

A leucine-rich repeat protein of carrot that exhibits antifreeze activity

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Abstract A gene encoding an antifreeze protein (AFP) was isolated from carrot (*Daucus carota*) using sequence information derived from the purified protein. The carrot AFP is highly similar to the polygalacturonase inhibitor protein (PGIP) family of apoplastic plant leucine-rich repeat (LRR) proteins. Expression of the AFP gene is rapidly induced by low temperatures. Furthermore, expression of the AFP gene in transgenic *Arabidopsis thaliana* plants leads to an accumulation of antifreeze activity. Our findings suggest that a new type of plant antifreeze protein has recently evolved from PGIPs.

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Key words: Leucine-rich repeat protein; Polygalacturonase inhibitor protein; Antifreeze protein; Apoplast; *Arabidopsis thaliana*; *Daucus carota*

1. Introduction

Antifreeze protein (AFP) activity has been detected in organisms that are subjected to low temperatures ranging from arctic fish and insects to plants, fungi and bacteria [1–7]. AFPs exhibit different degrees of thermal hysteresis, that is they decrease the temperature at which ice is formed, but do not affect the melting point of water. This effect is the result of AFP adsorption to the non-basal planes of ice at the ice-water interface [8]. Adsorption of the AFP to the surface of the growing ice crystal results in a concentration-dependent effect on ice crystal morphology. In the presence of low AFP concentrations, the pyramidal ice crystal surfaces are expressed and the crystal appears hexagonal in shape. At higher AFP concentrations ice crystal growth along the *a* axis is nearly completely suppressed and growth only occurs along the *c* axis resulting in the generation of hexagonal bi-pyramids or narrow spicules [5,8–10]. Furthermore, very low concentrations of AFPs can still function as inhibitors of ice recrystallisation, i.e. the growth of large ice crystals at the expense of smaller ones. Ice recrystallisation occurs during the thawing process and prolonged incubations at high sub-zero temperatures. Ice-recrystallisation is considered to be a source of frost-induced damage via mechanical disruption of the cell by the growing ice crystals [11].

AFPs exist as a diverse variety of polypeptides with unrelated compositions and structures. There are four classes of

AFPs in arctic fish. Type I AFPs are low molecular weight proteins consisting of alanine-rich amphipathic helices [12]. Type II AFPs are similar to the carbohydrate-recognition domain of C-type lectins, are extensively disulphide bonded and contain two α helices and two β sheet regions [13]. Type III AFPs are small, non-helical, globular proteins with a large hydrophobic core several short β sheets and one helical turn [14,15]. Antifreeze glycoproteins (AFGPs) are polymers of an Ala-Ala-Thr repeat motif that is glycosylated at every threonine hydroxy group with a disaccharide residue that is essential for AFP activity [16]. AFGPs have recently evolved from trypsinogen precursor molecules in two phylogenetically distant arctic fish species [17,18]. An insect AFP was recently described consisting largely of 12 amino acid repeats that are rich in cysteine and threonine [3].

Animal AFPs exhibit significant differences in the levels of thermal hysteresis (TH). This ranges from 1–2°C for fish AFPs to 6°C for the recently identified AFP from *Tenebrio molitor* [3]. This finding suggests that the major function of these proteins is the suppression of the freezing of the extracellular fluids. This is in stark contrast to the attributes of antifreeze activities that recently have been identified in a wide variety of plant species [5,7,19]. Plant AFPs characteristically have low levels of TH (0.2–0.6°C) [5,7]. This is not surprising since freezing tolerant plants differ from arctic fish or cold tolerant insects in that they have developed the ability to tolerate the freezing of water in their extracellular space [20]. Hence it is more likely that plant AFPs function to modify the freezing of water in the apoplastic space, perhaps via the modification of ice crystal shape or the inhibition of ice recrystallisation [21].

In plants AFP activity has been studied in detail in winter rye (*Secale cereale*) and the bitter sweet nightshade (*Solanum dulcamara*). Winter rye accumulates six distinct AFPs during cold acclimation. Based on their N-terminal protein sequence these proteins show similarity to three different classes of pathogenesis-related (PR) proteins [22]. The AFP of *Solanum dulcamara* is an unrelated 67 kDa protein that is glycosylated and possesses an unusually high glycine content [23].

We report here the isolation and characterisation of a gene encoding an AFP from carrot that was recently also cloned in an independent study [24]. The carrot AFP shows significant levels of similarity (50–65%) to the polygalacturonase inhibitor family of plant leucine-rich repeat (LRR) proteins. In this paper we demonstrate that AFP gene expression is virtually absent in non-acclimated plant tissues and is rapidly and dramatically upregulated in response to low temperature exposure. Furthermore, we demonstrate that the expression of the AFP in transgenic *Arabidopsis thaliana* plants results in the accumulation of apoplastic antifreeze activity, illustrating that an active form of the carrot AFP can be expressed in heterologous plants.

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2. Materials and methods

2.1. Plant material

Carrot plants (Newmarket F1, Mr. Forthergill's seeds, Kentford, UK) were germinated and grown for 4 months in soil at ambient temperature under greenhouse conditions. To obtain in vitro-grown carrot seedlings, carrot seeds were sterilised, germinated and grown for 25 days under sterile conditions on standard plant growth media [25], in a controlled environment under a 16 h light/8 h dark photoperiod at $100 \mu\text{E m}^{-2} \text{s}^{-1}$ at 21°C .

Arabidopsis thaliana plants of the ecotypes Columbia (Col) and RLD were cultivated under a 16 h light/8 h dark photoperiod at $100 \mu\text{E m}^{-2} \text{s}^{-1}$ at 22°C , on standard plant growth media or in a soil/perlite mixture.

2.2. Assay of antifreeze activity

The assay is based on the characteristic modification of ice crystal morphology in the presence of antifreeze proteins. The procedure has been adapted from [22] with a number of modifications. One microliter of protein sample was applied to the centre of a temperature-controlled freezing stage (Model THM 600, Linkham Scientific Instruments, Surrey, UK) on a circular glass cover. The freezing stage was fitted onto the stage of a conventional microscope and was connected to a pressured air supply that was cooled by liquid N_2 . The stage temperature was controlled by a programming unit (Model TMS 90 Linkham Scientific Instruments, Surrey, UK). After sample application, the stage was heated to 20°C and then cooled to -40°C at a rate of 99°min^{-1} . The freezing stage was then heated at the same rate to -5°C . The temperature was subsequently increased at 5°min^{-1} to a temperature at which controlled thawing took place. Thawing was allowed to proceed until a single ice crystal was generated. Subsequently the temperature was lowered until slow ice crystal growth could be observed. Under these conditions, high levels of AFP activity were indicated by a multi-faceted or bi-pyramidal and spear-like shape of the ice crystal whereas low levels of AFP activity were indicated by a flat, hexagonal shape of the growing ice crystal. In the absence of AFP activity ice crystals grew as spherical discs.

