation rate and/or efficiency.

If one were to downregulate the expression of the gene, one could insert the antisense sequence and the appropriate promoter into the vehicle. These techniques are known to those skilled in the art. The nucleic acid molecule or gene fragment may be either isolated from a native source (in sense or antisense orientations), synthesized, or it may be a mutated native or synthetic sequence or a combination of these.

Examples of regulatory elements include a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecule. Other regulatory regions that may be used include an enhancer domain and a termination region. The regulatory elements described above may be from animal, plant, yeast, bacterial, fungal, viral, avian, insect or other sources, including synthetically produced elements and mutated elements.

In addition to using the expression vectors of Example 28, the polypeptide may be expressed by inserting a recombinant nucleic acid molecule in a known expression system derived from bacteria, viruses, yeast, mammals, insects, fungi or birds. The recombinant molecule may be introduced into the cells by techniques such as *Agrobacterium tumefaciens*-mediated transformation, particle-bombardment-mediated transformation, direct uptake, microinjection, coprecipitation , transfection and electroporation depending on the cell type. Retroviral vectors, adenoviral vectors, DNA virus vectors and liposomes may be used. Suitable constructs are inserted in an expression vector, which may also include markers for selection of transformed cells. The construct may be inserted at a site created by restriction enzymes.

In one embodiment of the invention, a cell is transformed with a nucleic acid molecule of the invention or a fragment of a nucleic acid molecule inserted in an expression vector to produce cells expressing an AFP. The gene or gene fragment may be either isolated from a native source (in sense or antisense orientations), synthesized, or it may be a mutated native or synthetic sequence or a combination of these.

Another embodiment of the invention relates to a method of transforming a cell with the nucleic acid molecule of the invention or a fragment of the nucleic acid molecule, inserted in an expression vector to produce a cell expressing the AFP. The invention also relates to a method of expressing the polypeptides of the invention in the cells.

Transformed plant cells are then used to produce tissue cultures, seeds or whole plants. The methods and compounds for producing mature plants from cells are known in the art.

Successful transformation and plant regeneration can be achieved in monocots and dicots. Plants (and plant cells) that may be transformed with the AFP nucleic acid molecules or treated with compositions comprising AFPs include asparagus, arabidopsis, potato, tomato, brassica (e.g. canola), cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, rice, corn, wheat, orchard grass, rye, barley, atriplex, salicornia oat, barley, hops, sorgum, alfalfa, sunflower, alfalfa, beet, pepper, tobacco, melon, squash, pea, cacao, hemp, coffee plants, sugar cane, cucumbers and grape vines. Trees may also be transformed. Such trees include maple, birch, pine, eucalyptus, spruce, oak and poplar, as well as fruit trees like peach, nectarine, cherry, apple, pear, plum, apricot, olive, fig, lemon, lime, orange, tangerine and grapefruit. Decorative flowering plants such as carnations and roses may also be transformed with the gene of the invention. Plants bearing nuts such as peanuts, walnuts, pecans, and cashews may also be transformed with an antifreeze protein nucleic acid molecule. Plants used as spices and for herbal and medicial purposes, such as ginseng, ginger, gingko, lemongrass, cinnamon, nutmeg, pepper and cumin, may also be transformed with an antifreeze protein nucleic acid molecule.

In a preferred embodiment of the invention, transformed plant tissue cells or cultures which demonstrate cold tolerance are selected and plants which express antifreeze proteins and are cold tolerant are regenerated from these cultures. These plants may be reproduced, for example by cross pollination with a plant that is cold tolerant or a plant that is not cold tolerant. If the plants are self-pollinated, homozygous cold tolerant progeny may be identified from the seeds of these plants, for example by growing the seeds in a cold environment, using genetic markers or using an assay for cold tolerance or antifreeze protein activity. Seeds obtained from the mature plants resulting from these crossings may be planted, grown to sexual maturity and cross-pollinated or self-pollinated.

The nucleic acid molecule is also incorporated in some plant species by breeding methods such as back crossing to create plants homozygous for an AFP cold resistance nucleic acid molecule.

A plant line homozygous for the AFP nucleic acid molecule may be used as either a male or female parent in a cross with a plant line lacking the AFP tolerance

nucleic acid molecule to produce a hybrid plant line which is uniformly heterozygous for the nucleic acid molecule. Crosses between plant lines homozygous for the AFP resistance nucleic acid molecule are used to generate hybrid seed homozygous for the resistance nucleic acid molecule.

5 The nucleic acid molecule of the invention may also be used as a marker in transformation experiments with plants. A cold sensitive plant may be transformed with an antifreeze protein nucleic acid molecule and a nucleic acid molecule of interest which are linked. Plants transformed with the nucleic acid molecule of interest will grow in a cold environment in which the non-transformed plants are 10 unable to grow.

The nucleic acid molecules of invention may also be targeted for expression in only a certain part of the plant. For example, nucleic acid molecule expression may occur only in the fruit or harvested part of the plant to improve its quality during and after storage at low or freezing temperatures and to increase the shelf or 15 storage life of the product. The nucleic acid molecule of invention may also be expressed only in the temperature sensitive part of the plant such as flowers that bloom in spring when frost may occur or rootstocks that are sensitive to freezing in overwintering plants.

The nucleic acid molecules of invention may also be expressed at lower 20 levels in plants already endowed with the nucleic acid molecules by using antisense technology or cosuppression to reduce expression of wild-type and introduced nucleic acid molecules when the strategy is to increase sensitivity of plants such as weedy species to freezing or low temperature diseases to decrease survival.

Example 35: Biologically Functionally Equivalent Peptides, Polypeptides, and 25 Proteins

The present invention includes not only the chitinase-like AFP encoded by sequences presented in Figures 21 and 22 and the proteins described in the Examples, but also "biologically functional equivalent peptides, polypeptides and proteins" that exhibit the same or similar antifreeze activity as AFPs described in this 30 application. The phrase "biologically functional equivalent peptides, polypeptides, and proteins" denotes peptides, polypeptides, and proteins that exhibit the same or similar AFP activity as one or more of the AFPs of the invention when assayed as described in this application (see Example 5 for antifreeze assay). By "the same or similar AFP activity" is meant the ability to perform the same or similar function as 35 the AFPs of the invention. These peptides, polypeptides, and proteins can contain a

region or moiety exhibiting sequence identity to a corresponding region or moiety of the AFPs described in the application, but this is not required as long as they exhibit the same or similar AFP activity. Identity refers to the similarity of two polypeptide (or nucleotide sequences) that are aligned so that the highest order match is obtained. Identity is calculated according to methods known in the art, such as the Gap or BestFit programs, described below. For example, if a polypeptide (called "Sequence A") has 90% identity to a portion of the polypeptide in Figures 21 or 22, then Sequence A will be identical to the referenced portion of the polypeptide in Figures 21 or 22, except that Sequence A may include up to 10 point mutations ,such as deletions or substitutions with other amino acids , per each 100 amino acids of the referenced portion of the polypeptide in Figures 21 or 22. Peptides, polypeptides, and proteins biologically functional equivalent to the AFPs of the invention can occur in a variety of forms as described below.

As described in Example 18, the AFPs described in the application are also useful as an antigen for the preparation of antibodies that can be used to purify or detect AFPs or other AFP-like proteins. Monoclonal and polyclonal antibodies are prepared according to other techniques known in the art. For examples of methods of the preparation and uses of monoclonal antibodies, see U.S. Patent Nos. 5,688,681, 5,688,657, 5,683,693, 5,667,781, 5,665,356, 5,591,628, 5,510,241, 5,503,987, 5,501,988, 5,500,345 and 5,496,705. Examples of the preparation and uses of polyclonal antibodies are disclosed in U.S. Patent Nos. 5,512,282, 4,828,985, 5,225,331 and 5,124,147. Antibodies recognizing AFPs can be employed to screen organisms containing AFPs or AFP-like proteins. The antibodies are also valuable for immuno-purification of AFPs and AFP-like proteins from crude extracts.

A) Conservative Amino Acid Changes in AFP Sequences

Peptides, polypeptides, and proteins biologically functionally equivalent to AFP protein include amino acid sequences containing amino acid changes in an AFP sequence. The biologically functional equivalent peptides, polypeptides, and proteins have at least about 40% sequence identity, preferably at least about 60%, at least about 80%, at least about 90% or at least about 95% sequence identity, to the naturally occurring polypeptide, or corresponding region. Most preferably, the biologically functional equivalent peptides, polypeptides, and proteins have at least 97%, 98% or 99% sequence identity to the naturally occurring protein, or corresponding region or moiety. "Sequence identity" is determined by the Gap or BestFit. BestFit aligns the best segment of similarity between two sequences.

Alignments are made using the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482-489. The algorithm of Needleman and Wunsch (1970 J Mol. Biol. 48:443-453) is used in the Gap program.

B) Fragments and Variants of Antifreeze Proteins

5 Fragments and variants of AFPs possessing the same or similar AFP activity as that of the AFPs of the invention are also encompassed by the present invention.

C) AFP Fragments

The invention includes fragments of the polypeptides of the invention which 10 retain the same or similar activity as the polypeptides of the invention. Such peptides preferably consist of at least 5 amino acids. In preferred embodiments, they may consist of 6 to 10, 11 to 15, 16 to 25 or 26 to 50 amino acids of the polypeptides of the invention. Fragments of the AFPs can be created by deleting one or more amino acids from the N-terminus, C-terminus or an internal region of the protein (or 15 combinations of these), so long as such fragments retain the same or similar AFP enzymatic activity as the AFPs disclosed in the application. These fragments can be natural mutants of the AFPs, or can be produced by restriction nuclease treatment of an encoding nucleotide sequence. Fragments of the polypeptide may be used in an assay to identify compounds that bind the polypeptide. Methods known in the art 20 may be used to identify agonists and antagonists of the fragments.

Fragments and variants of AFPs encompassed by the present invention should preferably have at least about 40% sequence identity, preferably at least about 60%, at least about 80%, at least about 90% or at least about 95% sequence identity, to the naturally occurring protein, or corresponding region or moiety. Most 25 preferably, the fragments have at least 97%, 98% or 99% sequence identity to the naturally occurring polypeptide, or corresponding region. Sequence identiy is preferably measured with either the Gap or BestFit programs.

The invention also includes fragments of the polypeptides of the invention which do not retain the same or similar activity as the polypeptides but which can be 30 used as a research tool to characterize the polypeptides of the invention.

Example 36: AFP Variants

Variants of the AFPs of the invention may be created by laboratory techniques such as mutagenesis (as described in Example 32) or splicing. Variants can also be naturally occurring mutants of the AFPs disclosed in the application.

Variants of the proteins of the invention may be made, for example, with protein engineering techniques such as site-directed mutagenesis which are well known in the art for substitution of amino acids. A combination of techniques known in the art may be used to substitute, delete or add amino acids. For example, a hydrophobic residue such as methionine can be substituted for another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. An aromatic residue such as phenylalanine may be substituted for tyrosine. An acidic, negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine. Modifications of the proteins of the invention may also be made by treating a polypeptide of the invention with an agent that chemically alters a side group, for example, by converting a hydrogen group to another group such as a hydroxy or amino group.

Peptides having one or more D-amino acids are contemplated within the invention. Also contemplated are peptides where one or more amino acids are acetylated at the N-terminus. Those skilled in the art recognize that a variety of techniques are available for constructing peptide mimetics (i.e. a modified peptide or polypeptide or protein) with the same or similar desired biological activity as the corresponding peptide compound of the invention but with more favorable activity than the peptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis. See for example, Morgan and Gainor, *Ann. Rep. Med. Chem.*, 24:243-252 (1989).

The invention also includes hybrid genes and peptides, for example where a nucleotide sequence from the gene of the invention is combined with another nucleotide sequence to produce a fusion peptide. For example a domain from an AFP nucleic acid molecule or other molecule of interest may be ligated to an AFP nucleic acid molecule encoding an AFP described in this application. Fusion genes and peptides can also be chemically synthesized or produced using other known techniques.

The variants preferably retain the same or similar AFP activity as the naturally occurring AFPs of the invention. The AFP activity of such variants can be assayed by techniques described in this application.

Variants produced by combinations of the techniques described above but which retain the same or similar AFP activity as the naturally occurring AFPs of the

invention, are also included in the invention (for example, combinations of amino acid additions, deletions, and substitutions).

Example 37: Biologically Functionally Equivalent Nucleic Acid Molecules

The invention also includes nucleotide sequences that are biologically functional equivalent to the sequences in Figures 21 or 22 or other sequences of the invention. Biologically functional equivalent nucleotide sequences are DNA and RNA (such as genomic DNA, cDNA, synthetic DNA, and mRNA nucleotide sequences), that encode peptides, polypeptides, and proteins having the same or similar AFP activity as the AFPs shown in Figures 21 or 22 or disclosed in the application. Biologically functional equivalent nucleotide sequences can encode peptides, polypeptides, and proteins that contain a region having sequence identity to a region of an AFP in this application.

A) Nucleic Acid Molecules Encoding Conservative Amino Acid Changes in AFPs

The invention includes biologically functional equivalent nucleotide sequences that encode conservative amino acid changes within an AFP amino acid sequence and produce silent amino acid changes in the AFP sequences of the invention.

