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A Carrot Leucine-Rich–Repeat Protein That Inhibits Ice Recrystallization

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Many organisms adapted to live at subzero temperatures express antifreeze proteins that improve their tolerance to freezing. Although structurally diverse, all antifreeze proteins interact with ice surfaces, depress the freezing temperature of aqueous solutions, and inhibit ice crystal growth. A protein purified from carrot shares these functional features with antifreeze proteins of fish. Expression of the carrot complementary DNA in tobacco resulted in the accumulation of antifreeze activity in the apoplast of plants grown at greenhouse temperatures. The sequence of carrot antifreeze protein is similar to that of polygalacturonase inhibitor proteins and contains leucine-rich repeats.

Living organisms have developed diverse strategies to enable them to survive freezing conditions. One strategy that has evolved repeatedly is the expression of antifreeze proteins (AFPs) (*1*, *2*). AFPs cause thermal hysteresis (TH) and inhibit ice recrystallization. TH, in which the freezing temperature is lower than the melting temperature, allows freeze-avoiding organisms such as fish to supercool in the presence of ice. Ice recrystallization, the growth of large ice crystals at the expense of smaller ones, is one cause of tissue damage in freeze-tolerant organisms. Both TH and recrystallization inhibition (RI) activity are thought to result from interaction of AFPs with ice crystal surfaces (*2*, *3*).

TH activity has been detected in at least 26 species of higher plants (*4*), and some candidate proteins have been purified (*5*–*7*). However, the TH values exhibited by these extracts are low (0.2° to 0.6°C) in the context of the environmental temperatures that plants encounter. Plant AFPs are therefore unlikely to function to lower the temperature at which ice crystallizes in the apoplast but rather to inhibit the potentially damaging process of ice recrystallization. Here, we describe purification of an active AFP from cold-acclimated carrot tap roots and the cloning and expression of the corresponding cDNA.

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Using biochemical separation techniques (*8*), we isolated a 36-kD glycoprotein from cold-acclimated carrot tap root that copurified with RI activity (Fig. 1). The purified AFP was assayed for TH, and a value of 0.35°C was detected (*9*).

The carrot AFP was found to be N-glycosylated (Fig. 2) (*10*); however, enzymic removal of the small glycan side chain did not affect its RI activity. This result contrasts with the *Solanum* active or the fish antifreeze glycor protein (AFGP), which lost activity on removal of their glycan groups (*6*, *11*).

The amino acid sequence of internal peptides was obtained (*12*), and the coding region corresponding to the purified protein was isolated from a cold-acclimated carrot root cDNA library (*13*) (Fig. 3A). The predicted features of the deduced AFP sequence correlate well with those determined empirically for the purified carrot protein. The apparent relative molecular mass (M_r) of the native protein on SDS–polyacrylamide gel electrophoresis (PAGE) was 36 kD, and its isoelectric point was 5.0, compared with 34 kD and 4.8 for the deduced protein. The deduced protein had three potential N-glycosylation sites, at least one of which appears to be occupied in the native protein, and a putative signal peptide.

The AFP cDNA was fused to a double cauliflower mosaic virus *35S* promoter, and

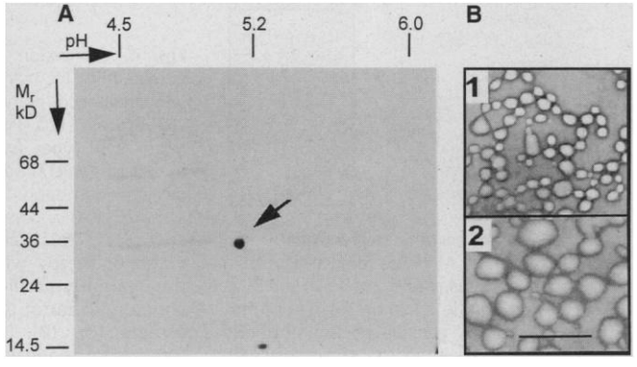


Fig. 1. RI activity of purified carrot AFP. (**A**) Two-dimensional PAGE separation of purified carrot AFP. Carrot antifreeze activity, purified as described previously (*8*), was separated by isoelectric focusing– and SDS-PAGE (*27*), and the gel was stained with Coomassie blue. (**B**) The same material was adjusted to a protein concentration of 1.5 μg/ml and assayed for RI (*8*). Ice crystals remained small in the sample containing purified carrot AFP (panel 1) compared with ice crystals in a control sample (panel 2). Scale bar, 100 μm.

the construct (*35S-AFP*) was introduced into tobacco (*Nicotiana tabacum* L. var. SR1) (*14*). Nine independent transgenic lines were produced and appeared phenotypically normal when grown under greenhouse conditions.

Protein immunoblot analysis of leaf extracts (Fig. 4A) with an affinity-purified antiserum (*8*) raised against the native, deglycosylated carrot AFP revealed a 36-kD polypeptide in eight of the lines carrying the transgene. Thus, the recombinant protein is stable in plants grown at greenhouse temperatures. In contrast, fish AFPI cannot be stably expressed in transgenic tobacco (*15*).