2.3. Purification and amino acid sequencing of the AFP

The carrot antifreeze protein was purified from apoplastic extracts of cold acclimated carrot tap roots purchased from the local supermarket. The tap roots were cut into cubes, washed in deionised water and vacuum infiltrated for 20 min with the AFP extraction buffer (50 mM Tris-HCl pH 8, 100 mM KCl, 20 mM CaCl_2). The surfaces of the infiltrated cubes were dried and apoplastic extracts were collected by centrifugation at $3500 \times g$ for 30 min at room temperature. Approximately 50 ml of apoplastic extract containing about 2 mg of total protein were extensively dialysed against deionised water at 4°C .

The AFP was purified using preparative isoelectric focussing (IEF) (Rotofor System, Bio-Rad Laboratories, Herts, UK) according to the manufacturer's instructions. The chamber was run for 3.5 h at 15 W using Bio-Lyte ampholytes, pH 4–6 at a final concentration of 0.5%. The IEF provided 2 ml fractions that were directly analysed by SDS-PAGE and assayed for AFP activity. Active fractions were lyophilised, dissolved in 1 M NaCl and ultimately desalted and concentrated using ultrafiltration. Protein fractions were further separated by reverse phase HPLC using a linear acetonitrile, 0.09% TFA gradient at a flow rate of 1.3 ml min^{-1} and a Delta-Pak C18 (300 Å, $5 \mu\text{m}$) $3.9 \times 150 \text{ mm}$ reverse phase column (Waters Ltd., Watford, UK) on a Waters 626 HPLC system. Following HPLC, IEF fractions with high levels of AFP activity were found to contain one predominant polypeptide, which eluted from the column with a retention time of 23.5 min. HPLC fractions corresponding to this peak were pooled, lyophilised and resuspended in tricine sample buffer and separated by SDS-PAGE (10–20% gradient gels). Protein was electroblotted onto polyvinylidene difluoride membrane which was subsequently washed in deionised water and stained with 0.1% Coomassie blue G250 in 50% methanol.

When separated by PAGE, pooled fractions of the HPLC run that eluted between 23 and 24 min produced a single diffuse band with a molecular weight of about 36 kDa. Five prominent bands of this protein and blank PVDF membrane from the same blot were excised, dried and cut into small pieces. The PVDF-bound protein was digested with modified porcine trypsin (Promega, Southampton, UK) according to [26]. Digestion products were extracted from the mem-

brane by sonication with enzyme-free digestion buffer followed by 0.1% TFA. Extracts were pooled and separated on the HPLC system described above using a RP 8 Symmetry Shield column with the dimensions $2.1 \times 150 \text{ mm}$ (100 Å, $5 \mu\text{m}$) at a flow rate of 0.4 ml min^{-1} using an acetonitrile, 0.09% TFA gradient. HPLC fractions containing peptides with the following retention times 25–25.5 min, 42–42.5 min, 46–46.5 min, 48–48.5 min, 52–52.5 min were pooled individually and subjected to sequencing from precycled Biobrene-treated glass fibre discs using the standard gas phase program on a Model 491 Procise protein sequencer (Perkin Elmer-Applied Biosystems, Warrington, UK).

2.4. Isolation of the AFP gene

The amino acid sequences IPPEISA and DIFGNFA derived from two peptide fragments of the purified AFP protein were reverse-translated into the DNA sequences 5'-ATHCCNGARGARRATHWSN-GC-3' and 5'-GCRAARTTNCRAADATRTC-3' respectively, in which R indicates A/G, W indicates A/T, S indicates C/G, D indicates A/G/T, H indicates A/C/T and N indicates A/G/C/T. Degenerate, synthetic oligonucleotides (Genosys Biotechnologies, Pampisford, UK) with these DNA sequences were used to amplify a fragment of the carrot AFP gene using the polymerase chain reaction under the following conditions in a $100 \mu\text{l}$ PCR reaction: 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.1% Triton X-100, 2.5 mM MgCl_2 , 0.2 mM dNTPs, 1 μM primers, 5 units AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA), 100 ng genomic carrot DNA 1.5 min 94°C , 1.5 min 45°C , 3 min 72°C , 35 cycles. $3 \mu\text{l}$ of a 1:100 dilution of the reaction products were amplified under the same conditions. The products of this reaction were separated by electrophoresis and DNA fragments of a size between 200 and 300 bp were cloned into pSKII⁺ (Stratagene, Cambridge, UK) using standard protocols. Several plasmid clones with an insert of 241 bp were recovered and sequenced for both strands.

A genomic library of carrot was generated in the cosmid vector pBIC20 [27] using DNA isolated from leaf tissue of carrot plants. DNA was partially digested to fragments of 10–25 kb with the restriction enzyme *Hind*III and ligated to the *Hind*III-linearised and dephosphorylated cosmid vector. Ligation products were introduced into *Escherichia coli* NM 554 cells (Stratagene) using Gigapack Gold in vitro packaging extracts (Stratagene) according to manufacturer's instructions. Roughly 1.2×10^6 clones were generated and grown on selective media and the average insert size of the genomic library was determined to be 17.7 kb, based on restriction analysis of plasmid DNA isolated from 48 randomly chosen colonies. Based on an estimated size of the haploid carrot genome of $4.73 \times 10^5 \text{ kb}$ [28], the library represents about 44 equivalents of the carrot genome.

Approximately 700 000 clones of the library were screened according to standard protocols, using a radiolabelled probe corresponding to the PCR derived AFP gene fragment. A cosmid clone with a genomic insert of 22.5 kb was found to hybridise to the probe. The insert DNA of the cosmid was excised by *Hind*III restriction. Two fragments with sizes of 2.2 and 0.6 kb, respectively were subcloned into pSKII⁺ and sequenced for both strands. Furthermore a 3 kb *Bam*HI restriction fragment was subcloned from the cosmid and used to sequence the junction between the two *Hind*III restriction fragments to determine their relative orientation in the carrot genome.

The 5' and 3' boundaries of the transcript corresponding to the AFP gene were determined using the 5'RACE and 3'RACE procedure (Gibco BRL, Paisley, UK). Synthesis of first strand cDNA was conducted according to manufacturer's conditions using the primer 5'-GATATATGTCCGGGATCCAGC-3' and total carrot RNA (isolated from roots of 25 day old in vitro-grown seedlings that had been cold acclimated at 4°C for 72 h) as template. Using this cDNA as template 5'RACE products with a size of 300 bp were generated by PCR using the 5' anchor primer (Gibco BRL) and the primer 5'-CCTGAGGTGGGATTTGGCCGG-3' according to the manufacturer's conditions. 3'RACE was conducted using cDNA derived from the same RNA sample. 3'RACE products with a size of 650 bp were generated by PCR according to the manufacturer's conditions using the 3' anchor primer (Gibco BRL) and the primer 5'-GCTACCG-GGTTTGTTC-3'. RACE products were cloned into pSKII⁺ and sequenced for both strands.