B) Nucleotide Sequences Encoding Non-Conservative Amino Acid Substitutions, Additions or Deletions in AFPs

The invention includes nucleotide sequences that are biologically functional equivalent to the sequences in Figures 21 or 22. Biologically functional equivalent nucleotide sequences are DNA and RNA (such as genomic DNA, cDNA, synthetic DNA, and mRNA nucleotide sequences) that encode peptides. polypeptides, and proteins having non-conservative amino acid substitutions, additions, or deletions but which also retain the same or similar AFP activity as the AFPs shown in Figures 21 or 22 or disclosed in the application. The DNA or RNA can encode fragments or variants of the AFPs of the invention. The AFP or AFP-like activity of such fragments and variants is identified by assays as described above. These biologically functional equivalent nucleotide sequences. Fragments and variants of AFPs encompassed by the present invention should preferably have at least about 40% sequence identity or preferably at least about 60%, at least about 80%, at least about 90% or at least about 95% sequence identity to the naturally occurring polypeptide, or corresponding region or moiety. Most preferably, the fragments have at least 97%, 98% or 99% sequence identity to the naturally occurring polypeptide, or corresponding region.

Sequence identiy is preferably measured with either the Gap or BestFit programs.

Nucleic acids biologically functionally equivalent to the AFPs in Figures 21 or 22 include:

(1) DNAs originating from winter rye, and other antifreeze proteins. For example,
5 the sequence shown in Figure 21(a) may have its length altered by natural or artificial mutations such as partial nucleotide insertion or deletion, so that when the entire length of the coding sequence within Figure 21(b), is taken as 100%, the biologically functional equivalent nucleotide sequence has an approximate length of about 60-120% thereof, preferably about 80-110% thereof; or

10 (2) nucleotide sequences containing partial (usually 80% or less, preferably 60% or less, more preferably 40% or less of the entire length) natural or artificial mutations so that such sequences code for different amino acids, but wherein the resulting protein retains the same or similar AFP enzymatic activity as that of a naturally occurring antifreeze protein. The mutated DNAs created in this manner should
15 preferably encode a protein having at least about 40%, preferably at least about 60%, at least about 80, and more preferably at least about 90% or 95%, and most preferably 97%, 98% or 99% sequence identity to the amino acid sequence of the AFPs in Figures 21 or 22. Sequence identity can be assessed by the Gap or BestFit programs.

20 C) Genetically Degenerate Nucleotide Sequences

Since the genetic code is degenerate, those skilled in the art will recognize that the nucleic acid sequence in Figures 21 or 22 and described in the application are not the only sequences which may be used to code for a peptide having antifreeze, glucanase, chitinase or thaumatin activity. This invention includes nucleic
25 acid sequences that have the same essential genetic information as the nucleic acid molecules described in the Figures 21 or 22. Nucleic acid molecules (including RNA) having one or more nucleic acid changes compared to the sequences described in this application and which result in production of a polypeptide shown in Figure 21 or 22 are within the scope of the invention. Genetically degenerate forms
30 of any of the other nucleic acid sequences discussed in this application are also within the invention.

Also included in the invention are nucleic acid molecules that have one or more nucleic acid changes that replace one or more of the amino acids shown in Figures 21 or 22 with a chemically similar amino acid.

D) Biologically Functional Equivalent Nucleic Acid Sequences Detected by Hybridization

Several nucleotide sequences encoding AFPs are shown in Figures 21 or 22 or easily determined by using the methods described in this application. Other biologically functional equivalent forms of AFP-encoding nucleic acids can be easily isolated using conventional DNA-DNA or DNA-RNA hybridization techniques. Thus, the present invention also includes nucleotide sequences that hybridize to one or more of the sequences in Figures 21 or 22 and their complementary sequences, and that encode expression for peptides, polypeptides, and proteins exhibiting the same or similar activity as that of the AFPs produced by the DNA in Figures 21 or 22 or its variants. Such nucleotide sequences preferably hybridize to one or more of the sequences in Figures 21 or 22 under moderate to high stringency conditions (see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Preferable conditions and are described Example 25.

The present invention also encompasses nucleotide sequences that hybridize to genomic DNA, cDNA, or synthetic DNA molecules that encode the amino acid sequence of the AFPs of the invention, or genetically degenerate forms thereof due to the degeneracy of the genetic code, under salt and temperature conditions equivalent to those described in this application, and that code on expression for a peptide, polypeptide, or protein that has the same or similar antifreeze enzymatic activity as that of the antifreeze proteins of the invention.

The nucleotide sequences described above are considered to possess a biological function substantially equivalent to that of the AFP genes of the present invention if inhibition of recrystallization or modification of ice crystal growth can still be observed in a solution when the encoded polypeptide is present at a concentration of 10 g /L or less, preferably 1 g/L or less , and more preferably 100 mg/L or less.

Example 38: Probes

The invention also includes oligonucleotide probes made from the cloned APF nucleic acid molecules described in Example 25 or other nucleic acid molecules of the invention. The probes may be 15 to 30 nucleotides in length and are preferably at least 30 or more nucleotides. Preferred probes are described in Example 25. The probes are useful to identify nucleic acids encoding antifreeze peptides, polypeptides and proteins from plants other than those described in the

application, as well as peptides, polypeptides, and proteins biologically functionally equivalent to the AFPs described in this application. The oligonucleotide probes are capable of hybridizing to one or more of the sequences shown in Figures 21 or 22 or the other sequences of the invention under stringent hybridization conditions. A
5 nucleic acid molecule encoding a peptide of the invention may be isolated from other organisms by screening a library under moderate to high stringency hybridisation conditions with a labeled probe. The activity of the peptide, polypeptide, or protein encoded by the nucleic acid molecule is assessed by cloning and expression of the DNA as described in Example 25. After the expression product is isolated, the
10 peptide, polypeptide or protein is assayed for antifreeze activity as described in this application. The expression product may also be assayed for glucanase, chitinase or thaumatin activity.

Biologically functional equivalent AFP genes from other plants, or equivalent AFP-encoding cDNAs or synthetic DNAs, can also be isolated by amplification using
15 Polymerase Chain Reaction (PCR) methods. Oligonucleotide primers, including degenerate primers, based on the amino acid sequence of the sequences in Figures 21 or 22 can be prepared and used in conjunction with PCR technology employing reverse transcriptase (E. S. Kawasaki (1990), In Innis et al., Eds., PCR Protocols, Academic Press, San Diego, Chapter 3, p. 21) to amplify biologically functional
20 equivalent DNAs from genomic or cDNA libraries of other organisms.

Alternatively, the oligonucleotides, including degenerate nucleotides, can be used as probes to screen cDNA libraries.

Example 39: Heterologous overexpression of AFPs

The expression vectors of Example 25 provide high levels of protein
25 expression. Plants and cell cultures transformed with the nucleic acid molecules of the invention are useful as research tools. Plants and cell cultures are used in research according to numerous techniques known in the art. A cell line (either an immortalized cell culture or a primary cell culture) may be transformed with a vector containing an AFP nucleic acid molecule (or variants) to measure levels of
30 expression of the nucleic acid molecule and the activity of the nucleic acid molecule. A polypeptide of the invention may be used in an assay to identify compounds that bind the polypeptide. Methods known in the art may be used to identify agonists and antagonists of the polypeptides. One may obtain plants that do not express AFPs and use them in experiments to assess AFP gene expression. Experimental groups
35 of plants may be transformed with vectors containing different types of AFP genes

(or genes similar to AFPs or fragments of AFP nucleic acid molecules) to assess the levels of protein produced, its functionality and the phenotype of the plants produced. The polypeptides are also useful for *in vitro* analysis of AFP activity. For example, the protein produced can be used for microscopy or X-ray crystallography studies.
5 Other expression systems can also be utilized to overexpress the AFPs in recombinant systems.

Example 40: Cloning of Nucleic Acid Molecules encoding Additional AFPs from Winter Rye

DNA and RNA molecules encoding glucanase-like and thaumatin-like AFPs
10 from winter rye are cloned using the inventive method described in Example 25. A cDNA library produced from mRNA isolated from healthy tissues of cold-acclimated winter rye is screened as described in Example 25 using heterologous probes obtained from a monocot, preferably a member of the Poaceae family, which encodes a glucanase or a thaumatin-like protein and which has been shown by
15 Northern blotting to hybridize with transcripts of the cold-induced rye AFPs under moderate stringency as described in Example 25.1. The positive clones identified by hybridization with these probes are characterized and sequenced as described in Example 25. The cDNAs are expressed by transformation of plants as described in Examples 27 and 39 or in other expression systems as described in Examples 28,
20 29, 30, 31 and 34. Biologically functionally equivalent peptides, polypeptides and proteins, variants of these AFPs, and biologically functionally equivalent nucleic acids of glucanase-like and thaumatin-like AFPs and their nucleic acid sequences as described in Examples 35, 36 and 37 are also included in this invention. This invention also includes probes for nucleic acids encoding glucanase-like and
25 thaumatin-like AFPs as described in Example 38.

Example 41: Production of AFPs for Use as Additives in Foods or Storage of Biological Materials

Peptides, polypeptides or proteins encoding AFPs or biologically functionally equivalent AFPs synthesized in a prokaryotic or eukaryotic expression system may
30 be produced as a crude extract, a partially purified extract or a purified peptide, polypeptide or protein for use in a food product or biological material. For example, the inventive DNA in Fig. 21 or 22 may be expressed in a plant and then a total soluble extract as described in Examples 18 and 24 or an extracellular extract as described in Examples 1, 15,19 and 20 may be obtained from the plant and used in
35 a food product or biological material. The peptide, polypeptide or protein may be

purified by nickel chelation, by colloidal chitin affinity chromatography, by immunoaffinity chromatography or by any standard liquid chromatographic technique. If the peptide, polypeptide or protein produced in a prokaryotic or eukaryotic cell line has been secreted into the growth medium or retained internally within the cells, then a total soluble extract, partially purified extract or purified peptide, polypeptide or protein is prepared as an additive for a food product or biological material.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope thereof. For example, where polypeptides are described, it will be clear that peptides and proteins can often be used.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety

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In order to facilitate discussion of already known aspects of frost tolerance in plants and the contribution of the subject invention, several journal articles are referred to herein, in accordance with the following index numbers for the group I, II, and III
5 listings of references.

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We claim:

1 A nucleic acid molecule isolated from a monocot, encoding an antifreeze protein.

2 The molecule of claim 1, comprising the DNA sequence in Figure 21(a), Figure 21(b), Figure 22(a) or Figure 22(b).

3 The molecule of claim 1, wherein the sequence comprises at least 40% sequence identity to all or part of the DNA sequence Figure 21(a), Figure 21(b), Figure 22(a) or Figure 22(b).

4 The molecule of claim 1 which is selected from a group consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded DNA and double-stranded DNA.

5 Nucleic acid molecules encoding the same amino acid sequence as the nucleic acid molecules of claim 2.

6 A nucleic acid molecule that encodes chitinase-like antifreeze polypeptide that hybridizes to the nucleic acid molecule of the coding strand from positions 340 to 744 of Figure 22 (a) under a wash stringency of 0.2X SSC to 2X SSC, 0.1% SDS, at 42°C.

7 A nucleic acid molecule that encodes chitinase-like antifreeze proteins that hybridizes to the nucleotide sequence of the coding strand from positions 541 to 745 of Figure 21 (a) under a wash stringency of 0.2X SSC to 2X SSC, 0.1% SDS, at 42°C.

8 A polypeptide encoded by the nucleic acid molecule of claim 1.

9 A mimetic of the purified and isolated polypeptide of claim 8.

10 The polypeptide of claim 8, having the amino acid sequence in Figure 21(a), Figure 21(b), Figure 21(c), Figure 21(d), Figure 22(a), Figure 22(b), Figure 22(c) or Figure 22(d).

11 The polypeptide of claim 8, which has at least 40% sequence identity to all or part of the amino acid sequence in Figure 21(a), Figure 21(b), Figure 21(c), Figure 21(d), Figure 22(a), Figure 22(b), Figure 22(c) or Figure 22(d)..

12 A cold-induced antifreeze polypeptide isolated from a monocot.

13 A cold-induced pathogenesis-related protein with antifreeze activity isolated

from a monocot.

14 The polypeptide of claim 12, wherein the monocot is from the family Poaceae.

15 The polypeptide of claim 14, wherein the monocot is selected from the group consisting of barley, wheat and rye, spring rye, spring wheat, winter wheat, winter barley, and spring oats.

16 A mimetic of the polypeptide of claim 12 or claim 13.

17 An isolated polypeptide having antifreeze activity, selected from the group consisting of barley, wheat and rye, spring rye, spring wheat, winter wheat and winter barley, winter canola, spring oats and kale.

18 The polypeptide of claim 12 or claim 13, selected from the group consisting of chitinase, thaumatin and glucanase.

19 The polypeptide of claim 12 or claim 13 for a use selected from a group consisting of producing antifreeze protein, increasing freezing tolerance in plants and microorganisms, increasing field survival and yields of plants, animals and microorgansims exposed to subzero temperatures, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

20 A recombinant DNA comprising a DNA molecule of any of claim 1 to claim 7 and a promoter region, operatively linked so that the promoter enhances transcription of said DNA molecule in a host cell.

21 A system for the expression of a chitinase gene, comprising an expression vector and chitinase cDNA inserted in the expression vector.

22 The system of claim 21, wherein the expression vector comprises a plasmid.

23 The system of claim 22, wherein the plasmid comprises an *E. coli* vector.

24 The system of claim 23, wherein the *E. coli* vector comprises pET12a.

25 The system of claim 21, wherein the expression vector comprises a *Pichia* vector.

26 The system of claim 25, wherein the *Pichia* vector comprises pGAPZαA.

27 The system of claim 21, wherein the chitinase DNA comprises all or part of the DNA sequence in Figure 21(a), Figure 21(b), Figure 22(a) or Figure 22(b).

28 A plant, plant cell, animal, animal cell, bacterium or yeast transformed by the system of any of claim 1 to claim 27.

29 A method for expressing chitinase comprising: transforming an expression host with a chitinase DNA expression vector; and culturing the expression host.

5 30 The method of claim 29, wherein the expression host is selected from the group consisting of a plant, plant cell, bacterium, yeast, fungus, protozoa, algae, animal and animal cell.

