Isolation and characterization of a novel antifreeze protein from carrot (*Daucus carota*)

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A modified assay for inhibition of ice recrystallization which allows unequivocal identification of activity in plant extracts is described. Using this assay a novel, cold-induced, 36 kDa antifreeze protein has been isolated from the tap root of cold-acclimated carrot (*Daucus carota*) plants. This protein inhibits the recrystallization of ice and exhibits thermal-hysteresis activity. The polypeptide behaves as monomer in solution and is N-glycosylated. The corresponding gene is unique in the carrot genome and induced by cold. The antifreeze protein appears to be localized within the apoplast.

Key words: apoplast, cold-on-regulated gene, ice recrystallization inhibition, polygalacturonase inhibitor protein, thermal hysteresis.

INTRODUCTION

A subzero-tolerance mechanism which has evolved repeatedly in organisms adapted to low temperatures is expression of antifreeze proteins (AFPs) (reviewed in [1]). AFPs have two related effects on aqueous solutions, thermal hysteresis (TH) and ice recrystallization inhibition (RI). TH is the non-colligative depression of the freezing temperature of solutions below their melting temperature. Ice recrystallization is the growth of large ice crystals at the expense of smaller ones in partially frozen solutions. AFPs inhibit this process. The mechanism for both TH and RI is thought to involve direct interaction between the protein and specific ice-crystal surfaces [2].

Antifreeze activity is widely distributed among organisms adapted to live at low temperatures, including prokaryotes, fungi, insects, plants and fish [1,3]. Five structurally distinct (glyco)proteins possessing antifreeze activity have been identified in fish [1,4] and other structurally unrelated proteins have been isolated from the mealworm beetle (*Tenebrio molitor*) [5] and spruce budworm (*Choristoneura fumiferana*) [6]. Although it has been observed that some plant gene products that are expressed in response to cold, such as KIN1, possess sequence similarity with previously characterized fish AFPs [7,8], expression studies have not confirmed the antifreeze function [9].

Plant antifreeze activity is cold-induced [10,11] and is located in the extracellular compartment where ice crystallizes in freeze-tolerant species. Apoplastic extracts of cold-acclimated winter rye (*Secale cereale*) have been reported to possess TH activity [12] and this activity has been attributed to cold-induced pathogenesis-related proteins (PRPs) [13]. A glycine-rich glycoprotein has been shown to co-purify with TH activity from bittersweet nightshade (*Solanum dulcamara*) [14]. It has been speculated that RI may be the most relevant activity for the *in planta* function of AFPs and RI may also be important for many industrial applications of AFPs.

Here we describe a modification of the Splat assay for RI [15] which allows unequivocal identification of RI activity in crude homogenates of plant tissues. We have used this assay in the isolation and biochemical characterization of a glycoprotein from cold-acclimated cultivated carrot (*Daucus carota*) which exhibits both RI and TH activity. The gene encoding the antifreeze is unique in the carrot genome and is induced by low temperature.

EXPERIMENTAL

Plant material

Carrot plants (*Daucus carota* cv Autumn King) were sown in pots and cultivated for 12 weeks in the greenhouse prior to transfer to a cold room held at 4 °C or a Conviron growth chamber (set at 6 °C for 8 h in the light, 2 °C for 16 h in the dark) for cold-acclimation. Carrot suspension cultures (kindly given by Dr. Paul Knox, School of Biology, University of Leeds, Leeds, U.K.) were cultured as described previously [16] and cold-acclimated in the same cold room.

Assay for antifreeze activity

In the sucrose-sandwich-splat assay, 2 μl of the solution under investigation in 30 (w/w) sucrose was squashed between 13 mm circular coverslips. The 'sandwich' was dropped into a bath of heptane held at −80 °C in a box of solid CO_2 and transferred into a glass viewing chamber containing heptane maintained at −6 °C by a circulating cooler (Grant). Ice crystals were viewed using a 20× objective on an Optiphot microscope (Nikon) and images captured after 30–60 min incubation at −6 °C using a video camera into an image analysis system

Abbreviations: AFP, antifreeze protein; TH, thermal hysteresis; RI, recrystallization inhibition; PRP, pathogenesis-related protein; Con A, concanavalin ABiP, binding protein; GST, glutathione S-transferase; ICF, Intercellular fluid; CA, cold-acclimated; NA, non-acclimated.