2.5. Generation of transgenic plants

The AFP gene of carrot was amplified as a 1.6 kb PCR fragment, using the oligonucleotide primers 5'-CTCGAAACATAATCC-3'

and 5'-CGGCACGGACTTTAATGC-3' and DNA of the cosmid clone containing the AFP gene. Conditions for a 100 µl PCR reaction were: 50 mM KCl, 10 mM Tris-HCl pH9, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM dNTPs, 1 µM primers, 5 units Pfu DNA polymerase (Stratagene, UK), 10 ng cosmid DNA, 1.5 min 94°C, 1.5 min 45°C, 2.5 min 72°C, 35 cycles. The product of the PCR was cloned into a modified pSKII⁺ vector that carried an enhanced version of the 35S promoter of the cauliflower mosaic virus [29]. The promoter had previously been inserted as a *HindIII/KpnI* fragment into pSKII⁺. The PCR amplified AFP gene was inserted into an *EcoRV* site located downstream of the CaMV 35S promoter in the modified pSKII⁺ vector. The AFP expression cassette was excised and cloned as a *SacII/KpnI* fragment into the binary plant transformation vector pGA482 [30], that carries the neomycin phosphotransferase (NPTII) gene as a plant selectable marker using standard protocols. The resulting construct carries 1634 bp of the AFP genomic sequence driven by the CaMV 35S promoter fused 27 bp upstream from the inferred ATG start codon. As a result, the CaMV 35S promoter drives the expression of the AFP gene using the transcription start site of the viral promoter and the polyadenylation signal present on the AFP genomic sequence. The binary vector carrying the AFP expression cassette was transformed into the *Agrobacterium tumefaciens* strain C58 C1 GV 3101 [31], by electroporation [32]. *Agrobacterium* cultures carrying the binary vector with the AFP expression construct were used to transform *Arabidopsis thaliana* plants of the RLD or Columbia ecotype using a published protocol of the vacuum infiltration technique [33]. Transgenic seedlings were identified under sterile conditions on standard plant growth media containing 50 mg l⁻¹ kanamycin (Sigma, UK) as a selectable agent. Kanamycin-resistant seedlings derived from independent infiltration experiments were grown in soil and permitted to set seed. Plants from seed stocks that segregated 3:1 for kanamycin resistance were plated on selective media and subsequently transferred to soil.

2.6. Analysis of nucleic acids

RNA was extracted from plant tissues [34], separated electrophoretically, transferred to Biodyne B Transfer Membranes (Pall Europe, Portsmouth, UK) and hybridised to radiolabelled probes prepared from plasmid templates according to standard protocols [35]. High molecular weight DNA was isolated from carrot leaf tissue according to [36]. Insert DNA of recombinant plasmids was sequenced by MWG Biotech (Milton Keynes, UK) using automated DNA sequencing and oligonucleotide sequences corresponding to the cloning vector.

2.7. Preparation of protein extracts from *Arabidopsis*

Arabidopsis seedlings grown under sterile conditions for 10 days were homogenised in AFP extraction buffer. The homogenate was cleared by centrifugation. The cleared homogenate was desalted and concentrated using ultrafiltration and adjusted to a protein concentration of 2.5 mg ml⁻¹. Apoplastic extracts were generated by vacuum-infiltrating leaves of 3 week old *Arabidopsis* plants with AFP extraction buffer containing 0.01% Triton X-100. Excess liquid was removed from the infiltrated leaves and extracts were collected by centrifugation as described above. Extracts were desalted, concentrated and adjusted to a concentration of 0.6 mg ml⁻¹. Protein concentrations were estimated by the Bradford method [37].

3. Results

3.1. Purification of the carrot AFP

The AFP was purified from apoplastic extracts of cold acclimated carrot tap roots. SDS-PAGE and AFP analysis of fractions generated by preparative IEF revealed that the abundance of a polypeptide with a size of approximately 36 kDa correlated with AFP activity (Fig. 1). When further purified by HPLC, AFP-enriched samples of the preparative IEF contained protein that eluted as one major peak from the column. This peak again produced a 36 kDa band when separated by SDS-PAGE. Direct sequencing of the HPLC-purified protein by Edman degradation was not successful, presumably due to chemical blockage of the N-terminus. Tryptic

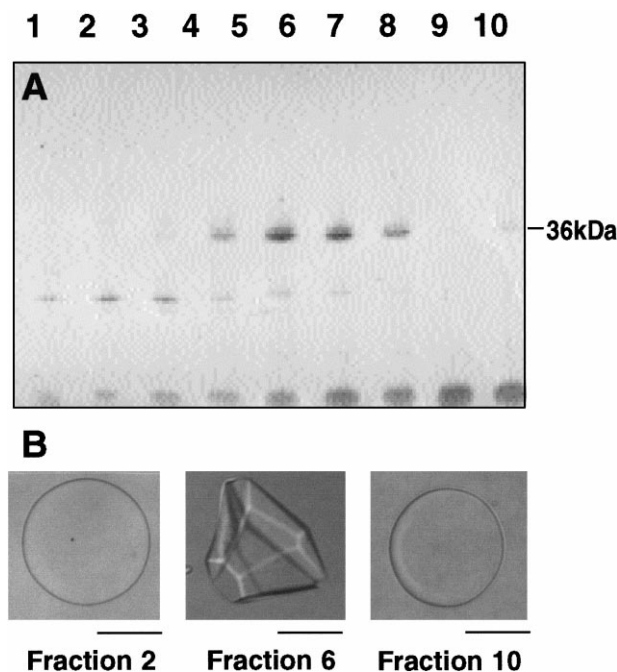


Fig. 1. Purification of the AFP from apoplastic carrot tap root extracts by isoelectric focusing. A: SDS-PAGE analysis of the first 10 fractions of a typical IEF run. (Fraction 1 is the most acidic fraction proximal to the anode chamber.) 0.15 µl of each fraction were separated on a 10–15% gradient SDS-PAGE and stained with silver. B: Antifreeze activity in fractions of the preparative IEF. Antifreeze activity was determined in fractions 2, 6 and 10 respectively by observing the morphology of ice crystals grown in solution. All crystals were photographed under the same magnification. The bar on each photograph represents 50 µm.

digests however yielded internal sequence for five protein fragments.

3.2. Isolation of the carrot AFP gene

A PCR experiment based on amino acid sequence data of two internal peptide fragments allowed amplification of a 241 bp fragment of the putative AFP gene (see Section 2). The fragment was employed to identify a genomic clone with a 22.5 kb insert from a carrot cosmid library. Southern blot analysis indicated that the PCR-generated probe hybridised to a 2.2 kb restriction fragment of this cosmid clone (data not shown). This fragment, and an adjacent 0.6 kb restriction fragment, were sequenced. This ultimately permitted the assembly of a continuous strand of genomic DNA sequence comprising about 2.8 kb. This sequence comprises an open reading frame of 999 bp which is not interrupted by introns (Fig. 2A). This sequence can be conceptually translated to a protein of 332 amino acids with a predicted molecular weight of 36.8 kDa and a calculated isoelectric point of 4.9.

