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Accumulation of type I fish antifreeze protein in transgenic tobacco is cold-specific

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Abstract

Expression of fish antifreeze protein (AFP) genes in plants is a possible means of increasing their frost resistance and freeze tolerance. Initial work involved transfer into tobacco of an AFP gene from winter flounder which codes for the alanine-rich, α-helical Type I AFP. Plants were transformed with a gene construct in which the preproAFP cDNA was inserted between the cauliflower mosaic virus 19S RNA promoter and the nopaline synthetase polyadenylation site. Although transgenic plants produced AFP mRNA, no AFP was detected on western blots. Re-evaluation of AFP expression in these transgenic plants showed that AFP accumulated to detectable levels only after exposure of the plant to cold. Extracts of plants incubated at 4 °C for 24 h contained a protein which co-migrated with winter flounder proAFP and was cross-reactive to Type I AFP antisera. Two other minor protein bands of slightly higher apparent M_r also cross-reacted with the antisera and are thought to represent processing intermediates. The proAFP was unique to the transgenic plants and was absent in extracts taken prior to cold exposure. AFP levels increased over the first 48 h of cold incubation then remained stable. Since the α-helix content of Type I AFP has been shown to decrease markedly at warmer temperatures, we postulate that Type I AFP stability in transgenic plants is dependent on its secondary structure.

Introduction

Certain species of marine teleosts survive in ice-laden seawater at temperatures below the freezing point of their unprotected blood sera by producing antifreeze proteins (AFP) or glycoproteins (AFGP) [11, 6]. These proteins depress the serum freezing point by binding to ice crystals and inhibiting their growth until the temperature is lowered further. Based on this property alone it has been suggested that the introduction of fish AFP into plants through gene transfer might increase their freeze resistance [7]. Antifreeze proteins and glycoproteins (AF(G)P) have subsequently been shown to neutralize ice nucleators [28], to inhibit ice recrystallization [22] and to protect cells and tissues from damage at cryogenic and hypothermic temperatures [34]. The latter two AF(G)P activities might also benefit plants by increasing their freeze tolerance [21].

The alanine-rich Type I AFP produced by winter flounder [13] was the first fish AFP to be

structurally defined [40]. The mature protein is a single amphipathic α-helix molecule, 37 amino acids in length. It is initially translated as a preproprotein [31, 8] and is exported to the circulatory system where the pro peptide is removed [17]. Vacuum infiltration of mature Type I AFP into the extracellular spaces of cannola, potato and tobacco leaves was shown to result in an average 1.8 °C decrease in the spontaneous freezing temperature of treated tissue [5]. Antifreeze activity was manifested as a decreased rate of ice crystal formation and an overall decrease in the amount of freezable tissue water frozen at any given temperature.

To date Type I AFP has only been expressed in plant systems as a fusion protein. After electroporation of a Type I preAFP fusion gene into corn protoplasts, AFP was expressed as an N-terminal extension of chloramphenicol acetyl transferase (CAT) protein [16]. Based on CAT assays, ca. 25% of the protein was exported to the extracellular space of the cell. However, the artificial signal peptide cleavage site was suboptimal according to the algorithm of von Heijne [39]. Type I AFP analogues have also been expressed in transgenic tomato plants as C-terminal fusions to truncated staphylococcal protein A [19]. Gene constructs encoding unfused mature AFP analogue were expressed in transgenic tobacco at the RNA level but did not produce sufficient AFP for detection of ice recrystallization inhibition activity [19].

Type I AFP fusion proteins have different levels of AFP activity. The protein A-AFP fusion was shown to inhibit ice recrystallization in frozen extracts of the transgenic plant leaf [19]. However, this activity occurs at much lower AFP concentrations than those required for thermal hysteresis activity [22]. The natural pro-form of the AFP has 70% of the thermal hysteresis activity of the mature form of the protein [18]. But, proAFP expressed behind the first 289 amino acids of β-galactosidase (β-gal) was inactive until the β-gal extension was proteolytically removed [29].