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(LUCIA, Nikon). For semi-quantitative analysis serial 1:2 dilutions of samples were assayed to determine the lowest dilution at which altered ice-crystal size could be unequivocally detected. Recombinant fish AFPIII, produced in *Escherichia coli* [HPLC isoform 12 from ocean pout (*Macrozoarces americanus*) and kindly given by Professor Rod Hubbard, Department of Chemistry, University of York, York, U.K.] was used as positive control. TH values were estimated according to DeVries [17].

Protein analytical procedures

The Bio-Rad protein assay was used for determination of protein content according to the manufacturer's instructions with a standard curve of BSA. SDS/PAGE was carried out according to Laemmli [18] using the Bio-Rad Mini system. Molecular-mass marker kits (Sigma) were used according to the manufacturer's instructions. Silver staining was carried out using the Bio-Rad kit. Western blotting was carried out as described by Towbin [19]. Concanavalin A (Con A) overlay was as described by Faye and Chrispeels [20]. For immunodetection, nitrocellulose blots were blocked for 1 h in PBS (0.137 M NaCl/16 mM KCl/8 mM Na_2HPO_4,$12H_2O$/14.7 mM KH_2PO_4, pH 7.2) containing 3 (w/v) milk powder (Marvel) prior to an overnight incubation in a 1:1000 dilution of crude rabbit antiserum or 1:200 dilution of affinity-purified antiserum or a 1 h incubation in a 1:2000 dilution of monoclonal antibody YZ1/2.23 [21,22] or 1 h incubation in a 1:5000 dilution of binding protein (BiP) antibody [23]. After extensive washing in PBS containing 0.5 M NaCl and 0.05 Tween 20, bound antibody was detected using anti-rabbit or anti-rat immunoglobulin-horseradish peroxidase conjugate (Dako) and the enhanced chemiluminescence (ECL®) detection system (Amersham) or 4-chloro-1-naphthol chromogenic substrate.

Purification of carrot root antifreeze activity

Storage root tissue from carrots which had been cold-acclimated for a minimum of 6 weeks was homogenized in 2 vol. (w/v) ice cold buffer A (20 mM ascorbic acid/10 mM EDTA/50 mM Tris/HCl, pH 7.4) in Waring blender held at 4 °C and filtered through a layer of muslin. The homogenate was centrifuged at 100000 at 4 °C for 30 min and a precipitate prepared from the supernatant between 2.2 and 3.5 M $(NH_4)_2SO_4$. The resuspended precipitate was desalted into buffer B (50 mM Tris/HCl, pH 7.4) using Hitrap desalting columns (Pharmacia) and applied to a 30 ml Q-Sepharose Fast Flow ion-exchange column (Pharmacia) pre-equilibrated in buffer B using a Gradifrac low-pressure-chromatography system (Pharmacia) at 4 °C at a flow rate of 1 ml/min and the eluate monitored at 280 nm by a UV absorbance monitor (Monitor UV1; Pharmacia) recorded on a chart recorder (REC 102; Pharmacia) and fractions collected. When the A_{280} returned to zero a gradient of 0–0.4 M NaCl in buffer B was applied. Antifreeze-active fractions eluted between 0.15 and 0.3 M NaCl were pooled, concentrated and applied to a Superdex 75 gel-permeation column pre-equilibrated in buffer B containing 0.15 M NaCl and calibrated with gel-filtration molecular-mass markers (Sigma) using an FPLC system (Pharmacia). Antifreeze activity eluted coincident with an A_{280} peak with an apparent molecular mass of 40 kDa. Fractions containing antifreeze activity were pooled and exchanged into buffer C (50 mM Hepes, pH 6.8) and applied to a Mono Q column on a SMART chromatography system (Pharmacia) and activity eluted using a 0–0.2 M gradient of NaCl.

For affinity chromatography on Con A, 1.2 ml of 20 μg/ml purified carrot AFP in buffer D (20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$) was applied to a 1 ml Con A–agarose (Vector) column pre-equilibrated in buffer D. An aliquot of the column sample and the pass from the column was collected, exchanged into water and concentrated 10-fold in 10 kDa-cut-off Microcon ultrafiltration devices (Amicon) prior to analysis by SDS/PAGE or sucrose sandwich splat.