The RACE procedure was employed to identify the 5' and 3' boundaries of the putative AFP gene transcript (see Section 2). Complementary DNA-derived PCR products were generated that exactly corresponded to the genomic sequence. This allowed localisation of the transcription start site of the putative AFP gene to a position 32 bp upstream of the first in-frame ATG and the 3' end of the transcript, 208 bp downstream of the inferred stop codon. Hence the transcribed region of the AFP comprises 1238 bp of DNA sequence which is in close agreement with the empirically determined size of

A

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acaactcgaaacataatccaataattcaatATGAATATTGAATCATCTTTTCGCCCTAT 60
                               M N I E S S F C P I
TTTGTGCATATGCATGATTTTCCCTTCGCCCTTCCAAACCTCTCGCATCACAAAGATGCAA 120
L C I C M I F L C L P N L S A S Q R C N
CAACAACGACAAGCAAGCTTTACTCCAAATCAAACAGCCTTGAAAAACCCACCATTAC 180
N N D K Q A L L Q I K T A L K N P T I T
AGACTCATGGGTGCAGACGACGATTGTGTGGTGGGACCTAGTCGAATGTGACGAAAC 240
D S W V S D D D C C G W D L V E C D E T
CAGCAACCGCATAATTTCCCTCATAATTCAAGACGACGAGCTCTCACCAGGCCAAATCCC 300
S N R I I S L I I Q D D E A L T G Q I P
ACCTCAGGTGGAGACCTACCATACTCCAAGCCTTATGTTCCGTAACCTCCCAATCT 360
P Q V G D L P Y L Q A L W F R K L P N L
TTTCGGAATAATCCAGAAGAAATTTCTGCACCTCAAGACCTAAATCCCTCAGACTCAG 420
F G K P E E I S A L K D L K S L R L S
CTCGACCACTCTCAGTGGCCCTGTCCCTTTATTTCTCCCTCAGCTTACGAACTAATCTTG 480
S T S L S G P V P L F F P Q L T R L T C
TTTAGACTTATCGTTTAAACAACTTTTGGGTGAATCCCTCAGCTTTCCACTCTTCC 540
L D L S F N K L I I Q D D E A L T G Q I P
GAACCTTAAAGCCCTGCACCTTAGAAGCTAACGAACCTACCGGTGAAATCCCGCATATCTT 600
N L K A L H L E R N E L T G E I P D I F
TGGAATTTTGTGGATCCCGGACATATATCTTCGCATAACCAAGCTACCGGTTTGT 660
G N F A G S P D I Y L S H N Q L T G F V
TCCCAAACTTTTGTAGAGCAGATCCAAATAGGCTTCGACCTTCTCAGGGAACAGACTAGA 720
P K T F A R A D P I R L D F S G N R L B
AGGTGATATTTCATCTCTTGTGGGCTTAAAAACGCTTGGAATGCTAGATTTTTCAGG 780
G D I S F L F G P K K R L E M L D F S G
AAACGTGCTTGTTCATTTCTCCAGGTCGAGGATTTCCACCTCTTTGACATATCT 840
N V L S F N E S R V Q E F P P S L T Y L
AGACTTGAACCATTAACAGATCAGCGGAAGTCTGTGAGTGAATTGGCTAAATGGACCT 900
D L N H N Q I S G S L S S E L A K L D L
GCAGACATTTAACGTCAGTGAATAATCTCCGCGCAAGATTCCAACAGGGGGAACCT 960
Q T F N V S D N N L C G K I P T G G N L
CCAGAGATTCGACCGTACGGCTATCTCCACAACAGTGTGTGTGGTGTCTCATTTGCC 1020
Q R F D R T A Y L H N S C L C G A P L P
AGAAATGCTAGTtaccatgcataatgtgccttaagggttatcttggtaagtgaatatattat 1080
E C *
gcagctcaagcagtgcaataagtttctctaattttgttatagtaagatatattgtattt 1140
cacagaaagtgtctactaggattgttaatatattataattgtctcataattgtatctgttt 1200
aatctgtaatccaaaacaccttatgtattggtttgaca 1238

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B

CON P-----L--L--L-LS-N-L-G-I

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66  ECDETS N R I I S L I I Q D D E A L T G Q I
90  P P Q V G D L P Y L Q A L W F R K L N L F G K I
115 P E E I S A L K D L K S L R L S S T S L S G P V
139 P L F F P Q L T K L T C L D L S F N K L L G V I
163 P P Q L S T L P N L K A L H L E R N E L T G E I
187 P D I F G N F A G S P D I Y L S H N Q L T G F V
211 P K T F A R A D P I R L D F S G N R L E G D I
234 S F L F G P K K R L E M L D F S G N V L S F N F
258 S R V Q E F P P S L T Y L D L N H N Q I S G S L
282 S S E L A K L D L Q T F N V S D N N L C G K I

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Fig. 2. A: Nucleotide sequence of the carrot AFP gene (top) and inferred amino acid sequence of the carrot AFP protein (below). Amino acid sequence corresponding to the five proteolytic fragments generated from the purified AFP protein are boxed. The amino acid sequence of peptides which were conceptually reverse-translated to degenerate oligonucleotides for PCR amplification of a fragment of the AFP gene are printed in bold. The sequence of a putative signal peptide that is removed when the protein is secreted to the apoplast is underlined. DNA sequences corresponding to the untranslated region in the 5' or 3' region of the AFP transcript are printed in lower case. Sequence motifs that match the consensus sequence for N-glycosylation (NXS/T) are double underlined. B: Alignment of leucine-rich tandem repeats of the carrot AFP. Conserved amino acids are shown in bold. The sequence was condensed or expanded at three different positions for an optimal alignment of the consensus sequence. The consensus sequence was derived from amino acids present in a specific position in at least 50% of the repeats.

the AFP transcript of 1.2 kb (Fig. 4 and data not shown). Genomic DNA sequence corresponding to the transcribed region of the AFP gene has been submitted to the European Molecular Biology Laboratory (EMBL), accession number AJ131340.

3.3. Structure of the AFP protein

Sequence comparison of the putative AFP with proteins in the databases revealed a high level of similarity of the protein to the polygalacturonase-inhibitor class of plant LRR proteins (Fig. 3). These are apoplastic proteins that have been shown to inhibit polygalacturonases of fungal plant pathogens in vitro and are therefore thought to be implicated in the plant's defense against fungal attack. The deduced sequence of the putative AFP is most similar to PGIP from kiwi (56% identity, 66% similarity), followed by PGIP from pear [38] (51% identity, 63% similarity), tomato [39] (50% identity 60% similarity) and kidney bean [40] (40% identity 55% similarity) (Fig. 3).

In addition, like other PGIPs the carrot AFP shows significant similarity to other plant proteins containing leucine-rich repeats. These include proteins involved in signaling processes in plant pathogen defense [41], the control of plant development [42] or phytohormone signal transduction [43].

The deduced amino acid sequence of the putative AFP is characterised by a block of ten imperfect tandem repeats of leucine-rich sequence elements. The average length of these LRRs is 24 amino acids (Fig. 2B). The consensus sequence of these repeats is identical to the consensus sequence of LRRs of the PGIP protein family [39]. This in turn is related to other LRR consensus sequences that are found in proteins with divergent functions from insects, human, yeast and plants [44].