Since mature Type I AFP and its proprotein are both very active, we elected to express in transgenic plants the natural AFP in preference to a fusion protein form. Accordingly, we have generated a line of transgenic tobacco plants carrying a gene construct encoding flounder Type I preproAFP. The gene construct was placed under the control of the cauliflower mosaic virus (CaMV) 19S promoter and transformed into plants by *Agrobacterium tumefaciens* Ti plasmid-mediated DNA transfer. Transgenic plants were self-crossed to establish a transgenic plant line. We have found that these plants express AFP mRNA at normal temperatures but accumulate AFP only under cold conditions. This observation relates to the relative stability of Type I AFP and has implications in the usefulness of this AFP type in freeze protection of plants.

Materials and methods

Gene constructs

DNA coding for winter flounder preproAFP [8] was excised from cDNA clone C17 [32] by digestion with *Hgi* AI and *Hin*d III (Fig. 1). Clone C17 contains a full-length cDNA, corresponding to genomic clone 2A-7 [9], that was inserted into the *Eco* RV site of plasmid pBR322 by homopolymeric tailing [32]. *Hgi* AI cut the cDNA in the 5′-untranslated region whereas *Hin*d III cut in the vector beyond the 3′ end of the cDNA. The *Hgi* AI/*Hin*d III fragment was force cloned into *Pst* I/*Hin*d III-digested pUC 18 such that the *Pst* I and *Hgi* AI overhangs were ligated together. The cDNA was released from this intermediate vector (pUC 18 + WFA) by digestion with *Bam* HI which cut 5′ to the cDNA in the pUC 18 multiple cloning site and at a *Bam* HI site created by homopolymeric tailing at the *Eco* RV site of pBR322 [32]. The *Bam* HI cDNA fragment was ligated into the *Bgl* II site of vector pMON 237 which lies between the 19S CaMV promoter and the nopaline synthetase (NOS) 3′-poly(A) attachment site. To discriminate against resealed pMON 237 the ligation products were incubated with *Bgl* II prior to transformation into *Escherichia coli* JM83. Clones were isolated with AFP cDNA in the sense (pMON 237 15^+) and antisense orienta-

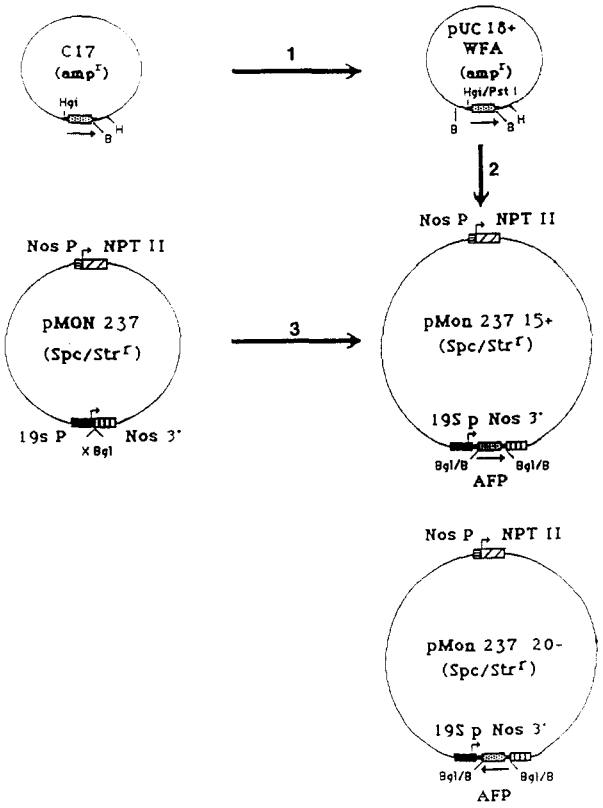

Fig. 1. Cloning strategy for generating Type I AFP gene constructs. The AFP cDNA is represented by the stippled bar, the 19S CaMV promoter by the black bar, and the NOS 3'-flanking DNA by the vertically stripped bar. Restriction sites for *Bam* HI (B), *Hin*d III (H), *Hgi* AI (Hgi) and *Bgl* II (Bgl) are indicated. Plasmid C17 containing the winter flounder AFP cDNA was digested (1) with *Hgi* AI and *Hin*d III and the released AFP cDNA fragment was force cloned into a pUC 18 vector cut with *Pst* I and *Hin*d III. The pUC 18-AFP construct (pUC 18 + WFA) was subsequently digested (2) with *Bam* HI and the AFP cDNA fragment ligated into a *Bgl* II-cut pMON 237 vector (3) and transformed into *E. coli*.

tions (pMON 237 20$^-$). Enzymes were used according to the manufacturers' recommendations. Recombinant DNA procedures were done as described by Maniatis *et al.* [25].