Preparation of intercellular-fluid (ICF) extracts

Four plants which had been cold-acclimated for at least six weeks were pooled and then 40 g used for whole homogenate or ICF extractions. ICF extracts were prepared by slicing a carrot root into matchstick-size pieces and washing thoroughly with 3 vol. of buffer A (50 mM Tris/HCl, pH 7.4). The 'matchsticks' were then vacuum-infiltrated at 0.45×10^5 Pa (0.45 bar) for 10 min in ice-cold buffer A. The matchsticks were then dried gently and inserted into 50 ml syringes which had the fine end blocked up with glass wool. The syringes were inserted into 50 ml tubes and centrifuged at 1000 for 5 min. The fluid produced in the bottom of the tube was termed ICF. If necessary the protein was concentrated by precipitation with 5 vol. of ice-cold acetone and the pellet resuspended in 50 mM Tris/HCl, pH 7.4. Homogenate extracts were prepared by homogenization in ice-cold buffer A, followed by filtering through two layers of muslin.

DNA and RNA gel-blot analyses

Carrot genomic DNA was isolated using the 'CTAB' (cetyltrimethylammonium bromide) method described by Draper et al. [24]. DNA (10 μg) was digested with the appropriate restriction enzyme, separated by agarose-gel electrophoresis and transferred to Zetaprobe membrane (Bio-Rad) by capillary blotting. An *Eco*RI/*Bam*HI fragment of the *AFP* coding region [25] was used for random primer labelling using the PRIME-IT II kit (Stratagene). The filter was hybridized and washed at 65 °C (high stringency) or 55 °C (low stringency) using the buffer system of Church and Gilbert [26] described in the Zetaprobe manual (Bio-Rad).

Total RNA was isolated from the leaves, stems or roots of carrot plants that had been cold-acclimated for 3 weeks or grown under non-cold conditions using the method of Verwoerd et al. [27]. RNA (10 μg) was denatured in 1 × TBE buffer (100 mM Tris base/90 mM boric acid/2 mM EDTA, pH 8.0) containing 25 formamide and heated to 65 °C for 5 min before separation by agarose-gel electrophoresis. The RNA was then blotted on to Zetaprobe membrane (Bio-Rad) by capillary transfer. The blot was hybridized with the *AFP* probe at 65 °C using the Church buffer system described above.

Expression of carrot AFP in *E. coli*

Two oligonucleotides were designed to amplify the mature *AFP* coding region from the cDNA and to create a fragment suitable for cloning, in-frame, into the pGEX-3x expression vector (Pharmacia). The N-terminal oligonucleotide, 5′-TTGAATTCGCAAAGATGCAACAACAACGAC-3′, incorporates the restriction enzyme site *Eco*RI. The C-terminal oligonucleotide, 5′-TTGAATTCTAGCATTCTGGCAATGG-3′, incorporates a stop codon and an *Eco*RI restriction enzyme site. The resulting PCR product was cloned as an *Eco*RI fragment into pGEX-3x in frame with the glutathione S-transferase (GST) protein coding region to produce the vector pGEX-3x-AFP. This vector was then used according to manufacturer's instructions to produce a

GST–AFP fusion protein. The fusion protein was sequestered into inclusion bodies which were isolated and used for antibody purification as described below.

Solubilizing and refolding protocol

Approx. 50 μg of inclusion bodies containing the GST–AFP fusion protein were solubilized in 50 μl of urea buffer [9 M urea/50 mM glycine (pH 10.8)/1 mM EDTA/2 mM β-mercaptoethanol]. This was added quickly to 500 μl of refolding buffer [50 mM potassium phosphate (pH 10.7)/100 mM NaCl], the solution mixed and centrifuged at 15000 for 5 min. The supernatant was desalted and the proteins concentrated by using a 10 kDa-cut-off Microcon ultafiltration device (Amicon). SDS/PAGE analysis of the soluble and insoluble fractions produced by the solubilizing and refolding protocol revealed that some of the GST–AFP fusion protein was present in the soluble phase. This was tested for RI activity in the sucrose-sandwich-splat assay.

Development of polyclonal antiserum specific for carrot AFP

For chemical deglycosylation of carrot AFP prior to use as immunogen, the Oxford GlycoSciences GlycoFree™ Kit was used following the manufacturer's instructions. Approx. 0.3 mg of purified, chemically deglycosylated carrot AFP was mixed 1:1 with Freund's complete adjuvant for the first subcutaneous injection into a male 16-week-old rabbit. Following four boosts with the same antigen mixed 1:1 with Freund's incomplete adjuvant at intervals of approx. 8 weeks, the rabbit was bled out and serum was prepared. For affinity purification, the antiserum was incubated with insoluble inclusion-body protein from *E. coli* transformed with recombinant vector and the antibody–inclusion-body complexes pelleted by centrifugation at 10000 for 5 min. The specific antibodies were dissociated using 0.1 M glycine, pH 2.5. Following adjustment of the pH to 7.5 with Tris and addition of BSA to 1 mg/ml, the partially purified antibody was incubated in the presence of non-recombinant vector inclusion bodies and insoluble complexes removed by centrifugation. The supernatant remaining after this step represented affinity purified AFP antibody. Aliquots were stored at -20 °C.