The AFP from carrot carries a stretch of 26 amino acids at the N-terminus that is rich in hydrophobic amino acids and very likely represents a signal peptide. Characteristics of this signal peptide are consistent with the features of presequences involved in transport of proteins across endoplasmic reticular membranes [45]. The putative site of signal peptide cleavage (SAS-QR) could be predicted using the SignalP package software and a neural network trained on eukaryotic protein sequences available at <http://www.cbs.dtu.dk/services/SignalP/> [46]. Additional support for the fact that this cleavage site is actually used is provided by the fact that the purified protein was found to be N-terminally blocked. N-terminal blockage is frequently the result of cyclisation of N-terminal glutamine to pyroglutamate. The predicted mature protein would have just such a N-terminal glutamine residue. Removal of the signal peptide would generate a protein of 306 amino acids with a calculated molecular weight of 34 kDa and a calculated isoelectric point (pI) of 4.97.

The calculated pI of the mature AFP agrees with the distribution of AFP activity in the preparative isoelectric focusing gradient: the activity was found to accumulate in fractions with a pH of 4.8–5.2. The isoelectric point of the mature carrot AFP is significantly lower than that of any PGIP characterised so far. The relative acidity of the AFP compared to the closely related PGIP family is predominantly the result of conservative substitutions of basic or uncharged amino acid residues by glutamic acid which primarily affect the LRR domain of the protein.

There is a slight deviation between the apparent molecular

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|--------|--------|--------|--------|--------|
| M | - | - | - | N | I | E | S | S | F | C | P | I | L | C | I | C | M | I | F | L | C | L | P | N | L | S | A | S | DCAFP | | |
| M | - | - | - | - | - | - | - | - | K | S | T | T | A | I | S | L | - | L | L | F | L | S | S | L | S | P | S | L | S | ACPGIP | |
| M | - | - | - | - | - | - | - | - | K | F | S | T | T | P | L | S | L | T | L | F | S | S | V | L | N | P | A | L | S | PCPGIP | |
| M | - | - | - | - | - | - | - | - | S | - | - | - | - | L | L | L | V | V | I | F | L | C | F | A | S | P | S | L | S | LEPGIP | |
| M | T | Q | F | N | I | P | V | T | M | S | S | S | S | S | I | I | L | V | I | L | V | S | L | R | T | A | L | S | PVPGIP | | |
| Q | R | C | N | N | N | D | K | Q | A | L | L | Q | I | K | T | A | L | K | N | P | T | I | T | D | S | W | V | S | DCAFP | | |
| D | R | C | N | N | P | N | D | K | K | V | L | L | R | I | K | Q | A | L | N | N | P | Y | L | L | A | S | W | N | P | ACPGIP | |
| D | L | C | N | P | D | D | K | K | V | L | L | Q | I | K | K | A | F | G | D | P | Y | V | L | A | S | W | K | S | PCPGIP | | |
| V | R | C | N | P | K | D | K | K | V | L | L | Q | I | K | K | D | L | G | N | P | Y | H | L | A | S | W | D | P | LEPGIP | | |
| E | L | C | N | P | Q | D | K | Q | A | L | L | Q | I | K | K | D | L | G | N | P | T | T | L | S | W | L | P | PVPGIP | | | |
| D | D | D | C | C | G | - | - | W | D | L | V | F | C | D | E | T | S | N | - | - | R | I | I | S | L | I | Q | DCAFP | | | |
| D | N | D | C | C | D | - | - | W | Y | N | V | D | C | D | L | T | T | N | - | - | R | I | I | A | L | T | I | F | ACPGIP | | |
| D | T | D | C | C | D | - | - | W | Y | C | V | T | C | D | S | T | T | N | - | - | R | I | N | S | L | T | I | F | PCPGIP | | |
| N | T | D | C | C | Y | - | - | W | Y | V | I | K | C | D | R | K | T | N | - | - | R | I | N | A | L | T | V | F | LEPGIP | | |
| T | T | D | C | C | N | R | T | W | L | G | V | L | C | D | T | D | T | Q | T | Y | R | V | N | N | L | D | L | - | PVPGIP | | |
| D | D | E | A | L | T | G | Q | - | - | I | P | P | Q | V | G | D | L | P | Y | L | Q | A | L | W | F | R | K | L | DCAFP | | |
| S | G | N | - | I | S | G | Q | - | - | I | P | A | A | V | G | D | L | P | Y | L | Q | T | L | I | F | R | K | L | S | ACPGIP | |
| A | Q | N | - | I | S | G | Q | - | - | I | P | A | A | V | G | D | L | P | Y | L | E | T | L | E | F | H | K | L | S | PCPGIP | |
| Q | A | N | - | I | S | G | Q | - | - | I | P | A | A | V | G | D | L | P | Y | L | E | T | L | E | F | H | H | V | LEPGIP | | |
| S | G | H | N | L | P | K | P | Y | P | I | P | S | S | L | A | N | L | P | Y | L | N | F | L | Y | I | G | G | I | PVPGIP | | |
| P | N | L | F | G | K | I | P | E | E | I | S | A | L | K | D | L | K | S | L | R | L | S | S | T | S | L | S | G | DCAFP | | |
| S | N | L | T | G | P | I | P | S | A | I | S | K | L | S | N | D | L | K | M | V | R | L | S | W | T | N | L | S | G | ACPGIP | |
| P | N | L | T | G | P | I | P | S | A | I | A | K | L | K | G | L | K | S | M | R | L | S | W | T | N | L | S | G | PCPGIP | | |
| T | N | L | T | E | F | T | I | P | S | A | I | A | K | L | T | N | L | K | M | L | R | L | S | F | T | N | L | T | G | LEPGIP | |
| N | N | L | V | G | P | I | P | S | A | I | A | K | L | T | Q | L | H | Y | L | Y | I | T | H | T | N | V | S | G | PVPGIP | | |
| P | V | P | L | F | F | F | Q | L | T | K | L | T | F | C | L | D | L | S | F | N | K | L | L | G | V | I | P | P | Q | DCAFP | |
| P | V | P | S | F | F | S | Q | L | K | N | L | T | F | L | D | L | S | F | N | K | L | L | G | V | I | P | P | S | S | ACPGIP | |
| S | V | P | D | F | L | S | Q | L | K | N | L | T | F | L | D | L | S | F | N | K | L | L | G | V | I | P | P | S | S | PCPGIP | |
| R | I | P | E | F | L | S | Q | L | K | N | L | T | F | L | D | L | S | F | N | K | L | L | G | V | I | P | P | S | S | LEPGIP | |
| A | I | P | D | F | L | S | Q | L | K | N | L | T | F | L | D | L | S | F | N | K | L | L | G | V | I | P | P | S | S | PVPGIP | |
| L | S | T | L | P | N | L | D | A | I | H | L | E | R | N | E | L | T | G | P | I | P | N | S | F | G | E | F | T | S | DCAFP | |
| L | S | K | L | T | N | L | D | A | I | H | L | E | R | N | E | L | T | G | H | I | P | I | P | N | S | F | G | E | F | T | ACPGIP |
| L | S | Q | L | P | N | L | L | A | M | Y | L | D | R | N | K | L | T | G | T | I | P | E | S | F | G | R | F | K | LEPGIP | | |
| I | S | L | P | N | L | G | I | T | F | D | G | N | R | I | S | G | A | I | P | D | S | Y | G | S | F | S | PVPGIP | | | | |
| G | S | - | - | P | D | I | Y | L | S | H | N | Q | L | T | G | F | V | P | K | T | F | A | R | A | D | P | I | R | DCAFP | | |
| G | - | Q | V | P | D | L | Y | L | S | H | N | Q | L | T | G | S | I | P | K | T | L | G | D | L | N | F | T | V | ACPGIP | | |
| G | - | N | V | P | D | L | Y | L | S | H | N | Q | L | T | G | S | I | P | K | T | L | G | D | L | N | F | T | V | PCPGIP | | |
| G | P | N | I | P | D | L | Y | L | S | H | N | Q | L | T | G | H | V | P | A | S | L | G | D | L | N | F | S | T | LEPGIP | | |
| - | K | L | F | T | A | M | T | I | S | R | N | R | L | T | G | K | I | P | T | F | A | N | L | N | L | F | A | S | PVPGIP | | |
| L | D | F | S | R | G | N | R | L | E | G | D | I | S | F | M | F | G | P | K | K | R | L | E | M | L | D | F | S | G | DCAFP | |
| I | D | V | S | R | N | M | L | S | G | D | A | I | S | F | L | F | G | S | N | K | T | I | Q | I | V | D | L | S | R | ACPGIP | |
| I | D | L | S | R | N | K | L | E | G | D | A | V | S | F | L | F | G | K | N | K | T | T | Q | I | V | D | L | S | R | PCPGIP | |
| L | D | F | S | R | N | K | L | E | G | D | V | S | F | L | F | G | K | N | K | T | S | Q | V | I | D | L | S | R | LEPGIP | | |
| V | D | L | S | R | N | M | L | E | G | D | A | S | V | L | F | G | S | D | K | N | T | K | K | I | H | L | A | K | PVPGIP | | |
| N | V | L | S | F | N | F | S | R | V | Q | E | F | P | P | S | L | T | Y | L | D | L | N | H | N | Q | I | S | G | DCAFP | | |
| N | K | F | Q | F | D | L | S | K | V | - | V | F | P | Q | S | L | T | S | L | D | I | N | H | N | K | I | Y | G | ACPGIP | | |
| N | L | L | E | F | N | L | S | K | V | - | E | F | P | T | S | L | T | S | L | D | I | N | H | N | K | I | Y | G | PCPGIP | | |
| N | S | L | A | F | D | L | G | K | V | - | G | L | S | K | N | L | N | G | L | D | L | R | N | N | R | I | Y | G | LEPGIP | | |
| N | S | L | S | E | L | A | K | L | D | - | L | Q | T | F | N | V | S | D | N | N | L | C | G | K | I | P | T | G | DCAFP | | |
| S | L | P | V | G | L | T | K | L | D | - | L | Q | T | F | N | V | S | D | N | N | L | C | G | K | I | P | T | G | ACPGIP | | |
| S | L | P | V | E | F | T | Q | L | N | - | F | Q | F | L | N | V | S | D | N | N | L | C | G | K | I | P | T | G | PCPGIP | | |
| S | L | P | P | G | L | K | D | V | P | - | L | Q | T | F | N | V | S | D | N | N | L | C | G | K | I | P | T | G | LEPGIP | | |
| T | L | P | Q | G | L | T | Q | L | K | F | L | Q | S | L | H | V | S | F | N | N | L | C | G | K | I | P | T | G | PVPGIP | | |
| G | N | L | Q | R | F | D | R | T | A | Y | L | H | N | S | C | L | C | G | A | P | L | P | E | C | . | . | . | DCAFP | | | |
| G | K | L | Q | G | F | D | Q | E | Y | S | Y | F | H | N | R | C | L | C | G | A | P | L | P | D | C | K | . | . | ACPGIP | | |
| G | K | L | Q | S | F | D | E | Y | S | Y | L | H | N | K | C | L | C | G | S | P | L | P | K | C | K | . | . | PCPGIP | | | |
| G | N | L | K | R | F | D | V | S | S | Y | A | N | N | K | C | L | C | G | S | P | L | P | S | C | - | T | . | . | LEPGIP | | |
| G | N | L | K | R | F | D | V | S | S | Y | A | N | N | K | C | L | C | G | S | P | L | P | S | C | - | T | . | . | PVPGIP | | |