31 The product of the expression system of any of claim 21 to 27 for a use selected from a group consisting of producing antifreeze protein, increasing
10 freezing tolerance in plants, animals and microorganisms, increasing field survival and yields of plants, animals and microorgansims exposed to subzero temperatures, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

15 32 A nucleotide sequence that targets protein secretion in plants consisting of the coding strand or its complement thereof shown in Figure 21 (a), positions 1 through 60.

33 A nucleotide sequence that targets protein secretion in plants consisting of the coding strand or its complement thereof shown in Figure 22 (a), positions 1
20 through 66.

34 A method of enhancing antifreeze activity, comprising combining antifreeze polypeptide with sugars to enhance the activity of antifreeze polypeptide to inhibit the recrystallization of ice and modify the normal growth of ice.

35 A composition comprising one or more antifreeze proteins combined with one
25 or more sugars.

36 The polypeptide of any of claims 8, 10-15 or 17-19 combined with a sugar for a use selected from a group consisting of producing antifreeze protein, increasing freezing tolerance in plants and microorganisms, increasing field survival and yields of plants, animals and microorgansims exposed to subzero
30 temperatures, inhibition of ice recrystallization in biological matter or food product, cryopreservation of cells, hypothermic protection of cells, cold protection of human platelets and killing tumor cells.

37 A polypeptide of any of claims 8, 10-15 or 17-19 for inhibition of the initiation or

progression of a disease or spoliage caused by a low temperature pathogen in a plant, a frozen food or any cryopreserved biological matter.

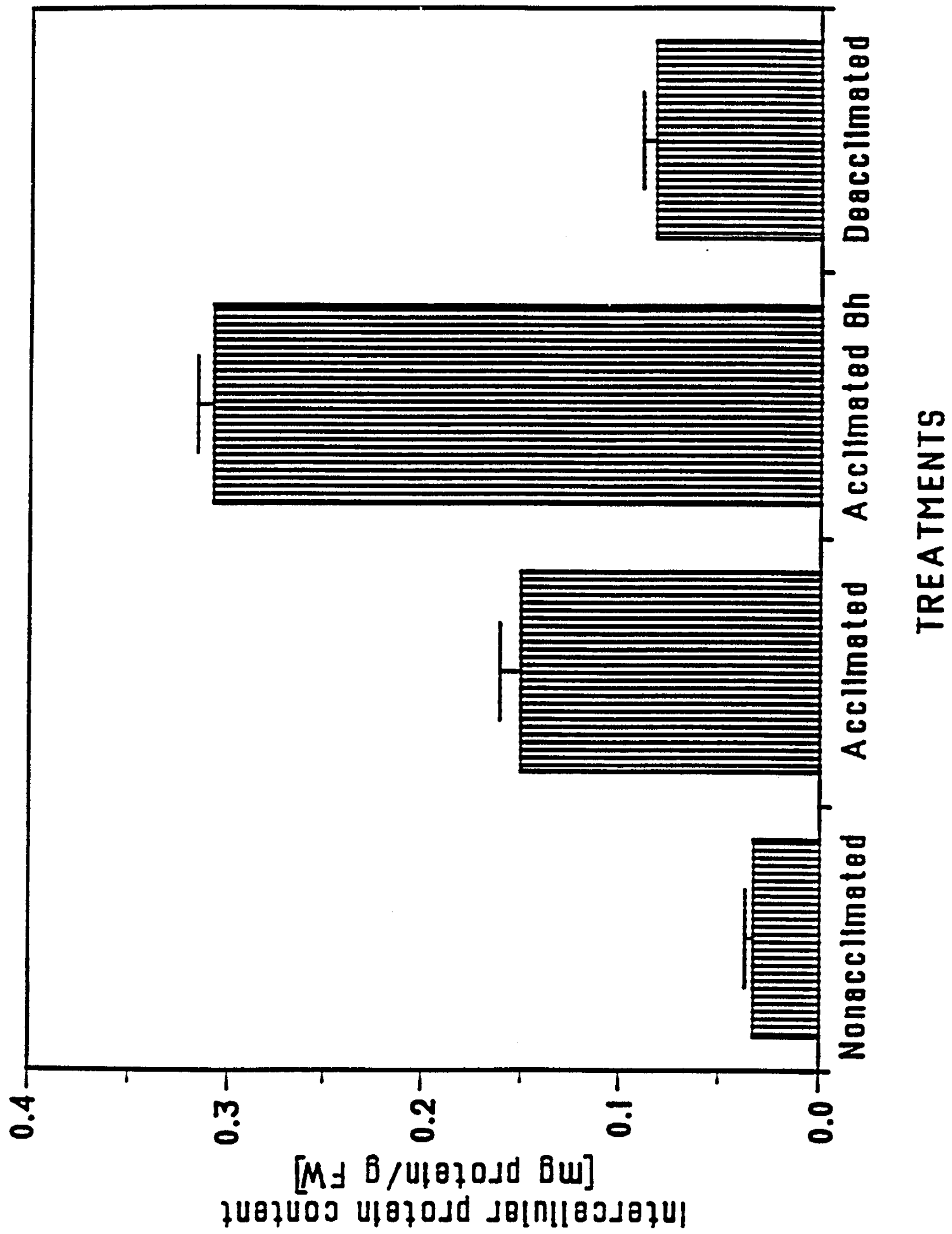


FIG.1.

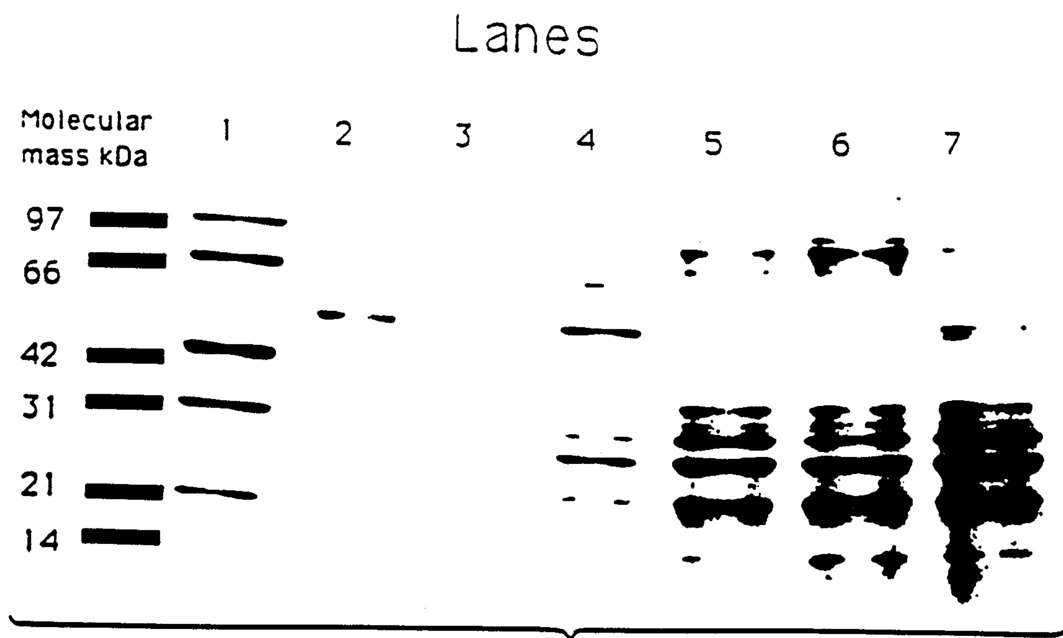


FIG.3.

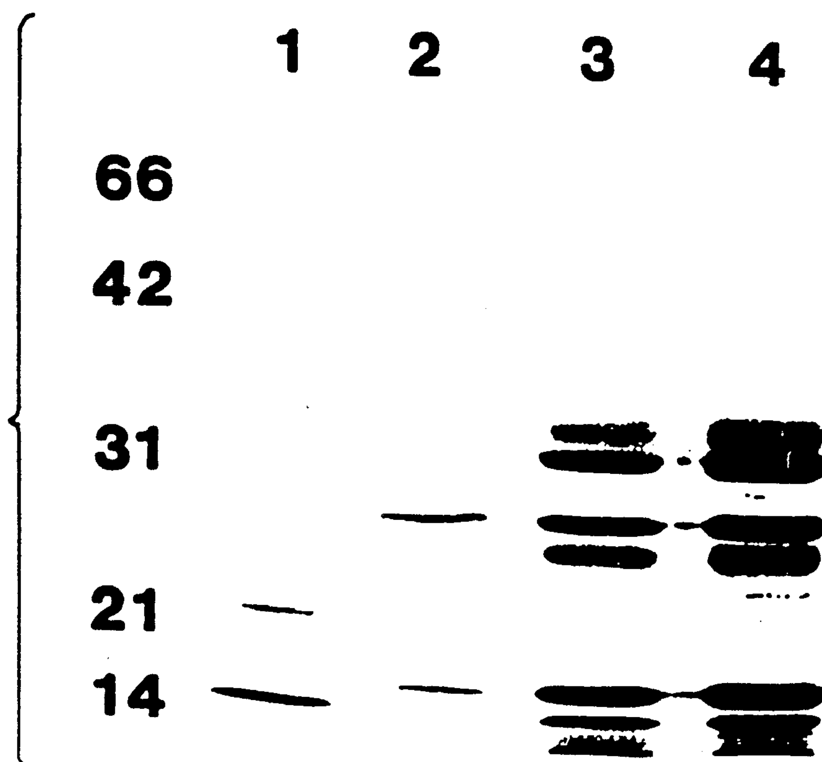


FIG. 2

SUBSTITUTE SHEET (rule 26)

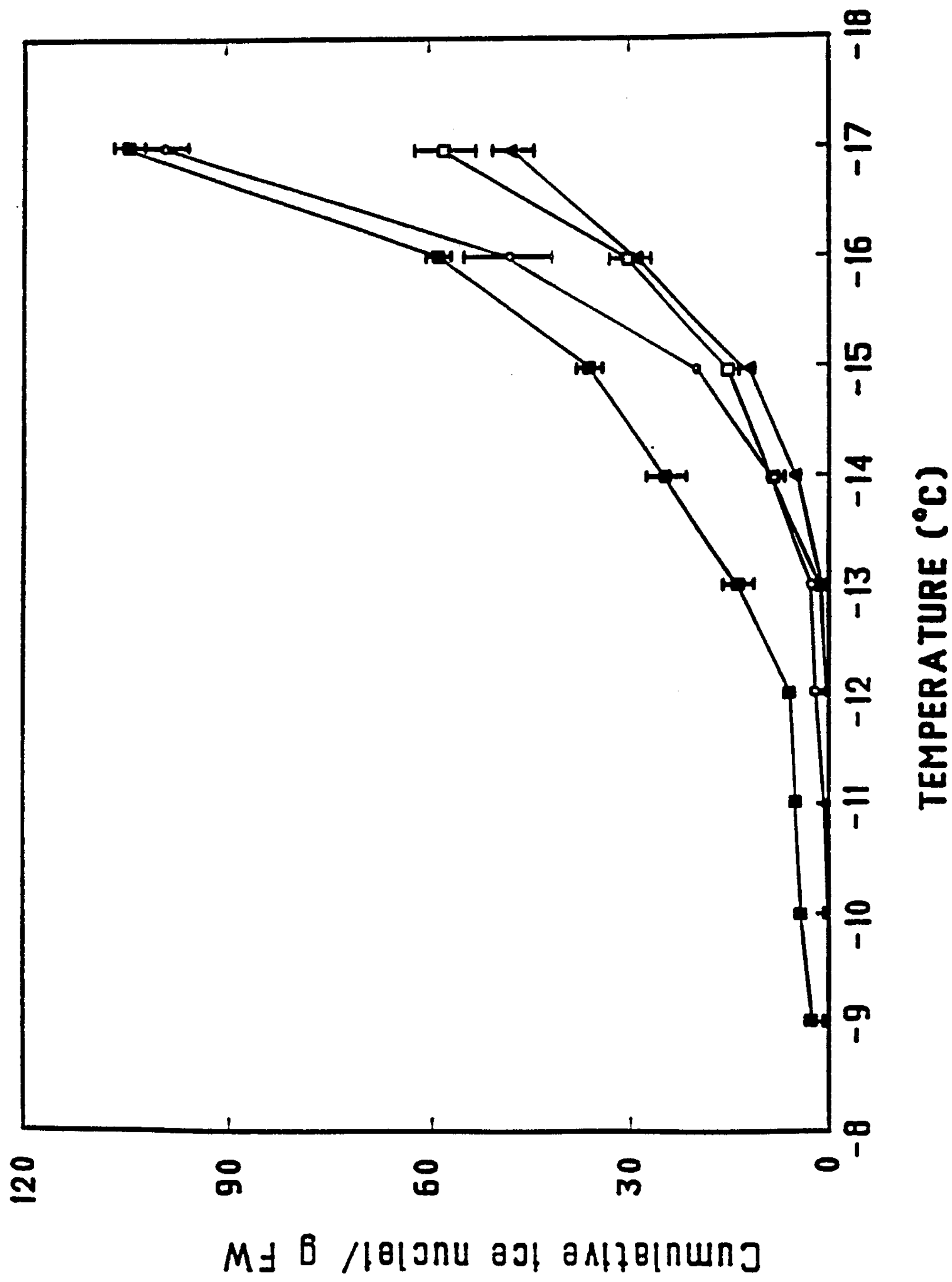


FIG.5.