RESULTS

A modified assay for RI

The essential features of Knight's splat assay for ice RI [15] are retained in the modified sucrose-sandwich-splat assay: small ice crystals are created by flash-freezing the solution and their growth is monitored microscopically. The modified assay allows inclusion of high levels of solute which is necessary to avoid boundary inhibition [28] and other non-specific effects on ice-crystal growth produced by solutes and particulate matter endogenous to the plant tissue under investigation. In the standard assay, when high levels of solute were used, samples were difficult to manipulate and opaque to view.

Figure 1 shows the appearance of ice crystals in a control solution containing no antifreeze (panel 1) and in a solution of recombinant fish AFPIII (panel 4) following a 40 min incubation at -6 °C. It illustrates that the ice crystals in the control solution have grown significantly, whereas those in the AFPIII sample have remained small. It is possible to render the assay semi-quantitative by preparing serial dilutions of samples and determining the concentration at which RI activity can no longer be detected.

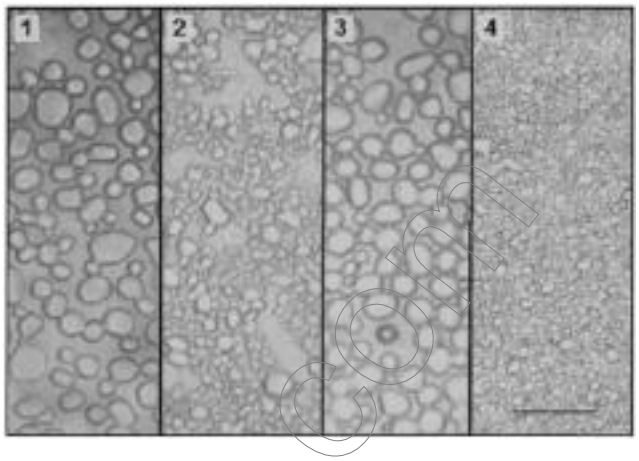

Figure 1 Sucrose-sandwich-splat assay for inhibition of ice recrystallization

Carrot root homogenates were adjusted to 1 mg/ml protein in buffer [50 mM Tris/HCl (pH 7.5)/30% (w/w) sucrose] and assayed by sucrose sandwich splat as described in the Experimental section. Images were captured after a 40 min incubation at -6 °C. Panel 1, control buffer; panel 2, cold-acclimated carrot root homogenate; panel 3, non-acclimated carrot root homogenate; panel 4, positive control recombinant AFPIII (100 μg/ml). The bar represents 50 μm.

Carrot tap root contains low-temperature-inducible antifreeze activity

Antifreeze activity is cold-induced in carrot. Figure 1 illustrates that ice crystals in the non-acclimated root sample are comparable with the control buffer (Figure 1, panel 3), whereas ice crystals in the cold-acclimated root extract are small (Figure 1, panel 2). Serial dilution of cold-acclimated carrot root homogenates showed that RI activity was detectable down to 150 μg/ml protein. The cold-acclimated tap-root homogenate exhibited a TH of 0.34 °C.

Purification of antifreeze activity

RI activity was purified from cold-acclimated carrot root tissue using the sucrose-sandwich-splat assay, illustrated in Figure 1, to follow activity through the purification protocol described in the Experimental section. Figure 2 illustrates that a polypeptide with an apparent molecular mass of 36 kDa on SDS/PAGE co-purified with RI activity. Careful observation of the gel illustrated in Figure 2 reveals that the 36 kDa AFP was resolved into two bands on SDS/PAGE. The 36 kDa polypeptide was eluted from a Superdex 75 gel-permeation column with an apparent molecular mass of 40 kDa, suggesting that the protein is monomeric.

Serial dilution of the purified protein showed that RI activity could still be detected at a protein concentration of < 1 μg/ml. This represents a 150-fold increase in specific activity over the crude cold-acclimated carrot extract. When the purified AFP was adjusted to 1 mg/ml protein, a TH of 0.35 °C was detected, a value similar to that found for the crude homogenate at 1 mg/ml protein.

RI activity was labile to heat (5 min at 95 °C) and exposure to pH < 4.0, but stable to periodate oxidation and exposure to dithiothreitol (10 mM, 1 h at room temperature).