Fig. 3. Amino acid sequence alignment of the carrot AFP (DCAFP) with PGIPs from *Actinidia deliciosa* (ACPGIP) (accession number Z49063), *Pyrus communis* (PCPGIP) (accession number L09264), *Lycopersicon esculentum* (LEPGIP) (accession number L26529) and *Phaseolus vulgaris* (PVPGIP) (accession number X64769). Shaded residues represent amino acids that are identical to those of the carrot AFP.

weight of the carrot AFP on SDS-PAGE gels (36 kDa) and the calculated MW (34 kDa) of the mature AFP. This could indicate that that protein is subjected to post-translational modification such as glycosylation. Indeed, the AFP sequence carries three sequence motifs for *N*-glycosylation (NXS/T) (Fig. 2A). An N-terminal, putative glycosylation site (NPT) is shared only with the tomato PGIP, whereas a second putative site (NFS) is only present in the AFP, and a third, carboxy-terminal site (NVS) is shared by the AFP protein with all PGIPs analysed. We have some evidence that the second *N*-glycosylation sequence (NFS) is used. This motif is part of a protein fragment that was generated from the purified AFP (Fig. 2A). When subjected to Edman degradation the predicted asparagine did not produce an identifiable

amino acid derivative whereas the following phenylalanine, serine and arginine residues did.

The carrot AFP shares seven cysteine residues outside the LRR domain with the other four PGIPs examined. This may suggest that the carrot AFP and the PGIP family have a common disulphide bond structure. There is, however, one cysteine residue in the LRR domain of the AFP that is not shared by any of the PGIP sequences published so far.