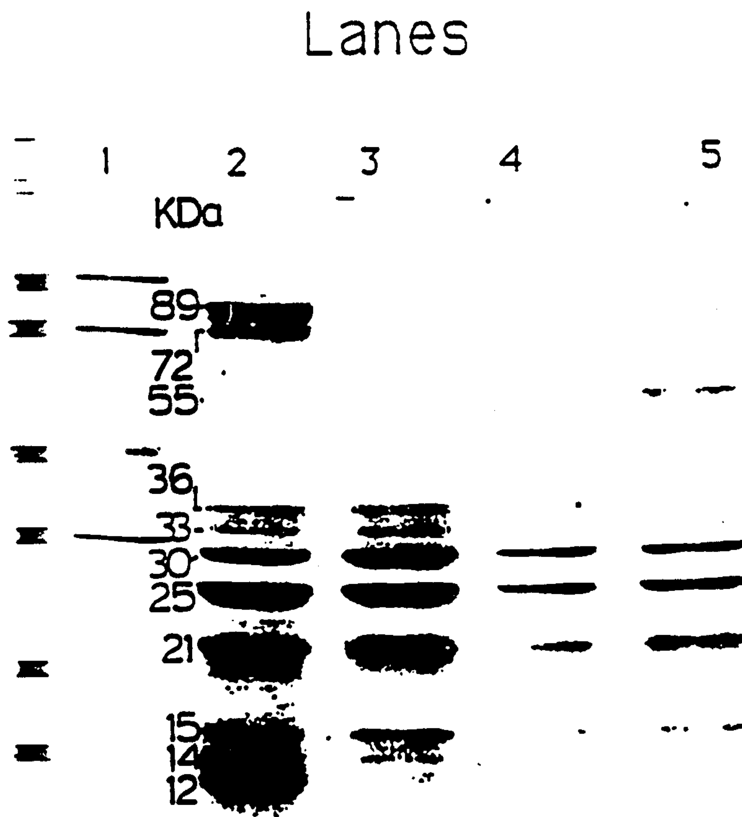


FIG.4.

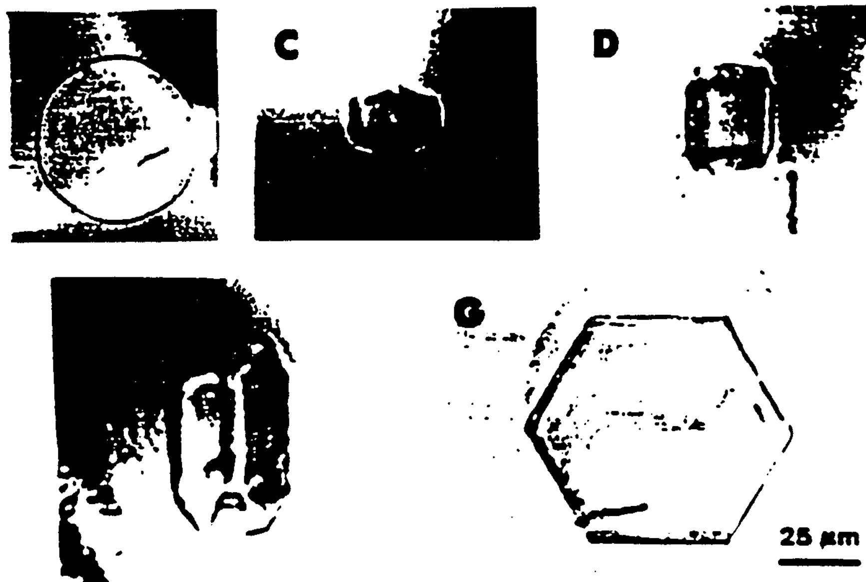


FIG. 6

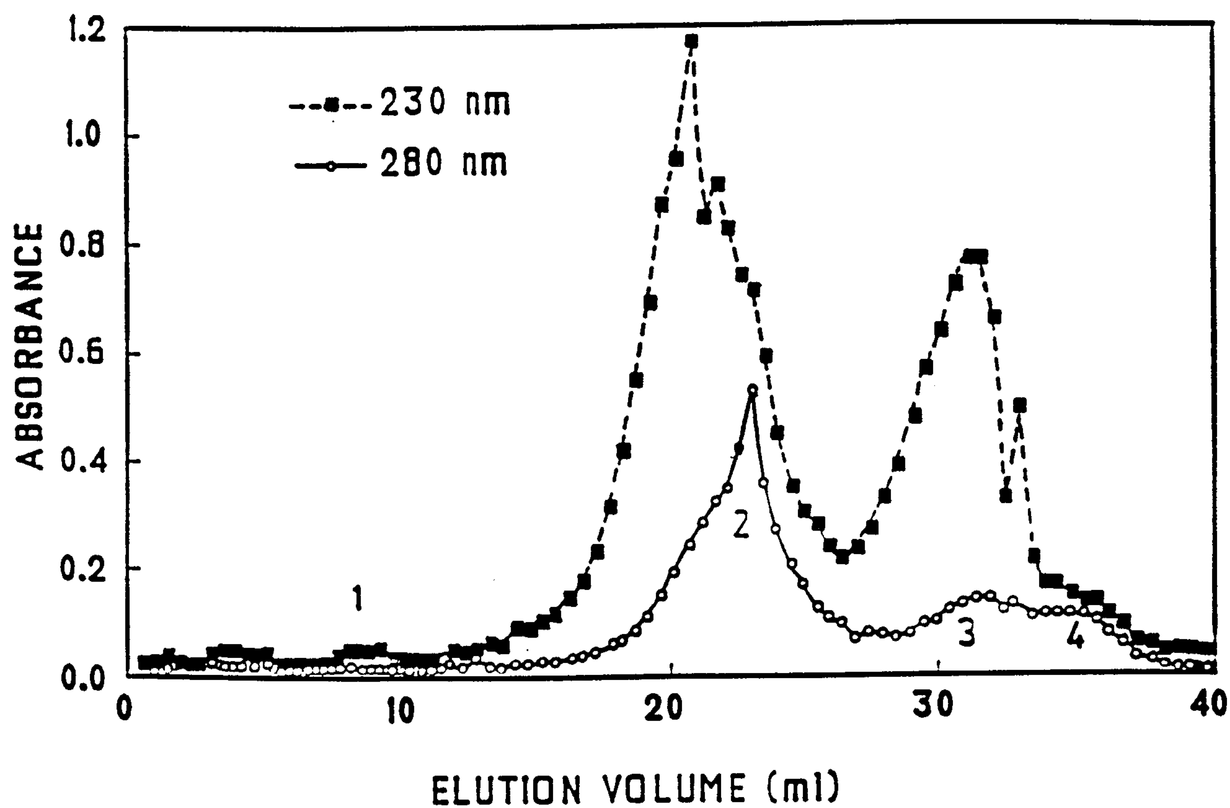


FIG.7.

SUBSTITUTE SHEET (rule 26)

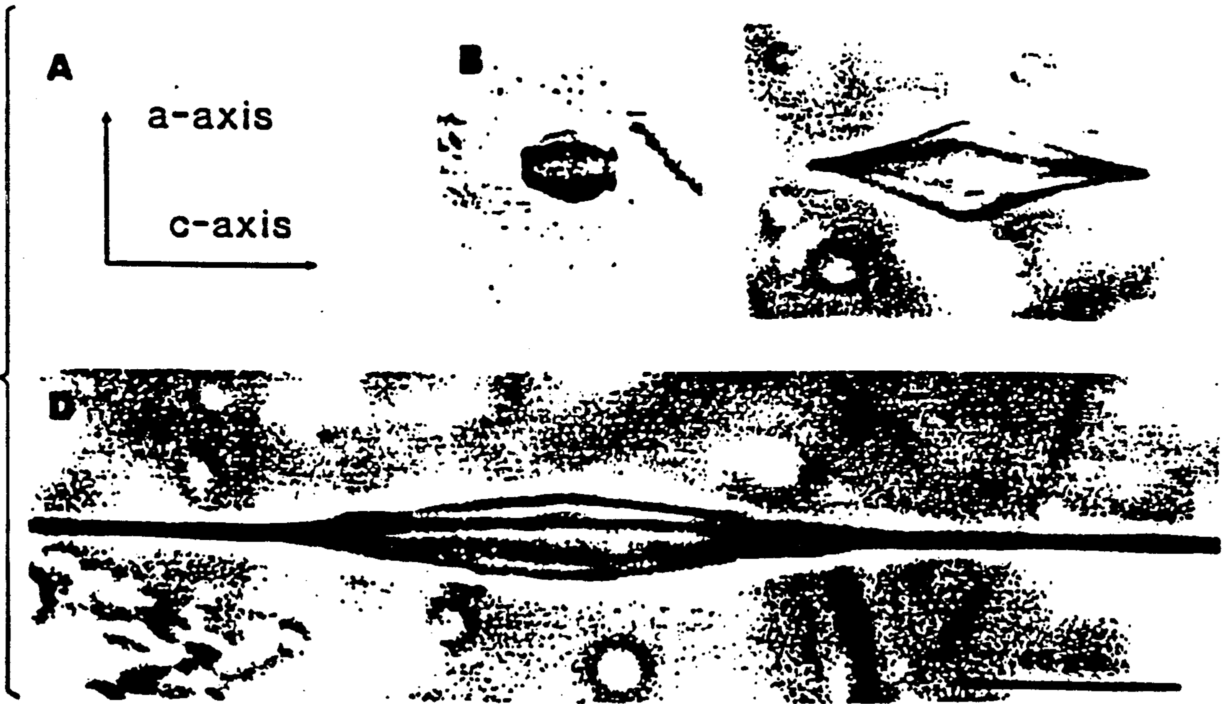


FIG.8.

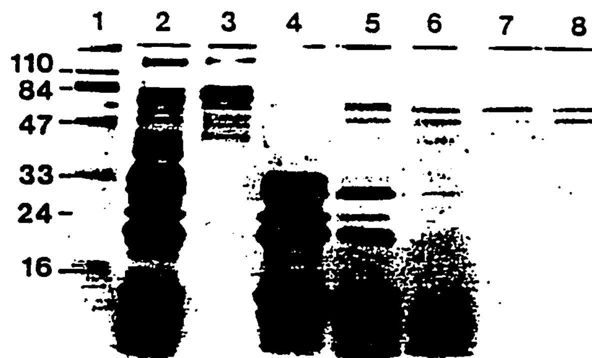


FIG 9.

SUBSTITUTE SHEET (rule 26)

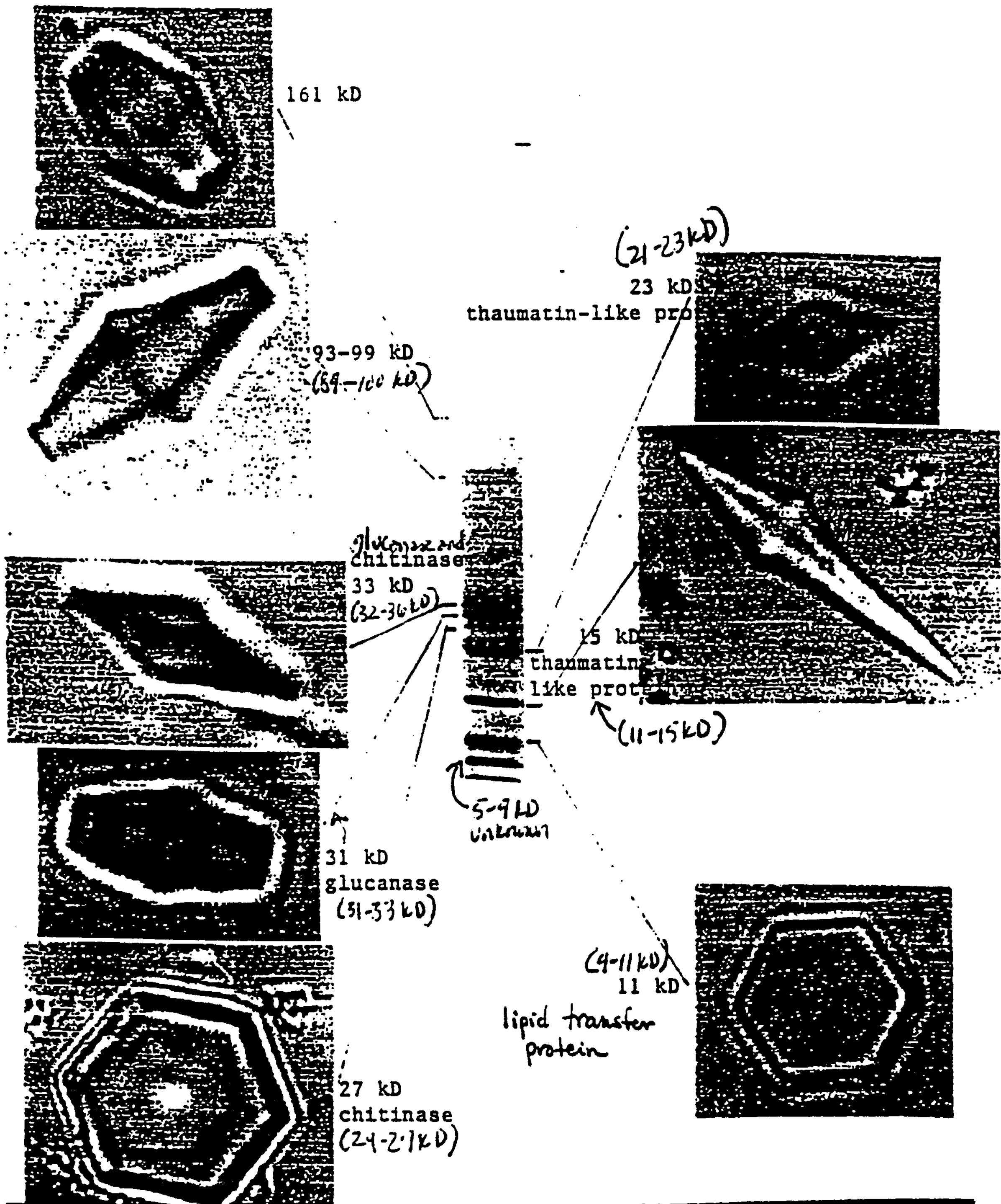


Figure 10

Immunodetection of chitinases in cold-acclimated winter rye

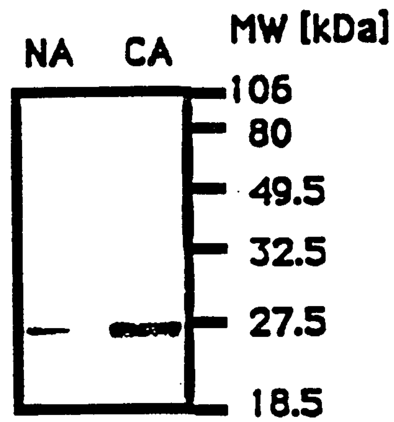


Figure 11

Immunodetection of chitinases induced in cold-acclimated winter rye at different ages