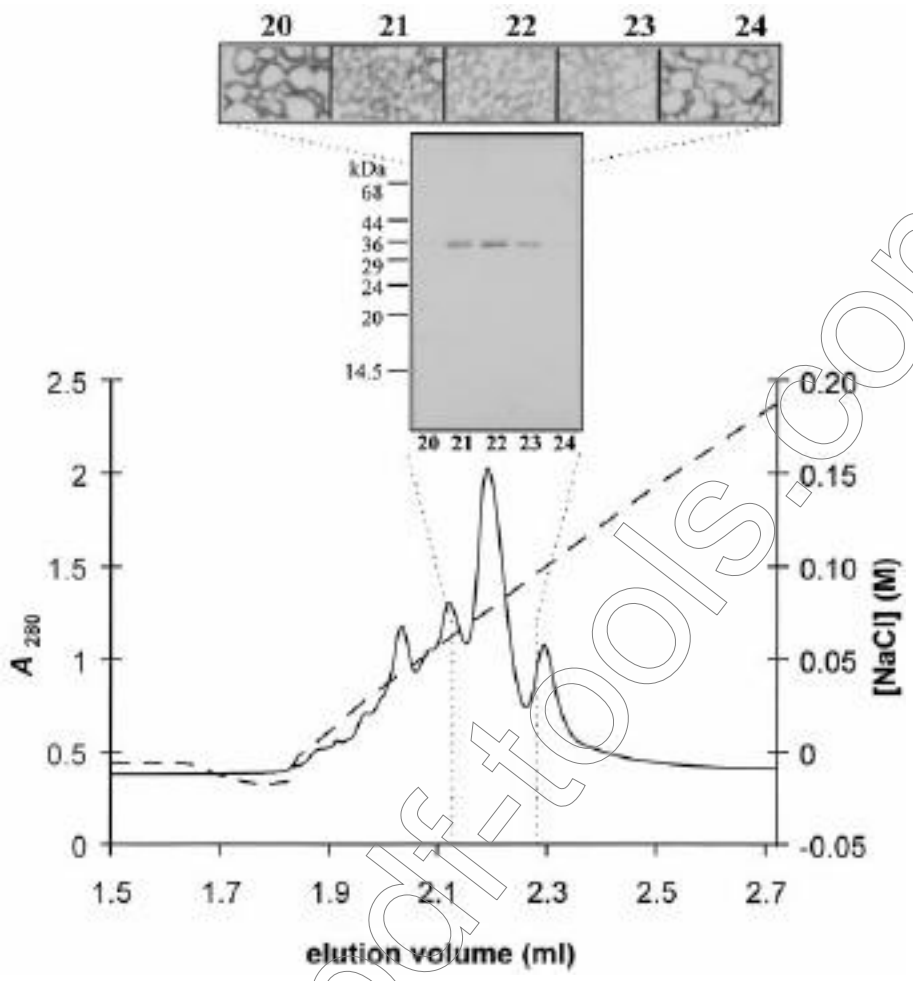

Figure 2 Final anion-exchange step in the purification of carrot AFP

RI activity, illustrated in the top panel, was eluted from a Q-Sepharose anion-exchange matrix in fractions 21–23, coincident with a polypeptide with an apparent molecular mass of 36 kDa on a Coomassie Blue-stained SDS/PAGE separation shown in the middle panel.

Glycosylation of carrot AFP

Western blots of purified AFP were prepared and probed with YZ1/2.23, a monoclonal antibody that binds to complex plant N-glycans containing a core α1,3-fucose residue [21,22], and with the lectin Con A, which binds to α-linked mannose residues typically in oligomannose-type glycans. The Western blot shown in Figure 3, lane 2, shows that YZ1/2.23 bound to the purified AFP. Interestingly, the Con A overlay (Figure 3, lane 3) reacted with a polypeptide with a slightly higher molecular mass than the AFP in the purified sample (compare the sizes of the Coomassie Blue-stained purified AFP and the YZ1/2.23-reactive protein with the Con A-reactive protein). Both YZ1/2.23 (lane 2) and Con A (lane 3) detected contaminants with an apparent molecular mass of approx. 50 kDa in the purified AFP used in this experiment.

The glycosylation status of the AFP was analysed further by application of the purified protein to a Con A column. Antifreeze (RI) activity passed through the matrix unhindered [Con A column pass (p) in Figure 3] as did the YZ1/2.23-reactive polypeptide (Figure 3, lane 4). However, it was clear from analysis of column pass with Con A overlay (Figure 3, lane 5) that Con A-reactive polypeptides had bound to the column. It was therefore concluded that the YZ1/2.23-reactive polypeptide possessed antifreeze activity.