3.4. Analysis of AFP gene expression in response to exposure to low temperatures

To further investigate the role of the AFP protein in the process of plant cold acclimation, induction kinetics of AFP gene expression in response to low temperatures were determined using RNA blot analysis. Initially experiments focused on long term kinetics of AFP induction in tap roots as well as in photosynthetic and non-photosynthetic tissues of seedlings (Fig. 4A). Expression of the carrot AFP was found to be dramatically induced by low temperatures (Fig. 4A). All of the tissues examined responded to exposure to low temperatures (4°C) with an increase in the steady-state levels of the AFP transcript by several orders of magnitude. Induction of

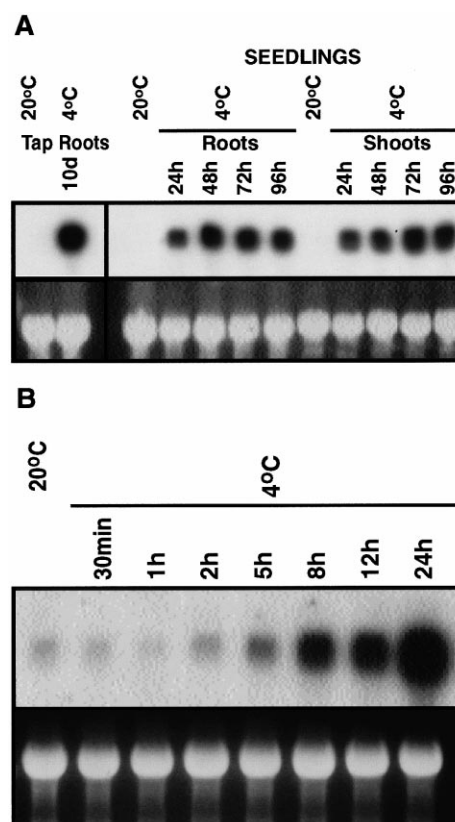


Fig. 4. Changes in AFP transcript levels in response to low temperature. A: Long term kinetics of AFP expression. Northern blot analysis of total RNA (15 µg) prepared from either tap roots of non- or cold acclimated 4 month old carrot plants or root and shoot tissues of 25 day old in vitro-grown seedlings exposed to 4°C for the indicated times. Blots were hybridised with a ³²P labeled probe corresponding to the untranslated 3' end of the AFP transcript. B: Short term kinetics of AFP expression. Northern blot analysis of total RNA (10 µg) prepared from 25 day old seedlings exposed to 4°C for the indicated times. Equal loading of lanes was verified by ethidium bromide staining of the 28S ribosomal RNA band.

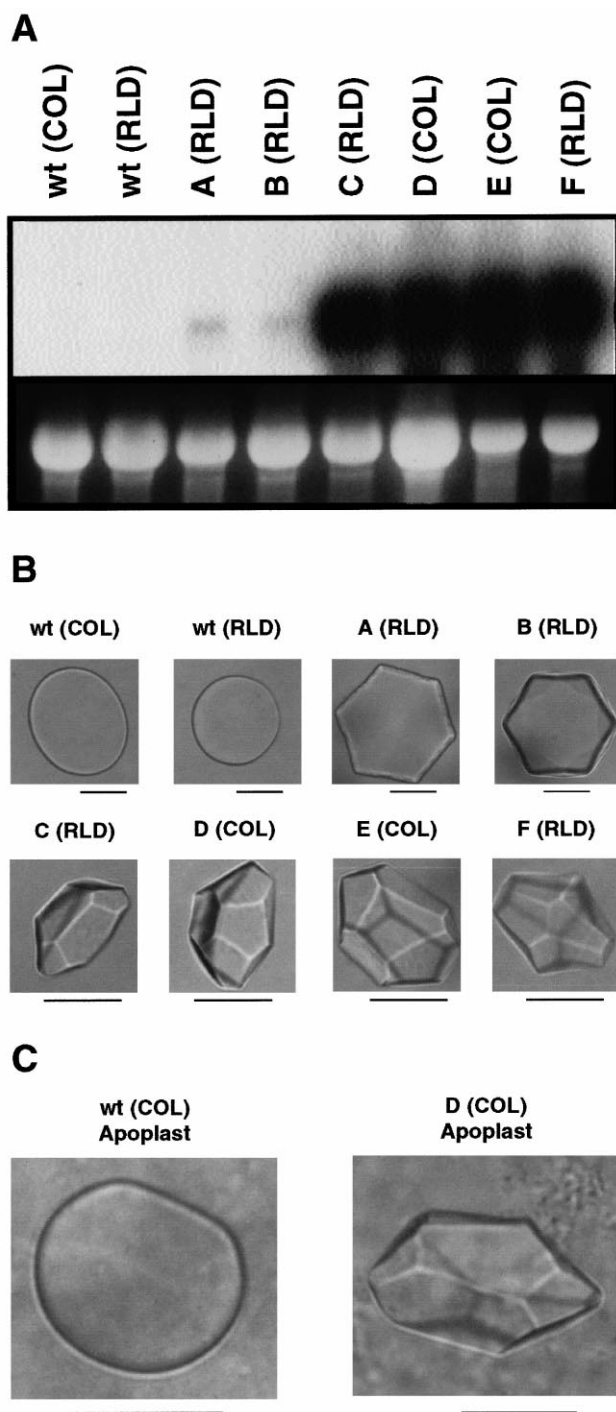


Fig. 5. Molecular and biochemical analysis of *Arabidopsis thaliana* plants expressing the carrot AFP. A: RNA blot analysis of AFP gene expression in non-acclimated *Arabidopsis* seedlings containing the CaMV 35S-AFP transgene. RNA (15 μ g) was isolated from in vitro-grown 14 day old seedlings of Col and RLD wild-type plants and two transgenic lines of the Col genetic background (D,E) and four transgenic lines of the RLD genetic background (A,B,C,F) respectively. The RNA blots were hybridised as in Fig. 4B. Antifreeze activity in total protein extracts (2.5 mg ml⁻¹) of wild-type and transgenic *Arabidopsis* plants. B: Antifreeze activity was determined by observing the morphology of ice crystals in solution. All crystals were photographed under the same magnification. The bar on each photograph represents 50 μ m. C: Antifreeze activity in apoplastic extracts (0.6 mg ml⁻¹) of the *Arabidopsis* wild-type and line D.

AFP expression in response to cold occurs within 24 h after which steady-state levels of the transcript reach a plateau level which is maintained in the presence of the cold stimulus. The AFP transcript is below detectable limits in both tap roots of mature plants and hypocotyl, cotyledon and leaf tissues of seedlings grown at ambient temperatures. There is however, some basal level of AFP expression in non-acclimated root tissues of seedlings, but similar to photosynthetic and tap root tissues, AFP transcript levels rise by several orders of magnitude in response to cold treatment. To further dissect the kinetics of AFP induction by cold, short time kinetics were determined using RNA that was isolated from entire seedlings (Fig. 4B). Transcript levels of the AFP gene were found to rise above basal levels 2 h after transfer to 4°C. Taken together, these data conclusively demonstrate that the expression of the putative AFP gene is a part of the cold acclimation response in a wide range of carrot tissues and organs.