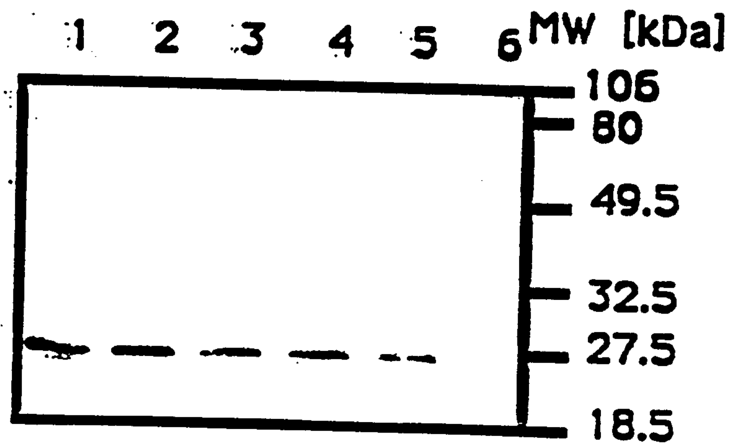
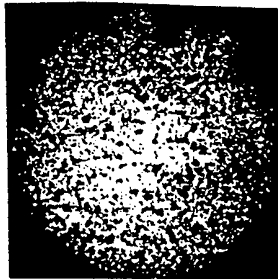


Figure 12

SUBSTITUTE SHEET (rule 26)