Carrot *AFP* gene is a single copy and induced by low temperature

Using the techiques of PCR and cDNA library screening the cDNA representing the carrot *AFP* has been cloned [25]. The number of *AFP*-related genes in the carrot genome was investigated by DNA-gel-blot analysis. Genomic DNA from carrot was digested with various restriction enzymes and hybridized at high or low stringency using a fragment from the *AFP* cDNA as a probe (Figure 4A). A single hybridizing fragment was observed for each DNA digest under both high- and low-stringency conditions. These results indicate that the *AFP* gene is unique within the carrot genome.

The steady-state levels of the *AFP* message in leaves, stems and roots of carrot plants were investigated by RNA-gel-blot analysis (Figure 4B). Although a low level of *AFP* transcript is visible in the non-acclimated leaf, the transcript is clearly more abundant in cold-acclimated material, and roots contain the greatest amount of message. RI activity was also found in leaf and stem

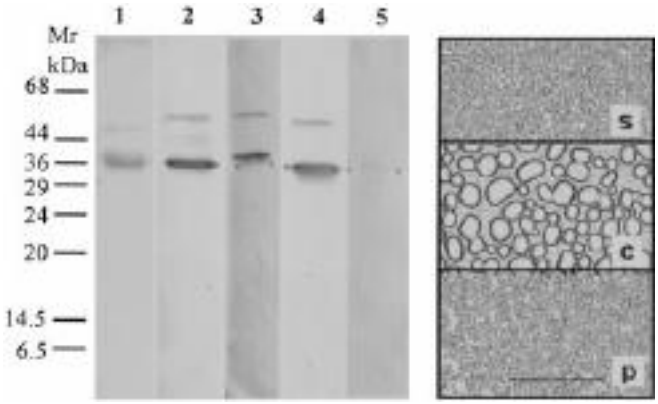

Figure 3 Glycosylation analysis of purified carrot AFP

Purified carrot AFP was separated by SDS/PAGE and either stained in the gel with Coomassie Blue (lane 1) or transferred to nitrocellulose and developed with anti-N-glycan monoclonal antibody YZ1/2.23 (lane 2) or Con A (lane 3). The purified protein was applied to a Con A–agarose column, and material which passed unhindered through this matrix was separated by SDS/PAGE, transferred to nitrocellulose and developed with YZ1/2.23 (lane 4) or Con A (lane 5). The RI activity of the purified protein (s) and the Con A column pass (p) were equivalent. Buffer control (c). The bar represents 100 μm.

tissue of cold-acclimated plants, but at lower levels than that found in the root.

Expression in *E. coli* and antibody purification

As a means of producing protein for antifreeze activity testing and antibody purification, the *AFP* coding region was expressed in *E. coli*. The *AFP* cDNA contained a putative N-terminal signal sequence [25]. Before the cDNA was expressed in *E. coli*, PCR was used to amplify a new fragment of the *AFP* coding region that lacked the signal sequence. The mature carrot *AFP* was expressed in *E. coli* as a fusion to the *GST* gene and the resulting protein was found to be sequestered into insoluble inclusion bodies (Figure 5A). When the GST–AFP fusion protein was solubilized and refolded and tested for antifreeze activity using the sucrose-sandwich-splat method, the protein did not exhibit RI activity. In contrast, when the complete *AFP* cDNA was expressed in tobacco (*Nicotiana tabacum*), both RI and TH activity were detected [25].

In order to develop a polyclonal antiserum specific for proteinaceous epitopes on the carrot AFP, chemically deglycosylated, purified carrot AFP was used as immunogen in rabbit. The resulting antiserum reacted with both deglycosylated and glycosylated forms of the protein (Figure 5B). The antiserum was affinity purified using the recombinant GST–AFP produced in *E. coli*. Using this purified antibody, a 36 kDa polypeptide was detected in a homogenate of cold-acclimated carrot tap root, but not in an equivalent extract from greenhouse-cultivated carrot tap root (Figure 5C).

The purified carrot AFP was often resolved into a doublet of two bands on SDS/PAGE (Figure 2). Figure 6A, lane H, shows that the anti-AFP antibody reacted with both members of the 36 kDa doublet in the homogenate. These data suggest that the two 36 kDa polypeptides are closely related at the protein level, since the antibody was affinity-purified on recombinant protein produced in *E. coli* and should therefore not react with glycan epitopes carried on the AFP.