3.5. Expression of the AFP in transgenic *Arabidopsis* plants

To directly test the hypothesis that the isolated gene encodes a polypeptide with antifreeze activity, transgenic plants were generated in which the AFP-candidate gene was over-expressed under the control of the CaMV 35S promoter [29]. The construct was transformed into *Arabidopsis thaliana* plants via *Agrobacterium*-mediated transformation. A total of 38 transgenic lines were generated. When grown under the same controlled conditions no obvious phenotypic difference between transgenic and wild-type plants could be detected. Six transgenic lines were analysed in detail. Levels of transgene expression were determined using Northern blots (Fig. 5A). The selection of transgenic lines examined comprised two low expressers and four lines that expressed high levels of the AFP transcript. This is in clear contrast to wild-type plants which did not express a transcript that cross-hybridised with the carrot AFP probe under high stringency conditions. Total protein extracts were prepared from the six transgenic lines and the corresponding wild-type controls. In contrast to protein extracts of non-acclimated wild-type *Arabidopsis* plants, extracts from transgenic lines clearly exhibited AFP activity, as demonstrated by either the hexagonal or the multi-faceted bi-pyramidal shape of ice crystals grown in the protein extracts (Fig. 5B). Furthermore, there was a clear correlation between the level of AFP transcript and AFP activity in the transgenic lines. Low levels of AFP transcript in lines A and B were found to be associated with flat, albeit hexagonal, ice crystal structures, whereas high levels of AFP transcript in lines C to F were clearly associated with multi-faceted or even bi-pyramidal ice crystal structures.

Apoplastic extracts were prepared from fully developed leaves of wild-type *Arabidopsis* plants and plants of transgenic line D. The apoplastic extracts contained about a quarter of the protein of the total extract of the corresponding transgenic line. However levels of AFP activity in total and apoplastic extracts of line D were indistinguishable (Fig. 5C) which indicated that a significant portion of the transgene-derived AFP accumulated in the apoplast of the transgenic plant.

In conclusion, the data on the transgenic lines provide unambiguous evidence that the carrot PGIP-like protein can be expressed in heterologous plant tissue and that this protein has antifreeze activity. Furthermore AFP activity in the transgenic plants is correlated with AFP transcript levels and a

significant portion of the transgene-derived protein seems to accumulate in the apoplast of the transgenics, which is similar to the situation in the host plant.

4. Discussion

4.1. Isolation of a gene from carrot that encodes a protein with antifreeze activity

A protein with antifreeze activity was purified from apoplastic extracts of cold acclimated carrot tap roots. Internal protein sequence information derived from the purified protein allowed isolation of the corresponding AFP gene.

Several independent lines of evidence provide conclusive evidence that the isolated gene encodes the AFP from carrot. First, the deduced amino acid sequence of the isolated gene contains all of the sequence information derived from the purified protein. Second, steady-state levels of the AFP transcript are dramatically induced by cold. These levels increase 2 h after exposure to cold and rise by several orders of magnitude to a plateau level that is maintained in the presence of the cold stimulus. This finding supports an important role for the gene in the process of cold acclimation. The ultimate proof that the gene encodes a polypeptide with AFP activity is provided by transgenic *Arabidopsis* plants in which the AFP gene is under the control of a constitutive promoter. This results in antifreeze activity in non-acclimated *Arabidopsis* plants that do not exhibit this trait.

4.2. The possible role of the carrot AFP in freezing tolerance

Plant defense mechanisms against freezing damage have recently been reviewed [20]. The major sources of freezing damage comprise cytoplasmic dehydration [47] and disruption of cell integrity [48] as the result of apoplastic freezing. Mechanisms to counteract dehydration-induced membrane damage include alterations in membrane lipid composition [49], the accumulation of small osmotically active molecules such as sugars [50], and the accumulation of certain hydrophilic polypeptides [20]. This and recent studies [4,5,7,19,21–24] indicate that an additional complementary frost protection mechanism has recently evolved in some plant species. This mechanism involves AFPs that target apoplastic freezing rather than its consequences. This finding emphasises the multigenic nature of plant frost acclimation. Low thermal hysteresis of the carrot AFP [7,24], suggests that the major function of this protein may not lie in the suppression of apoplastic freezing, but in the modification of ice crystal shape including the inhibition of ice recrystallisation.

The availability of the AFP gene will allow the study of its role in freezing resistance using a reverse genetics approach in carrot. The expression of the AFP in transgenic *Arabidopsis* plants that possess the ability to cold acclimate will allow us to test further hypotheses: first, the ability of the AFP to cooperate with endogenous frost protection mechanisms and second, the capacity of the protein to protect non-acclimated plant tissues against freezing damage. This will ultimately allow assessment of the efficacy of the carrot AFP for the improvement of frost resistance of commercially important crop plant species.

4.3. A leucine-rich repeat protein that binds to ice

The LRR domain of the AFP comprises about 74% of the

processed protein. It is thus tempting to speculate that it contributes at least partly to the ice binding domain of the AFP. LRRs have been found in the primary structure of a wide variety of proteins that function as receptors for extra- or intracellular ligands, enzyme subunits, enzyme inhibitors and structural cell adhesion proteins [44,51]. A recurrent theme is that the LRR domain seems to be the central interface of protein-protein interactions. In some LRR-containing molecules the LRR domain corresponds to a structural unit consisting of an amphipathic β strand and an α helix [44]. The alternating β strand and α helical regions are arranged in parallel to a common axis resulting in a non-globular, horse-shoe-shaped molecule [52]. Other LRR-containing proteins such as pectate lyase have been proposed to have a structure that is entirely composed of β strands [53]. The β - α conformation has recently been proposed as the possible structure of the LRR consensus motif that is common to all plant LRR proteins [51]. According to this model the carrot AFP would possess a region composed of solvent exposed β sheets that could be structurally related to the planar, exposed ice-binding domain of AFPs II and III from arctic fish species [13–15]. The proposed ice-binding domain of these two unrelated proteins is also at least partly composed of structural elements with β sheet conformation.

How could an AFP have evolved from a PGIP? A large class of LRR-containing proteins in plants comprise products of the resistance (R) gene loci which have been implicated as receptors of plant pathogen-derived extra or intracellular ligands and control pathogen specificity of plant defense mechanisms [54,55]. Allelic variation at resistance gene loci is responsible for differences in pathogen resistance of plant cultivars. This variation is mainly the result of variability of the LRR domain of the R genes. Hence it has been proposed that the ability of plants to constantly adapt and respond to new pathogens is the result of hypervariability of the LRR domain of R-genes [56–58]. It could be speculated that a mechanism of positive selection for variability has equally affected the LRR domain of PGIPs that have also been implicated as important determinants of the specificity of plant pathogen interactions [59]. Our and recent findings [24], further suggest that this evolutionary mechanism of divergent selection has at some stage produced a PGIP-derived protein that binds to the non-proteinaceous ligand ice. A PGIP gene would have provided the ideal template for the evolution of an AFP because it has already encoded a protein that was secreted to the extracellular space where freezing first occurs. In the scenario outlined above, it is reasonable to assume that the carrot AFP has recently evolved from a PGIP-like precursor via gene duplication. In this context it is interesting to note that during the course of this work we have isolated another PGIP-like protein from carrot that shares 75% amino acid sequence identity with the carrot AFP (K. Meyer and S. Crossley, unpublished data). In contrast to the carrot AFP expression of the AFP-like gene is, although cold-induced, characterised by significant expression levels in non-acclimated plant tissues. This could indicate that cold inducibility of AFP gene expression has evolved from a more general, stress-induced pattern of gene expression. We have cloned regulatory sequences of both genes and predict that analysis of these sequences will reveal important data on the evolution of transcriptional regulation of these genes.

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