INHIBITION OF ICE RECRYSTALLIZATION

PURE WATER



APOPLASTIC EXTRACT FROM WINTER RYE LEAVES

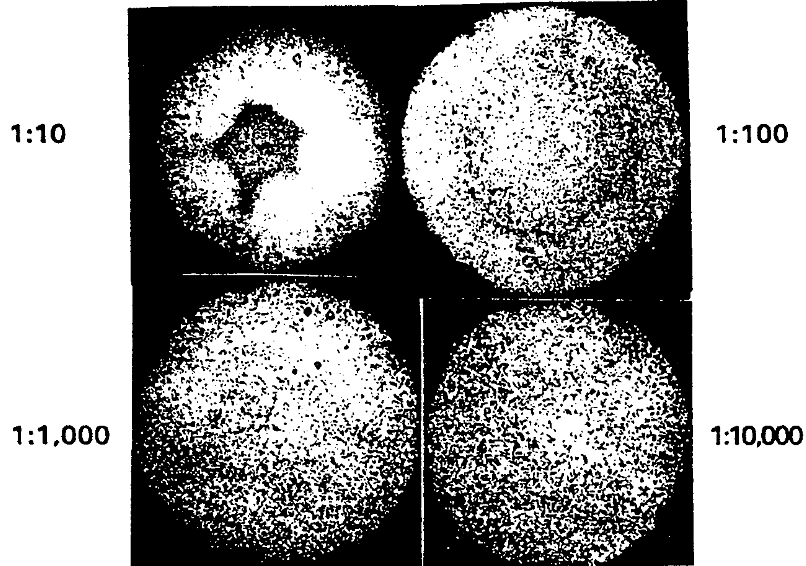


Figure 13

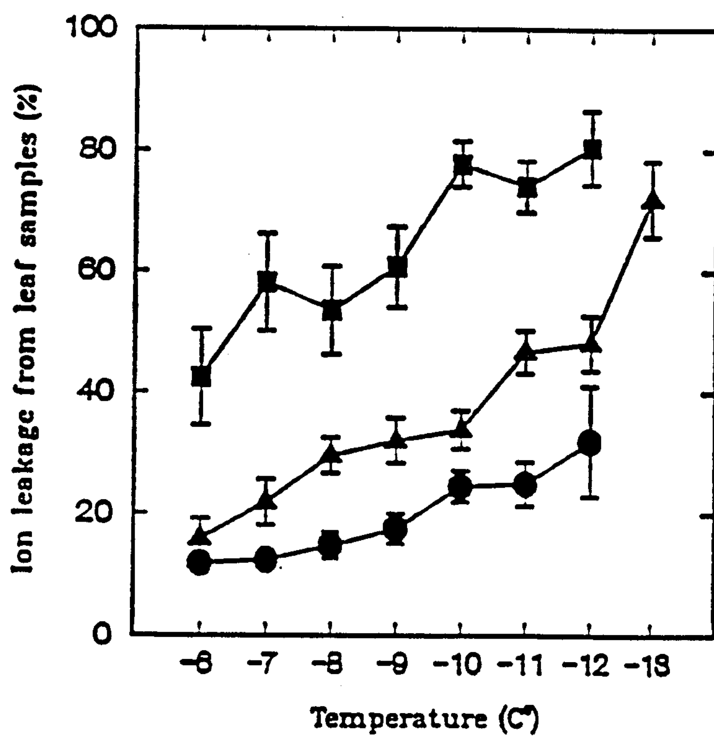


FIGURE 14

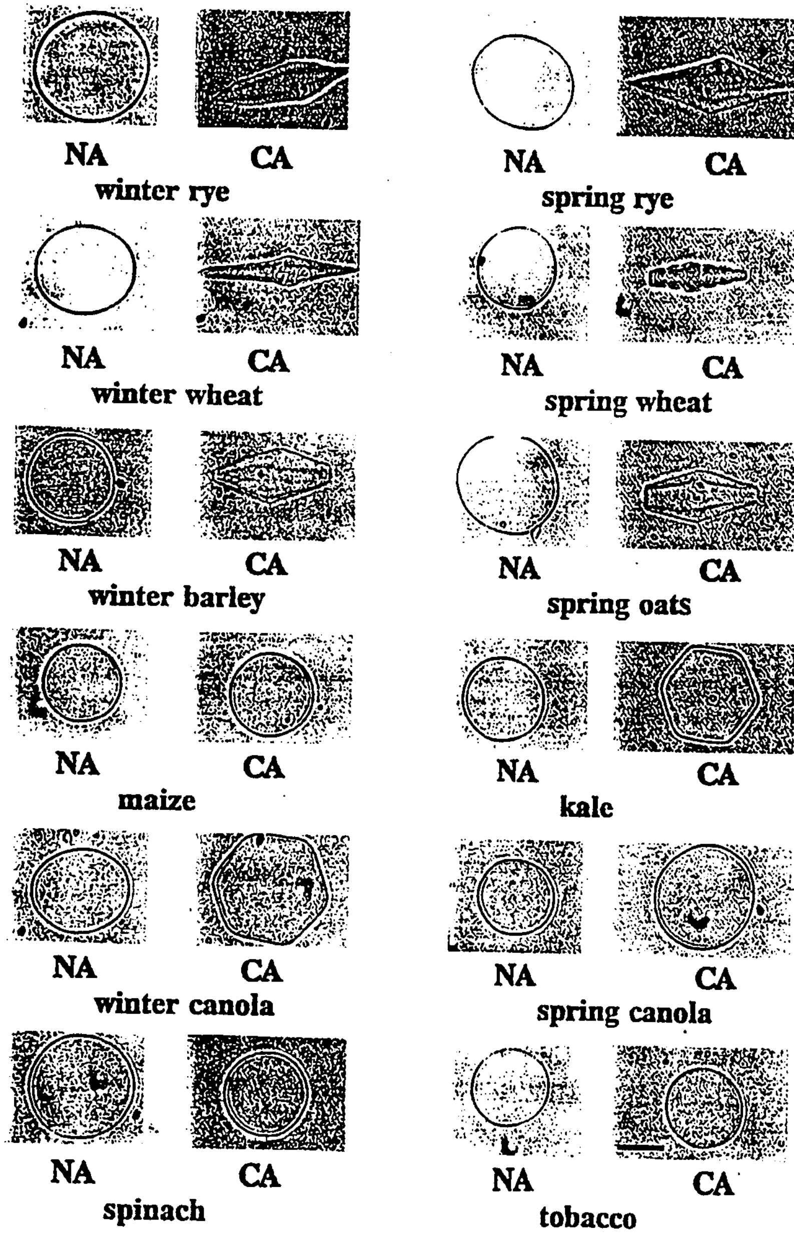


Figure 15

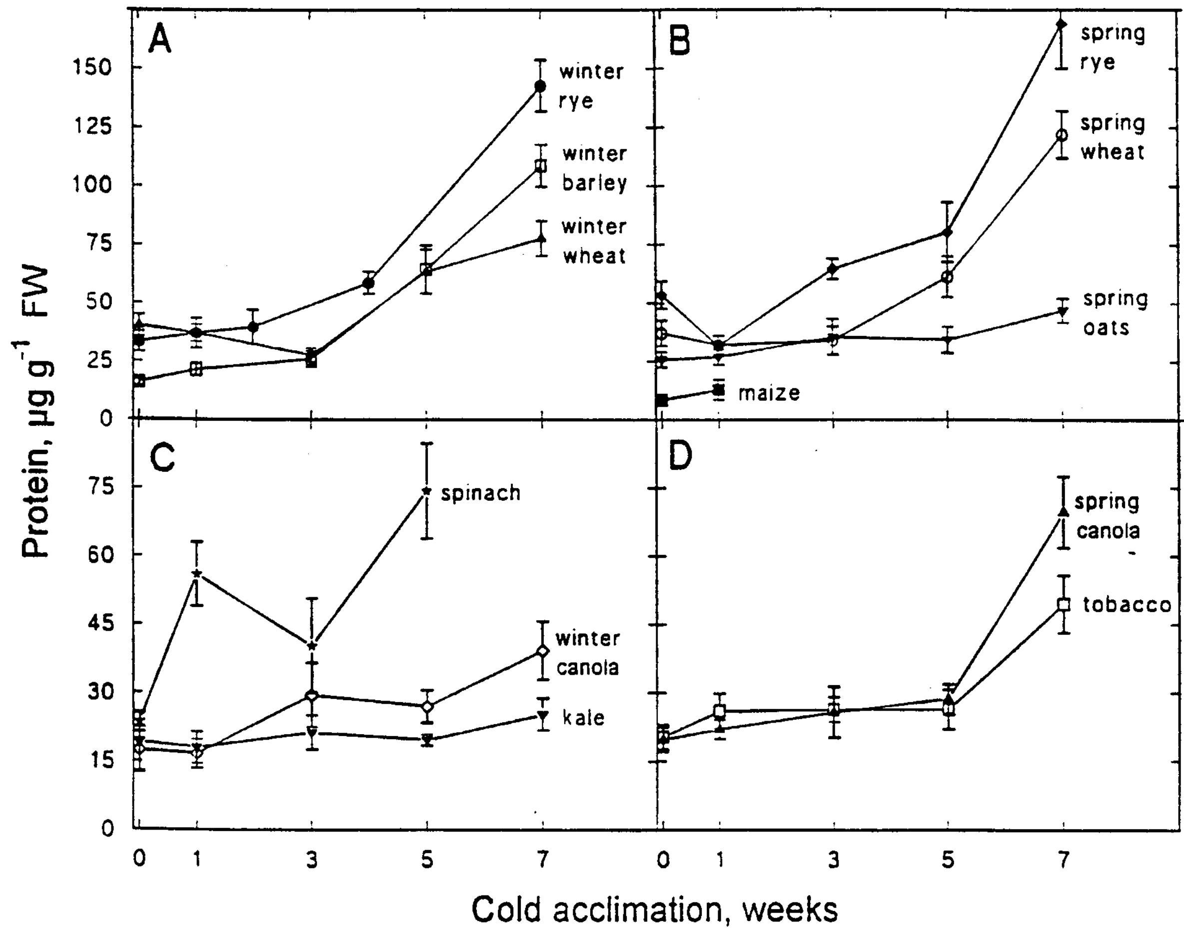


Figure 16

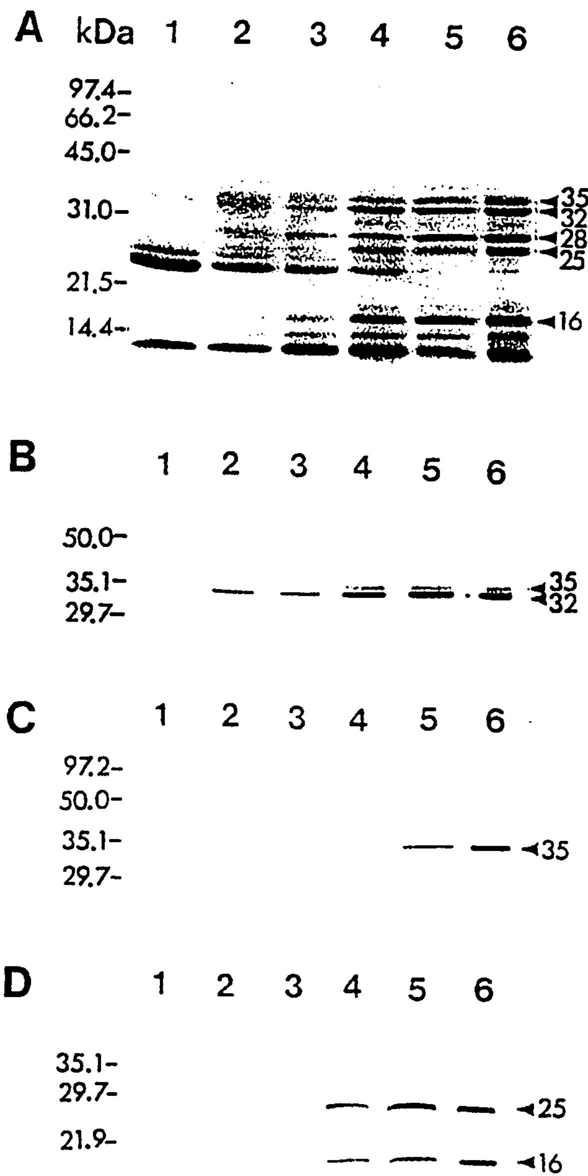


Figure 17

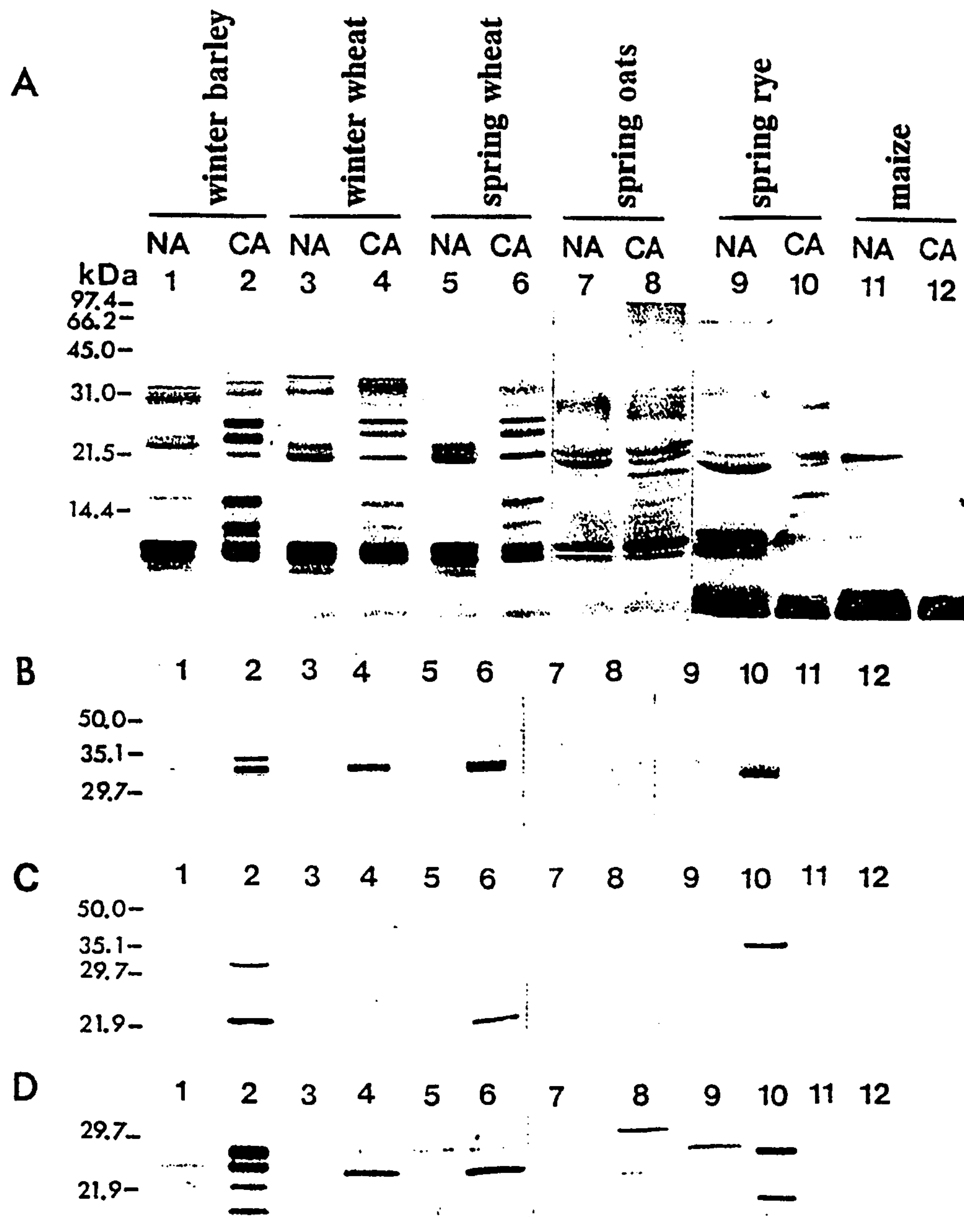


Figure 18

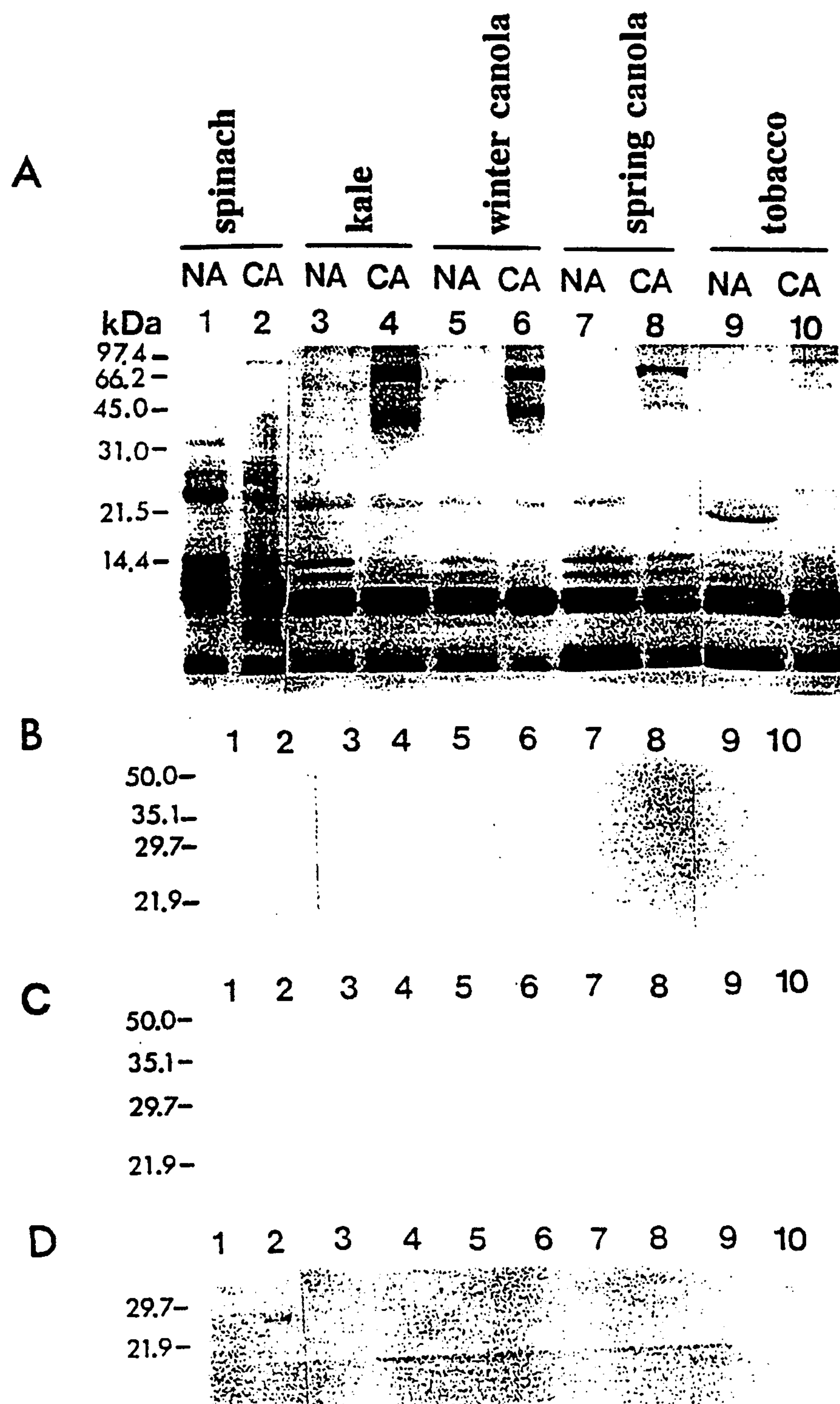


Figure 19

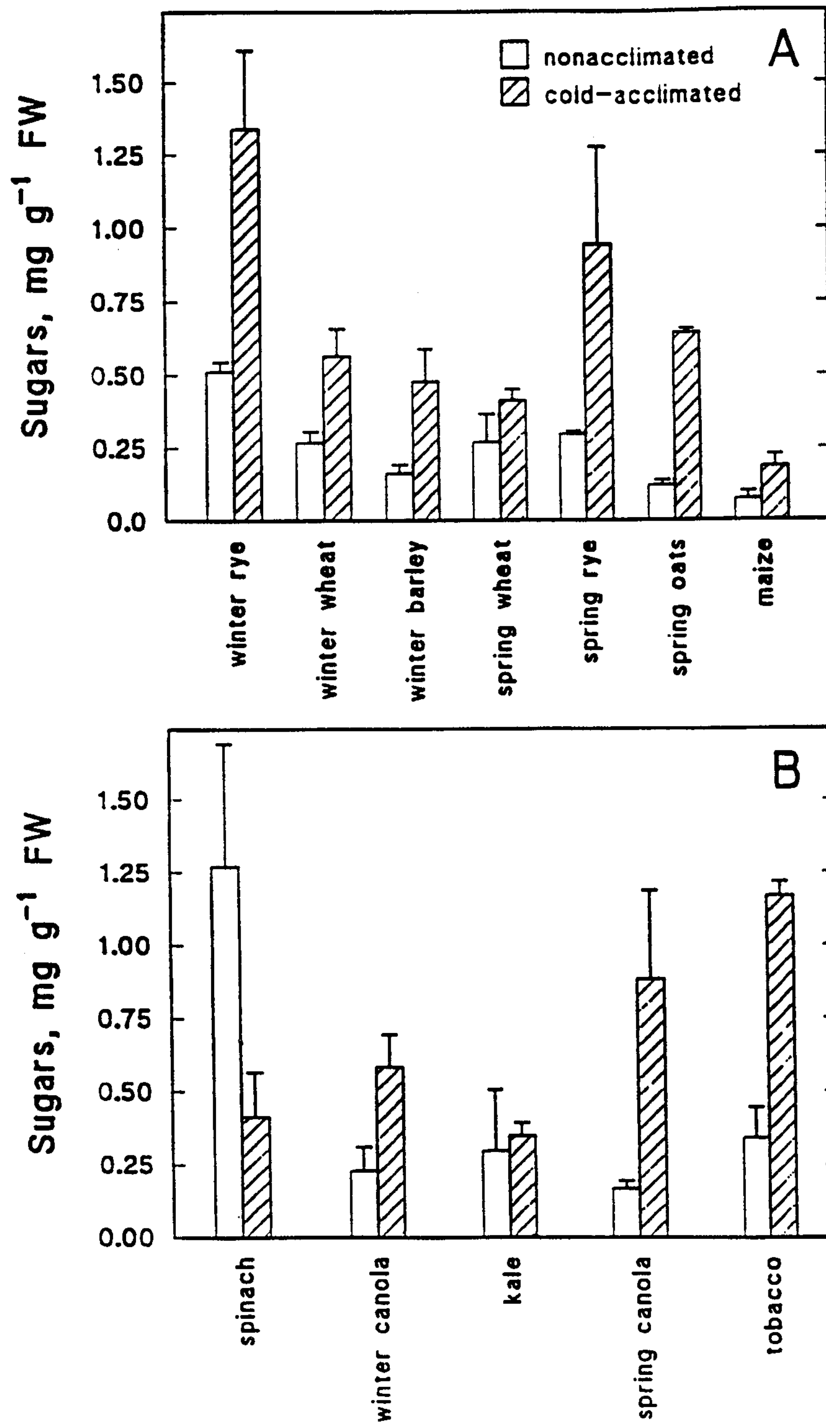


Figure 20

```
ch-9 preprotein ->   1-phase Translation

DNA sequence    954 b.p.   ATGAGAGGAGTT ... AGGCCGTTCGCA  linear

ch-9 full length sequence with 446=c, 876=g, 897=g, 917=t, 952=c, 953=t, 1037=c

1/1                                     31/11
ATG AGA GGA GTT GTG GTG GTG GCC ATG CTG GCC GCG GCC TTC GCC GTG TCT GCA CAC GCC
Met arg gly val val val val ala met leu ala ala ala phe ala val ser ala his ala
61/21                                   91/31
GAG CAG TGC GGC TCG CAG GCC GGC GGG GCG ACG TGC CCC AAC TGC CTC TGC TGC AGC AAG
glu gln cys gly ser gln ala gly gly ala thr cys pro asn cys leu cys cys ser lys
121/41                                  151/51
TTC GGC TTC TGC GGC TCC ACC TCC GAG TAC TGC GGC GAC GGC TGC CAG AGC CAG TGC AAC
phe gly phe cys gly ser thr ser glu tyr cys gly asp gly cys gln ser gln cys asn
181/61                                  211/71
CGC TGC GGC GGC ACA CCA GTA CCG GTA CCG ACC CCC ACC GGC GGC GGC GTG TCC TCC ATT
arg cys gly gly thr pro val pro val pro thr pro thr gly gly gly val ser ser ile
241/81                                  271/91
ATC TCG CAG TCG CTC TTC GAC CAG ATG CTG CTG CAC CGC AAC GAT GCG GCG TGC CTG GCC
ile ser gln ser leu phe asp gln met leu leu his arg asn asp ala ala cys leu ala
301/101                                 331/111
AAG GGG TTC TAC AAC TAC GGC GCC TTC ATC GCC GCC GCC AAC TCG TTC TCG GGC TTC GCG
lys gly phe tyr asn tyr gly ala phe ile ala ala ala asn ser phe ser gly phe ala
361/121                                 391/131
ACC ACG GGT GGC ACC GAC GTC AGG AAG CGC GAG GTG GCC GCG TTC CTA GCT CAG ACC TCC
thr thr gly gly thr asp val arg lys arg glu val ala ala phe leu ala gln thr ser
421/141                                 451/151
CAC GAG ACC ACC GGC GGG TGG CCC ACG GCG CCC GAC GGC CCC TAC TCG TGG GGC TAC TGC
his glu thr thr gly gly trp pro thr ala pro asp gly pro tyr ser trp gly tyr cys
481/161                                 511/171
TTC AAC CAG GAG CGC GGC GCC CCC TCC GAC TAC TGC TCG CCG AGC TCG CAG TGG CCG TGC
phe asn gln glu arg gly ala pro ser asp tyr cys ser pro ser ser gln trp pro cys
541/181                                 571/191
GCG CCG GGC AAG AAG TAC TTC GGG CGC GGG CCC ATC CAG ATC TCA TAC AAC TAC AAC TAC
ala pro gly lys lys tyr phe gly arg gly pro ile gln ile ser tyr asn tyr asn tyr
```