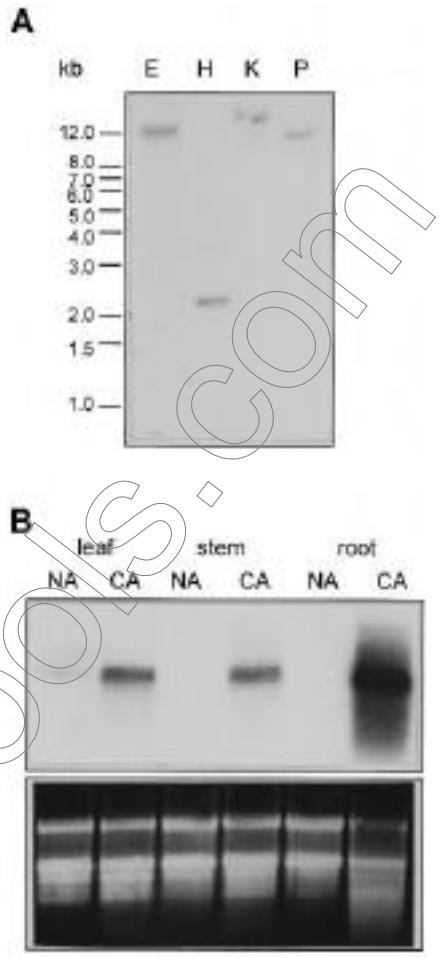

Figure 4 Analysis of *AFP* gene copy number and regulation

(**A**) Carrot genomic DNA gel blot. DNA was digested with *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K) or *Pst*I (P). The blot was hybridized and washed at low stringency (55 °C) using a fragment of the AFP cDNA as a probe. The positions of the molecular-mass markers are indicated at the side of the autoradiograph. (**B**) RNA-gel-blot analysis. Expression of the *AFP* gene in different organs (leaves, stems and tap root) of carrot plants grown in the greenhouse (NA) or for 3 weeks at low temperature (CA) is shown. Top panel, autoradiograph; bottom panel, RNA gel stained with ethidium bromide.

Carrot AFP is located in the apoplast

An investigation was made into whether the AFP was secreted into the intercellular matrix. ICF and homogenate extracts were prepared from the tap roots of cold-acclimated plants and analysed by Western blotting. Figure 6 (top panel) shows that the AFP is significantly enriched in ICF extract compared with the homogenate.

ICF extractions frequently result in some degree of cellular damage. Therefore, as a control to assess cytoplasmic leakage in the ICF preparations, the AFP blot was stripped and reprobed with an antibody raised against binding protein (BiP) [23], an ER resident protein (Figure 6A, lower panel). It is clear that, although some BiP signal is obtained in the ICF, the signal is stronger in the homogenate. This scenario is the opposite to that obtained with the AFP antibody.

Western analysis of carrot suspension-culture cells and their growth medium provided further evidence for the apoplastic

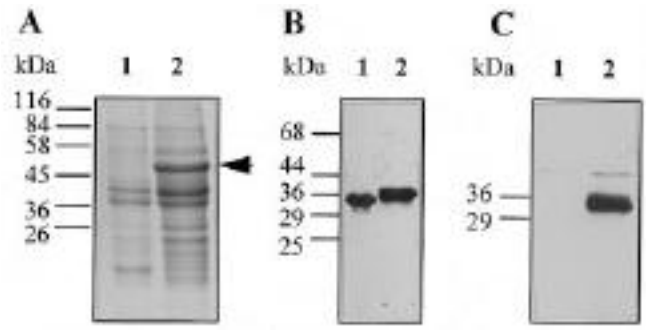

Figure 5 Affinity purification of AFP-specific antiserum

(**A**) Coomassie Blue-stained SDS/PAGE separation of polypeptides present in inclusion bodies from *E. coli* transformed with non-recombinant vector pGEX-3x (1) or recombinant vector pGEX-3x-AFP (2). The GST–AFP fusion protein is identified by an arrow. (**B**) Western blot of purified deglycosylated (1) and control (2) carrot AFP developed with crude polyclonal antiserum raised against deglycosylated carrot AFP. (**C**) Western blot of homogenate of tap root from greenhouse-cultivated (1) and cold-acclimated carrot (2) developed with antiserum affinity-purified on inclusion bodies (10 μg total protein was loaded for each sample).