Extracts from the transgenic tobacco lines constitutively expressing carrot AFP inhibited ice crystal growth (Fig. 4B). RI activity was detectable down to a total protein concentration of 3 μg/ml in intercellular fluid extracts (ICF) and a total protein concentration of 30 μg/ml in whole leaf homogenates of the 35-9-C2 line (*16*). This enrichment of activity in the ICF suggests that the AFP is secreted, and protein immunoblot analysis confirmed this probability (Fig. 4A). Whole homogenates from two of the lines were tested for TH and gave values of between 0.35° and 0.56°C (*9*).

Carrot AFP shares sequence similarity with polygalacturonase inhibitor proteins (PGIPs) (Fig. 3A). PGIPs belong to a glycoprotein family, many of which specifically inhibit fungal polygalacturonase (PG) activity in vitro (*17*). Carrot AFP did not inhibit PG extracted from ripe tomato fruit or from *Aspergillus niger* (*18*); however, sequence analyses (*19*) indicate that the PGIP sequences bear more similarity to each other than to the carrot AFP, suggesting that the carrot AFP may interact with a different range of proteins.

It appears that proteins have been coopted to antifreeze activity from other functions quite recently in evolution (*20*). In plants, pathogenesis-related proteins such as the β(1-3)endoglucanase and chitinase of winter rye (*5*) and the PGIP-related carrot protein have been recruited. The cell wall is modified in response to both low temperature and pathogen attack (*21*). Because ice crystallizes in the apoplast, proteins involved in such cell wall modification are well suited for cooption into antifreeze activity if their protein structures permit.

PGIPs belong to a large family of proteins known as the leucine-rich–repeat (LRR) proteins (*22*). LRR proteins contain 10 to 30 repeated units of a ~24–amino acid peptide containing regularly spaced leucine residues. The carrot AFP consensus sequence is similar to the motif found in other LRR proteins (Fig. 3B). One LRR protein exhibits an unusual nonglobular protein structure with a solvent-exposed parallel β sheet (*23*), and this structure has been compared with the related parallel β sheet found in pectin-degrading enzymes such as pectate lyase (*22*). In this context, it may be relevant that fish AFPIII contains a β sheet on its presumptive ice-binding face (*24*) and that the AFPII ice-binding face may also contain a β sheet structure (*25*).

The co-option of an LRR protein into antifreeze function in carrot suggests an additional common structural feature of AFPs. Of the seven AFPs known (*1*, *2*), four contain repeated sequences. Thus, a repetitive structure may correlate with antifreeze activity.

The carrot AFP can be stably produced in tobacco plants grown under normal greenhouse conditions. The RI properties of this protein may be useful for improving food storage and protecting crop plants against cold temperatures.

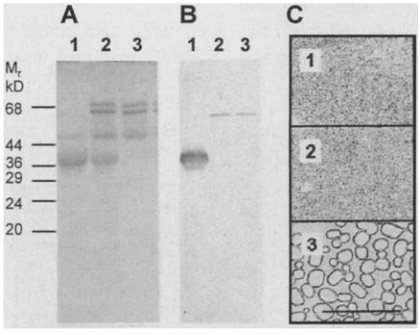
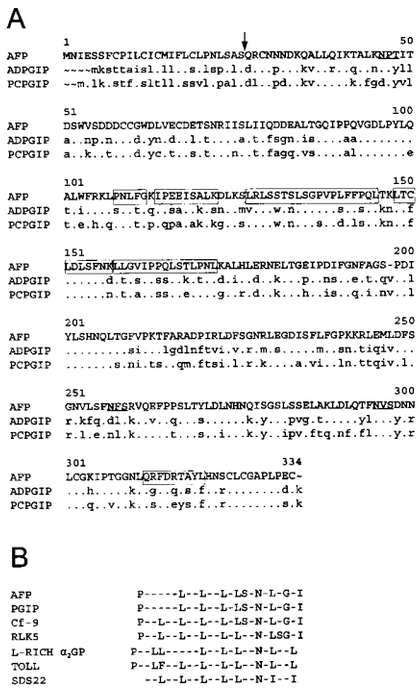


Fig. 2. The effect of enzymic deglycosylation on RI activity. Purified AFP was digested with a cocktail of glycosidases and analyzed by SDS-PAGE and Coomassie staining (**A**), immunoblotting with an *N*-glycan–specific antibody YZ1/2.23 (**B**), or assaying for RI activity (**C**). Lane 1, undigested AFP; lane 2, glycosidase-treated AFP; and lane 3, enzymes alone. Scale bar, 100 μm.