Figure 21a(to be continued)

```
601/201                                 631/211
GGG CCG GCG GGG CGG GCC ATC GGC ACG GAC CTA CTC AAC AAC CCA GAC CTC GTG GCC ACG
gly pro ala gly arg ala ile gly thr asp leu leu asn asn pro asp leu val ala thr
661/221                                 691/231
GAC GCC ACC GTG TCA TTT AAG ACG GCA CTG TGG TTC TGG ATG ACG CCG CAG TCA CCA AAA
asp ala thr val ser phe lys thr ala leu trp phe trp met thr pro gln ser pro lys
721/241                                 751/251
CCT TCG AGC CAC GAC GTG ATC ACG GGC CGG TGG AGC CCC TCG GGC GCC GAC CAG GCA GCG
pro ser ser his asp val ile thr gly arg trp ser pro ser gly ala asp gln ala ala
781/261                                 811/271
GGT AGG GTG CCT GGG TAC GGC GTG ATC ACC AAC ATC ATC AAC GGT GGG CTC GAG TGC GGG
gly arg val pro gly tyr gly val ile thr asn ile ile asn gly gly leu glu cys gly
841/281                                 871/291
CGC GGG CAG GAT GCT CGT GTC GCC GAC CGA ATC GGG TTC TAC AAG CGC TAC TGT GAC CTC
arg gly gln asp ala arg val ala asp arg ile gly phe tyr lys arg tyr cys asp leu
901/301                                 931/311
CTC GGC GTC AGC TAC GGC GAC AAT CTG GAC TGC TAC AAC CAG AGG CCG TTC GCA
leu gly val ser tyr gly asp asn leu asp cys tyr asn gln arg pro phe ala
```

Figure 21a continued

```
ch-9B.aa   ->    1-phase Translation

DNA sequence    894 b.p. GAGCAGTGCGGC ... AGGCCGTTCGCA    linear

this is the sequence lacking the predicted signal peptide coding region:
ch-9 full length sequence with 446=c, 876=g, 897=g, 917=t, 952=c, 953=t, 1037=c

1/1                                     31/11
GAG CAG TGC GGC TCG CAG GCC GGC GGG GCG ACG TGC CCC AAC TGC CTC TGC TGC AGC AAG
glu gln cys gly ser gln ala gly gly ala thr cys pro asn cys leu cys cys ser lys
61/21                                   91/31
TTC GGC TTC TGC GGC TCC ACC TCC GAG TAC TGC GGC GAC GGC TGC CAG AGC CAG TGC AAC
phe gly phe cys gly ser thr ser glu tyr cys gly asp gly cys gln ser gln cys asn
121/41                                  151/51
CGC TGC GGC GGC ACA CCA GTA CCG GTA CCG ACC CCC ACC GGC GGC GGC GTG TCC TCC ATT
arg cys gly gly thr pro val pro val pro thr pro thr gly gly gly val ser ser ile
181/61                                  211/71
ATC TCG CAG TCG CTC TTC GAC CAG ATG CTG CTG CAC CGC AAC GAT GCG GCG TGC CTG GCC
ile ser gln ser leu phe asp gln met leu leu his arg asn asp ala ala cys leu ala
241/81                                  271/91
AAG GGG TTC TAC AAC TAC GGC GCC TTC ATC GCC GCC GCC AAC TCG TTC TCG GGC TTC GCG
lys gly phe tyr asn tyr gly ala phe ile ala ala ala asn ser phe ser gly phe ala
301/101                                 331/111
ACC ACG GGT GGC ACC GAC GTC AGG AAG CGC GAG GTG GCC GCG TTC CTA GCT CAG ACC TCC
thr thr gly gly thr asp val arg lys arg glu val ala ala phe leu ala gln thr ser
361/121                                 391/131
CAC GAG ACC ACC GGC GGG TGG CCC ACG GCG CCC GAC GGC CCC TAC TCG TGG GGC TAC TGC
his glu thr thr gly gly trp pro thr ala pro asp gly pro tyr ser trp gly tyr cys
421/141                                 451/151
TTC AAC CAG GAG CGC GGC GCC CCC TCC GAC TAC TGC TCG CCG AGC TCG CAG TGG CCG TGC
phe asn gln glu arg gly ala pro ser asp tyr cys ser pro ser ser gln trp pro cys
481/161                                 511/171
GCG CCG GGC AAG AAG TAC TTC GGG CGC GGG CCC ATC CAG ATC TCA TAC AAC TAC AAC TAC
ala pro gly lys lys tyr phe gly arg gly pro ile gln ile ser tyr asn tyr asn tyr
541/181                                 571/191
GGG CCG GCG GGG CGG GCC ATC GGC ACG GAC CTA CTC AAC AAC CCA GAC CTC GTG GCC ACG
gly pro ala gly arg ala ile gly thr asp leu leu asn asn pro asp leu val ala thr
```

Figure 21b (to be continued)

```
601/201                                 631/211
GAC GCC ACC GTG TCA TTT AAG ACG GCA CTG TGG TTC TGG ATG ACG CCG CAG TCA CCA AAA
asp ala thr val ser phe lys thr ala leu trp phe trp met thr pro gln ser pro lys
661/221                                 691/231
CCT TCG AGC CAC GAC GTG ATC ACG GGC CGG TGG AGC CCC TCG GGC GCC GAC CAG GCA GCG
pro ser ser his asp val ile thr gly arg trp ser pro ser gly ala asp gln ala ala
721/241                                 751/251
GGT AGG GTG CCT GGG TAC GGC GTG ATC ACC AAC ATC ATC AAC GGT GGG CTC GAG TGC GGG
gly arg val pro gly tyr gly val ile thr asn ile ile asn gly gly leu glu cys gly
781/261                                 811/271
CGC GGG CAG GAT GCT CGT GTC GCC GAC CGA ATC GGG TTC TAC AAG CGC TAC TGT GAC CTC
arg gly gln asp ala arg val ala asp arg ile gly phe tyr lys arg tyr cys asp leu
841/281                                 871/291
CTC GGC GTC AGC TAC GGC GAC AAT CTG GAC TGC TAC AAC CAG AGG CCG TTC GCA
leu gly val ser tyr gly asp asn leu asp cys tyr asn gln arg pro phe ala
```

Figure 21b continued

SUBSTITUTE SHEET (rule 26)

ch-9 including a presequence of 20 aa that is removed in the plant cell (the final purified protein that has chitinase activity and antifreeze activity is lacking this presequence). total number of amino acids in this sequence =318.

MRGVVVVAMLAAAFAVSAHAEQCGSQAGGATCPNCLCCSKFGFCGSTSEYCGDGCQSQCNRCGGTPVPVPTPTGGGVSSI
ISQSLFDQMLLHRNDAACLAKGFYNYGAFIAAANSFSGFATTGGTDVRKREVAAFLAQTSHETTGGWPTAPDGPYSWGYC
FNQERGAPSDYCSPSSQWPCAPGKKYFGRGPIQISYNYNYGPAGRAIGTDLLNNPDLVATDATVSFKTALWFWMTPQSPK
PSSHDVITGRWSPSGADQAAGRVPGYGVITNIINGGLECGRGQDARVADRIGFYKRYCDLLGVSYGDNLDCYNQRPFA

Figure 21c

ch-9 predicted amino acid sequence lacking a presumptive signal sequence of 20 amino acids. Total amino acids in this sequence: 298.

EQCGSQAGGATCPNCLCCSKFGFCGSTSEYCGDGCQSQCNRCGGTPVPVPTPTGGGVSSIISQSLFDQMLLHRNDAACLA
KGFYNYGAFIAAANSFSGFATTGGTDVRKREVAAFLAQTSHETTGGWPTAPDGPYSWGYCFNQERGAPSDYCSPSSQWPC
APGKKYFGRGPIQISYNYNYGPAGRAIGTDLLNNPDLVATDATVSFKTALWFWMTPQSPKPSSHDVITGRWSPSGADQAA
GRVPGYGVITNIINGGLECGRGQDARVADRIGFYKRYCDLLGVSYGDNLDCYNQRPFA

Figure 21d

```
ch-46B.aa ->    1-phage Translation

DNA sequence    690 b.p.   AGCGTGGGCTCC ... AACTTCGCTAGC       linear

     have removed untranslated sequences and the putative presequence

1/1                                     31/11
AGC GTG GGC TCC GTC ATC ACG CAG TCC ATG TAC GCG AGC ATG CTG CCC AAC CGC GAC AAC
ser val gly ser val ile thr gln ser met tyr ala ser met leu pro asn arg asp asn
61/21                                   91/31
TCG CTG TGC CCG GCC AGG GGG TTC TAC ACG TAC GAC GCC TTC ATC GCC GCC GCC AAC ACC
ser leu cys pro ala arg gly phe tyr thr tyr asp ala phe ile ala ala ala asn thr
121/41                                  151/51
TTC CCG GGC TTC GGC ACC ACC GGC AGC ACG GAC GAC GTC AAG CGC GAG GTC GCC GCC TTC
phe pro gly phe gly thr thr gly ser thr asp asp val lys arg glu val ala ala phe
181/61                                  211/71
TTC GGC CAG ACC TCA CAC GAG ACC ACC GGA GGG ACT AGA GGC GCC GCC GAC CAG TTC CAG
phe gly gln thr ser his glu thr thr gly gly thr arg gly ala ala asp gln phe gln
241/81                                  271/91
TGG GGC TAC TGC TTC AAG GAG GAG ATA AAC AAG GCC ACG TCT CCA CCC TAC TAC GGA CGG
trp gly tyr cys phe lys glu glu ile asn lys ala thr ser pro pro tyr tyr gly arg
301/101                                 331/111
GGA CCC ATC CAA TTG ACA GGG CGG TCC AAC TAC GAT CTC GCC GGG AGA GCC ATC GGG AAG
gly pro ile gln leu thr gly arg ser asn tyr asp leu ala gly arg ala ile gly lys
361/121                                 391/131
GAC CTG GTG AGC AAC CCG GAC CTG GTG TCC ACG GAC GCG GTG GTT TCC TTC AGG ACG GCC
asp leu val ser asn pro asp leu val ser thr asp ala val val ser phe arg thr ala
421/141                                 451/151
ATG TGG TTC TGG ATG ACG GCG CAG GGC AAC AAG CCA TCG TCC CAC GAC GTC GCC CTC CGC
met trp phe trp met thr ala gln gly asn lys pro ser ser his asp val ala leu arg
481/161                                 511/171
CGC TGG ACG CCG ACG GCT GCC GAT AAC GCT GCG GGT CGG GTC CCT GGG TAC GGC GTA ATC
arg trp thr pro thr ala ala asp asn ala ala gly arg val pro gly tyr gly val ile
541/181                                 571/191
ACC AAT ATC ATC AAC GGC GGG CTC GAG TGC GGC ATG GGC CGG AAC GAC GCC AAC GTC GAC
thr asn ile ile asn gly gly leu glu cys gly met gly arg asn asp ala asn val asp
```

Figure 22a (to be continued)

SUBSTITUTE SHEET (rule 26)

```
601/201                                         631/211
CGC ATC GGC TAC TAC ACA CGC TAC TGC GGC ATG CTT GGC ACG GCC ACC GGG GGC AAC CTC
arg ile gly tyr tyr thr arg tyr cys gly met leu gly thr ala thr gly gly asn leu
661/221
GAC TGC TAC ACC CAG CGA AAC TTC GCT AGC
asp cys tyr thr gln arg asn phe ala ser
```