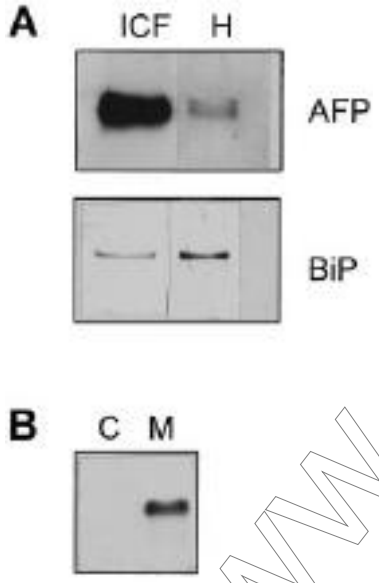

Figure 6 Western analysis of root and cell culture fractions

(**A**) Western blot of extracts prepared from cold-acclimated carrot tap root [homogenate (H) and ICF], as described in the Experimental section, probed with affinity-purified AFP or BiP antibody as indicated. (**B**) Western blot of protein extracted from the washed cells (C) or concentrated medium (M) of cold-acclimated carrot suspension culture probed with affinity-purified AFP antibody. Total protein (1.0 μg) was loaded for each sample.

location of carrot AFP. Figure 6(B) shows that AFP could be detected in the medium of suspension-culture cells which had been exposed to a temperature of 6 °C for 3 weeks but not in a homogenate of washed, cold-acclimated cells.

DISCUSSION

The specific activity of the purified carrot AFP is high compared with fish AFPs. RI activity can be detected at < 1 μg/ml pure carrot AFP compared with > 5 μg/ml recombinant fish AFPIII. Similarly a 1 mg/ml solution of AFPIII produces a TH of 0.23 °C compared with 0.35 °C for 1mg/ml solution of the carrot protein.

Interestingly, the pure carrot protein exhibited a 150-fold enrichment in RI activity over the crude carrot homogenate but almost no enrichment in terms of TH activity. Duman also found no increase in specific activity as measured by TH during isolation of an AFP from *Solanum dulcamara* [14]. These results suggest that cofactors involved with the TH activity of these two AFPs are present in the plant homogenates, but are lost during purification of the activity. There are precedents for co-factors which affect the TH activity of AFPs [29].

The carrot AFP is abundant: 0.5 mg of pure protein can be isolated from a 1 kg of fully acclimated carrot tap roots, representing approx. 10 of the total RI activity in the original homogenate. If there are no enhancing factors in the homogenate, the 150-fold increase in specific RI activity suggests that AFP represents in the order of 0.7 of the total soluble protein. This, in turn, implies a high concentration in the carrot root apoplast.

Carrot *AFP* message was found to be more abundant in the tap root compared with the leaf and stem tissue of cold-treated plants. The carrot is a biennial plant, and preservation of the root tissue over winter is fundamental for survival as the plant has to regrow from the underground root storage organ to flower and set seed in the second year. The high levels of AFP in the roots compared with the leaves and stems may reflect the fact that roots are able to survive the winter, but aerial tissues are lost. The presence of AFP message in non-acclimated leaves suggests that the protein may have a different role in addition to antifreeze activity. The high degree of similarity of the carrot AFP with leucine-rich-repeat proteins and, more specifically, with polygalacturonase inhibitor proteins [25], may indicate that the AFP functions in protein–protein interactions.

When purified carrot AFP was analysed by SDS/PAGE it was resolved into a doublet of two bands representing two polypeptides with apparent molecular mass of approx. 36 kDa (Figure 2). Both members of the 36 kDa doublet reacted with antisera affinity-purified on recombinant carrot AFP (Figure 6A), suggesting that they are closely related to each other. The carrot *AFP* gene contains no introns within the coding region (results not shown) and therefore the doublet is unlikely to represent alternative splice variants. Given that the carrot gene is single-copy (Figure 4), it seems likely that these two closely related proteins represent glycoforms of the same gene product.

Preferential isolation of antifreeze activity from the apoplast of carrot leaves together with detection of antifreeze activity in the growth medium of carrot suspension-culture cells, suggest that the AFP is located in the apoplast *in planta*. Many plants tolerate crystallization of ice within certain regions of their apoplast, and regulation of crystal growth within this space may be important in limiting damage by ice. It is possible that the properties of plant AFPs may be rather different from those of fish AFPs, in which depression of the blood's freezing temperature is their critical property. Because of this, plant AFPs may be more effective than fish AFPs in protecting transgenic plants from frost damage. Similarly the properties of the plant proteins may render them more suitable for industrial uses as cryoprotectants or in modifying ice crystals in frozen food.

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