Fig. 3. Relationship of carrot AFP sequence to PGIPs and LRRs. (**A**) The deduced carrot AFP amino acid sequence (GenBank accession number AF055480) was aligned with kiwi PGIP (ADPGIP; GenBank Z49063) and pear PGIP (PCPGIP; GenBank L09264). Dots in the PGIP sequences indicate amino acids that are identical to the AFP sequence, and dashes indicate gaps introduced to maximize alignment. The positions of peptide sequences obtained from the native carrot protein are boxed, and potential N-glycosylation sites are underlined. The arrow indicates the putative signal sequence cleavage site. (**B**) Comparison of LRR motifs. The AFP consensus was derived from the AFP sequence in which amino acids were present in at least 50% of the repeats. The PGIP consensus was derived from the tomato, bean, and pear sequences (*28*). Cf-9, membrane-anchored receptorlike protein, tomato (GenBank U15936); RLK5, receptorlike kinase, *Arabidopsis thaliana* (GenBank M84660); leucine-rich α_2GP, LRR protein of unknown function, human (PIR NBHUA2); TOLL, transmembrane protein, *Drosophila melanogaster* (GenBank PO8953); SDS22, protein involved with mitosis, yeast (GenBank X836903).



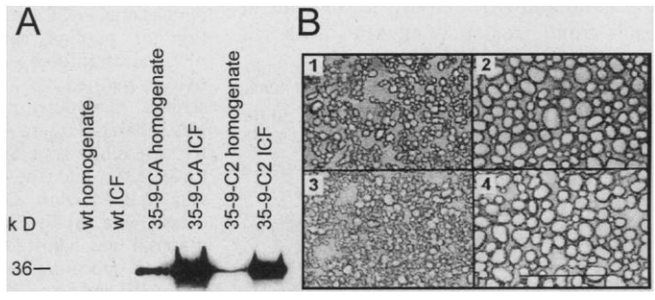


Fig. 4. Expression of active AFP in transgenic tobacco plants. (**A**) Protein (20 μg), from wild-type (wt) and transgenic tobacco leaf homogenates and ICF, was separated by SDS-PAGE and immunoblotted with the affinity-purified antibody to carrot antifreeze (*8*). (**B**) Extracts from the leaves of transgenic tobacco line 35S-AFP-CA (panel 1), wild-type tobacco (panel 2), or the storage roots of cold-acclimated (panel 3) or nonacclimated (panel 4) carrot (*14*) were adjusted to a protein concentration of 1 mg/ml before RI assay. Scale bar, 100 μm.

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13. First-strand cDNA was obtained by reverse transcription of total RNA from cold-acclimated carrot storage root with the oligonucleotide primer OG1 (5′-GAGAGAGGATCCTCGAGTTTTTTTTTTTTTT-3′) and the enzyme Superscript (Gibco BRL), according to the manufacturer's instructions. One percent of the cDNA was used as a template for polymerase chain reaction with the primers OG1 and cp3 (5′- GGICCIGTICCIYTITTYTTYCC-3′, where I is inosine and Y is T or C). The reaction was carried out in a thermal cycler with Taq DNA polymerase (Gibco BRL), for 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 74°C, according to manufacturer's instructions, with 2 mM $MgCl_2$ and primer concentrations of 1 μM. The ~800–base pair amplified fragment was cloned and used to screen a cold-acclimated carrot tap root cDNA library (ZAP cDNA kit; Stratagene) to obtain the full-length AFP clone. Seventeen hybridizing plaques were identified, seven were partially sequenced, and two were sequenced to completion, both of which contained identical coding regions representing the carrot AFP.
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Role for the Target Enzyme in Deactivation of Photoreceptor G Protein in Vivo

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Heterotrimeric guanosine 5′-triphosphate (GTP)–binding proteins (G proteins) are deactivated by hydrolysis of the GTP that they bind when activated by transmembrane receptors. Transducin, the G protein that relays visual excitation from rhodopsin to the cyclic guanosine 3′,5′-monophosphate phosphodiesterase (PDE) in retinal photoreceptors, must be deactivated for the light response to recover. A point mutation in the γ subunit of PDE impaired transducin-PDE interactions and slowed the recovery rate of the flash response in transgenic mouse rods. These results indicate that the normal deactivation of transducin in vivo requires the G protein to interact with its target enzyme.

In numerous signaling cascades, G proteins relay signals from seven-helix receptors to target effector molecules. G proteins become activated upon receptor-catalyzed binding of GTP to the α subunit and then modulate the activity of an effector until the bound GTP is hydrolyzed to guanosine diphosphate (GDP) (*1*). Two different mechanisms control the rate of GTP hydrolysis. First, the guanosine triphosphatase (GTPase) activities of at least two G proteins (transducin and G_q) can be increased by their target enzymes in vitro (*2, 3*). Second, the rate of GTP hydrolysis can be accelerated by members of the regulators of G protein signaling (RGS) protein family. RGS proteins are strong GTPase activators for a large spectrum of G proteins (*4*). The individual roles of the effector and the RGS protein in controlling G protein deactivation in vivo are not known.

The phototransduction cascade of retinal rods is a particularly accessible G protein signaling system (*5*). Upon absorption of a photon,

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