Figure 22a continued

SUBSTITUTE SHEET (rule 26)

```
ch-46 sequence.final ->   1-phase Translation

DNA sequence   756 b.p.   ATGGCGCGGTTT ... AACTTCGCTAGC      linear

     have removed untranslated sequences
1/1                                     31/11
ATG GCG CGG TTT GCT GCG CTC GCC GCG CTT CTT CTC GCC GTG GCG GTG GGC GGC GCC GCG
Met ala arg phe ala ala leu ala ala leu leu leu ala val ala val gly gly ala ala
61/21                                   91/31
GCG CAG AGC GTG GGC TCC GTC ATC ACG CAG TCC ATG TAC GCG AGC ATG CTG CCC AAC CGC
ala gln ser val gly ser val ile thr gln ser met tyr ala ser met leu pro asn arg
121/41                                  151/51
GAC AAC TCG CTG TGC CCG GCC AGG GGG TTC TAC ACG TAC GAC GCC TTC ATC GCC GCC GCC
asp asn ser leu cys pro ala arg gly phe tyr thr tyr asp ala phe ile ala ala ala
181/61                                  211/71
AAC ACC TTC CCG GGC TTC GGC ACC ACC GGC AGC ACG GAC GAC GTC AAG CGC GAG GTC GCC
asn thr phe pro gly phe gly thr thr gly ser thr asp asp val lys arg glu val ala
241/81                                  271/91
GCC TTC TTC GGC CAG ACC TCA CAC GAG ACC ACC GGA GGG ACT AGA GGC GCC GCC GAC CAG
ala phe phe gly gln thr ser his glu thr thr gly gly thr arg gly ala ala asp gln
301/101                                 331/111
TTC CAG TGG GGC TAC TGC TTC AAG GAG GAG ATA AAC AAG GCC ACG TCT CCA CCC TAC TAC
phe gln trp gly tyr cys phe lys glu glu ile asn lys ala thr ser pro pro tyr tyr
361/121                                 391/131
GGA CGG GGA CCC ATC CAA TTG ACA GGG CGG TCC AAC TAC GAT CTC GCC GGG AGA GCC ATC
gly arg gly pro ile gln leu thr gly arg ser asn tyr asp leu ala gly arg ala ile
421/141                                 451/151
GGG AAG GAC CTG GTG AGC AAC CCG GAC CTG GTG TCC ACG GAC GCG GTG GTT TCC TTC AGG
gly lys asp leu val ser asn pro asp leu val ser thr asp ala val val ser phe arg
481/161                                 511/171
ACG GCC ATG TGG TTC TGG ATG ACG GCG CAG GGC AAC AAG CCA TCG TCC CAC GAC GTC GCC
thr ala met trp phe trp met thr ala gln gly asn lys pro ser ser his asp val ala
541/181                                 571/191
CTC CGC CGC TGG ACG CCG ACG GCT GCC GAT AAC GCT GCG GGT CGG GTC CCT GGG TAC GGC
leu arg arg trp thr pro thr ala ala asp asn ala ala gly arg val pro gly tyr gly
```

Figure 22b (to be continued)

```
601/201                                      631/211
GTA ATC ACC AAT ATC ATC AAC GGC GGG CTC GAG TGC GGC ATG GGC CGG AAC GAC GCC AAC
val ile thr asn ile ile asn gly gly leu glu cys gly met gly arg asn asp ala asn
661/221                                      691/231
GTC GAC CGC ATC GGC TAC TAC ACA CGC TAC TGC GGC ATG CTT GGC ACG GCC ACC GGG GGC
val asp arg ile gly tyr tyr thr arg tyr cys gly met leu gly thr ala thr gly gly
721/241                                      751/251
AAC CTC GAC TGC TAC ACC CAG CGA AAC TTC GCT AGC
asn leu asp cys tyr thr gln arg asn phe ala ser
```

Figure 22b continued

Final aa sequence of clone 46 with the putative signal sequence removed.
Final sequence is 230 amino acids

SVGSVITQSMYASMLPNRDNSLCPARGFYTYDAFIAAANTFPGFGTTGSTDDVKREVAAFFGQTSHETTGGTRGAADQFQ
WGYCFKEEINKATSPPYYGRGPIQLTGRSNYDLAGRAIGKDLVSNPDLVSTDAVVSFRTAMWFWMTAQGNKPSSHDVALR
RWTPTAADNAAGRVPGYGVITNIINGGLECGMGRNDANVDRIGYYTRYCGMLGTATGGNLDCYTQRNFAS

Figure 22c

Final aa sequence: 252 aa (untranslated sequence has been removed)

MARFAALAALLLAVAVGGAAAQSVGSVITQSMYASMLPNRDNSLCPARGFYTYD
AFIAAANTFPGFGTTGSTDDVKREVAAFFGQTSHETTGGTRGAADQFQWGYCFKEEINKATSPPYYGRGPIQLTGRSNYD
LAGRAIGKDLVSNPDLVSTDAVVSFRTAMWFWMTAQGNKPSSHDVALRRWTPTAADNAAGRVPGYGVITNIINGGLECGM
GRNDANVDRIGYYTRYCGMLGTATGGNLDCYTQRNFAS

Figure 22d

ClustalW Multiple Sequence Alignment Results

```
                1             15 16            30 31            45 46            60 61            75 76            90
1 1         --------------- -----EQCGSQAGGA TCPNCLCCSRFGWCG STSDYCGDGCQSQCA GCGGGGTPVTPTPTP SGGGGVSSIVSRALF     70
2 rye       --------------- --------------- --------------- --------------- --------------- ----SVSSIISHAQF     11
3 pCHT-9    MRGVVVVAMLAAAFA VSAHAEQCGSQAGGA TCPNCLCCSKFGFCG STSEYCGDGCQSQCN RCGG--TPV-PVPTP TGG-GVSSIISQSLF     86
4 pCHT-46   --------------- --------------- --------------- -MARFAALAALLLAV AVGG----------- AAAQSVGSVITQSMY     33

                91           105 106           120 121           135 136           150 151           165 166           180
1 1         DRMLLHRNDGACQAK GFYTYDAFVAAAGAF PGFGTTGSTDTRKRE VAAFLAQTSHETTGG WATAPDGAFAWGYCF KQERGATSNYCTPSA    160
2 rye       DRMLLHRNDGACQAK GFYTYDAFVAAANAF PGFGATGSTDARKRD VAAFLAQTSHETTGG WATAPDGAFAWGYCF KQERGAAADYCTPSA    101
3 pCHT-9    DQMLLHRNDAACLAK GFYNYGAFIAAANSF SGFATTGGTDVRKRE VAAFLAQTSHETTGG WPTAPDGPYSWGYCF NQERGAPSDYCSPSS    176
4 pCHT-46   ASMLPNRDNSLCPAR GFYTYDAFIAAANTF PGFGTTGSTDDVKRE VAAFFGQTSHETTGG TRGAAD-QFQWGYCF KEEINKAT---SPP-    118

                181          195 196           210 211           225 226           240 241           255 256           270
1 1         QWPCAPGKSYYGRGP IQLSHNYNYGPAGRA IGVDLLRNPDLVATD PTVSFKTAMWFWMTA QAPKPSSHAVITGQW SPSGTDRAAGRVPGF    250
2 rye       QWPCAPGKRYYGRGP IQLSHNYNYGPAGRA IGVDLLRNPDLVATD PTVSFKTALWFWMTA QAPKPSSHAVITGKW SPSGADRAAGRAPGF    191
3 pCHT-9    QWPCAPGKKYFGRGP IQISYNYNYGPAGRA IGTDLLNNPDLVATD ATVSFKTALWFWMTP QSPKPSSHDVITGRW SPSGADQAAGRVPGY    266
4 pCHT-46   ---------YYGRGP IQLTGRSNYDLAGRA IGKDLVSNPDLVSTD AVVSFRTAMWFWMTA QGNKPSSHDVALRRW TPTAADNAAGRVPGY    199

                271          285 286           300 301           315 316           330
1 1         GVITNIVNGGIECGH GQDSRVADRIGFYKR YCDILGVGYGNNLDC YNQRPFA-        302
2 rye       GVITNIINGGLECGH GQDSRVADRIGFYKR YCDILGVGYGDNLDC YNQRPFA-        243
3 pCHT-9    GVITNIINGGLECGR GQDARVADRIGFYKR YCDLLGVSYGDNLDC YNQRPFA-        318
4 pCHT-46   GVITNIINGGLECGM GRNDANVDRIGYYTR YCGMLGTATGGNLDC YTQRNFAS        252
```

Figure 23 (to be continued)

Alignment Data (Fasta format)

```
>1
--------------------EQCGSQAGGATCPNCLCCSRFGWCGSTSDY
CGDGCQSQCAGCGGGTPVTPTPTPSGGGGVSSIVSRALFDRMLLHRNDG
ACQAKGFYTYDAFVAAAGAFPGFGTTGSTDTRKREVAAFLAQTSHETTGG
WATAPDGAFAWGYCFKQERGATSNYCTPSAQWPCAPGKSYYGRGPIQLSH
NYNYGPAGRAIGVDLLRNPDLVATDPTVSFKTAMWFWMTAQAPKPSSHAV
ITGQWSPSGTDRAAGRVPGFGVITNIVNGGIECGHGQDSRVADRIGFYKR
YCDILGVGYGNNLDCYNQRPFA-
>rye
--------------------------------------------------
-----------------------------SVSSIISHAQFDRMLLHRNDG
ACQAKGFYTYDAFVAAANAFPGFGATGSTDARKRDVAAFLAQTSHETTGG
WATAPDGAFAWGYCFKQERGAAADYCTPSAQWPCAPGKRYYGRGPIQLSH
NYNYGPAGRAIGVDLLRNPDLVATDPTVSFKTALWFWMTAQAPKPSSHAV
ITGKWSPSGADRAAGRAPGFGVITNIINGGLECGHGQDSRVADRIGFYKR
YCDILGVGYGDNLDCYNQRPFA-
>pCHT-9
MRGVVVVAMLAAAFAVSAHAEQCGSQAGGATCPNCLCCSKFGFCGSTSEY
CGDGCQSQCNRCGG--TPV-PVPTPTGG-GVSSIISQSLFDQMLLHRNDA
ACLAKGFYNYGAFIAAANSFSGFATTGGTDVRKREVAAFLAQTSHETTGG
WPTAPDGPYSWGYCFNQERGAPSDYCSPSSQWPCAPGKKYFGRGPIQISY
NYNYGPAGRAIGTDLLNNPDLVATDATVSFKTALWFWMTPQSPKPSSHDV
ITGRWSPSGADQAAGRVPGYGVITNIINGGLECGRGQDARVADRIGFYKR
YCDLLGVSYGDNLDCYNQRPFA-
>pCHT-46
----------------------------------------------MARF
AALAALLLAVAVGG-----------AAAQSVGSVITQSMYASMLPNRDNS
LCPARGFYTYDAFIAAANTFPGFGTTGSTDDVKREVAAFFGQTSHETTGG
TRGAAD-QFQWGYCFKEEINKAT---SPP----------YYGRGPIQLTG
RSNYDLAGRAIGKDLVSNPDLVSTDAVVSFRTAMWFWMTAQGNKPSSHDV
ALRRWTPTAADNAAGRVPGYGVITNIINGGLECGMGRNDANVDRIGYYTR
YCGMLGTATGGNLDCYTQRNFAS
```

Figure 23 continued

SUBSTITUTE SHEET (rule 26)

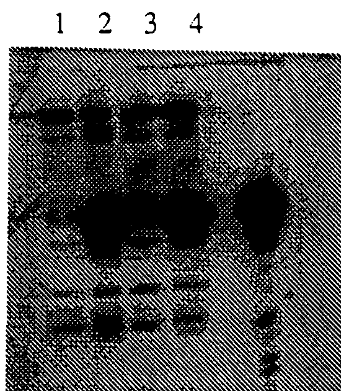


Figure 24

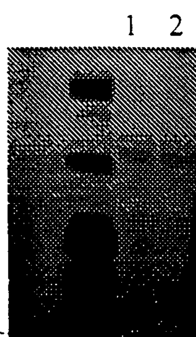


Figure 25a

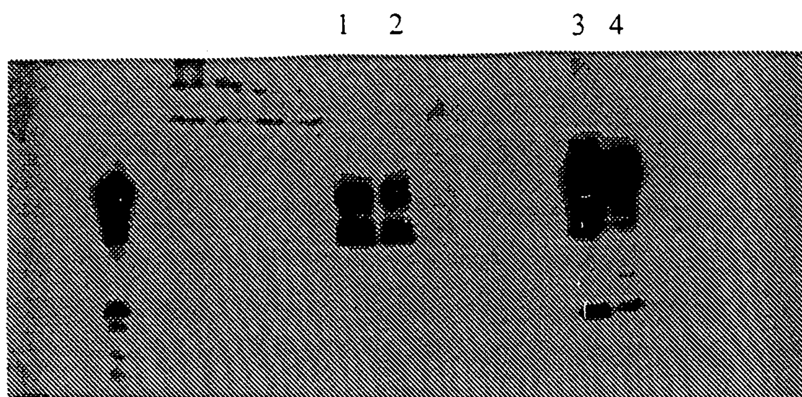


Figure 25b

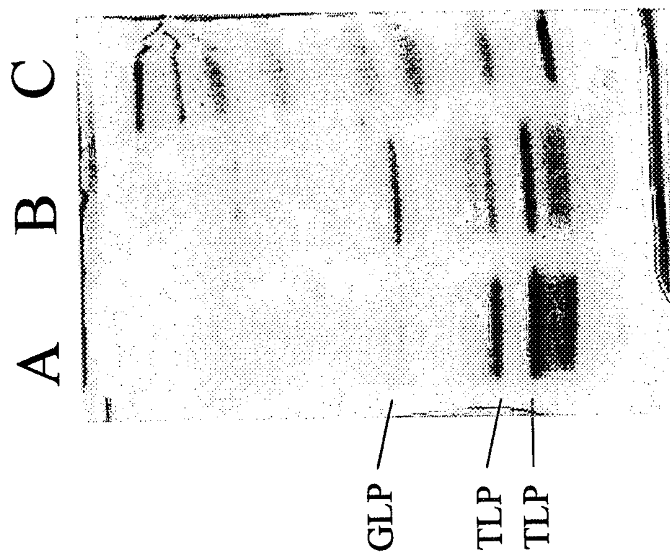


Figure 26