Agrobacterium-*mediated tobacco transformation*

The expression vectors pMON 237 15$^+$ and pMON 237 20$^-$ were transferred from *E. coli* to the disarmed *A. tumefaciens* vector (pGV3850, 60) by conjugation in the presence of the *E. coli* strain HB101 harbouring the mobilization plasmid pRK2013 [12]. *A. tumefaciens* containing the cointegrates were selected by growth on minimal media containing rifampicin (100 μg/ml), spectinomycin (100 μg/ml) and streptomycin (100 μg/ml), and were used to transform leaf discs from *Nicotiana tabacum* cv. Xanthi using the method of Horsch *et al.* [20].

Isolation of plant DNA and RNA

High-molecular-weight genomic DNA was isolated from 0.1 g or more of fresh or frozen tobacco leaf tissue using a scaled-down version of the Bernatzky and Tanksley method for tomato DNA isolation [2]. DNA yields were typically between 5–10 μg for each extraction. RNA was isolated by the method of Palmiter from leaves (6–10 g) removed from the top half of the plant [27].

Identification of transgenic plants by PCR

DNA amplification reactions were done in 1 × Taq buffer (Promega) containing 20 pmol of each PCR primer and 2.5 U of *Taq* DNA polymerase in a final volume of 50 μl. Each dNTP was present at 200 μM. Generally, 1 μg of template DNA was present per reaction but the amount was increased if the DNA sample seemed excessively sheared. DNA was subjected to 25 cycles of amplification where each cycle involved denaturation for 1 min at 96 °C, annealing for 1 min at 65 °C, and elongation for 2 min at 72 °C. Control amplification of the host acetolactate synthase (ALS) gene was conducted separately under the same conditions or in the same reaction.

Northern blotting

RNA samples were electrophoresed on 1.5% agarose gels at 4 V/cm in the presence of glyoxal

as a denaturant [38] and were blotted onto nylon membrane (zeta-probe GT, BioRad).

Protein analysis

Total protein extracts were prepared by the method of Gengenheimer from three to four leaves taken from the upper half of a healthy plant [15]. Protein extracts were prepared for western blotting by dialysis against 10 mM NH_4HCO_3 at 4 °C followed by lyophilization. Lyophilized protein samples were resuspended in Millipore-filtered water, and their concentrations estimated by the Bradford assay [3]. Western blots were prepared according to the method described by Burnette [4]. Aliquots of soluble protein extract (120 μg) were electrophoresed through a 4% SDS-polyacrylamide stacking gel and a 12–20% gradient separating gel [24]. The gel was trans-blotted overnight onto nitrocellulose (0.45 μM pore size, Mandel Scientific). Blots were treated with rabbit antisera to Type I AFP and goat anti-rabbit IgG peroxidase conjugate antibody (SIGMA). Protein antibody complexes were detected by chemiluminescence probing (Amersham).

Results

Identification of transgenic plants

Transformed plants were selected for growth on kanamycin. Resistance to this antibiotic was conferred by the cotransfer of a kan^r gene linked to the AFP gene. AFP gene transfer in surviving plants was established by the detection of AFP mRNA on northern blots (not shown). Progeny plants derived from self-crosses of transgenic parents were screened for the presence of the AFP gene by PCR (Fig. 2). Primers, 5′-ATGGCTCTCTCACTTTTCACTGT-3′ (sense) and 5′-CATCAAGACGACCACGATCCTTAAC-3′ (antisense), amplified a 268 bp fragment representing the entire AFP coding region between the initiation and termination codons (underlined) (Fig. 2A). Type I AFP cDNA is very GC-rich

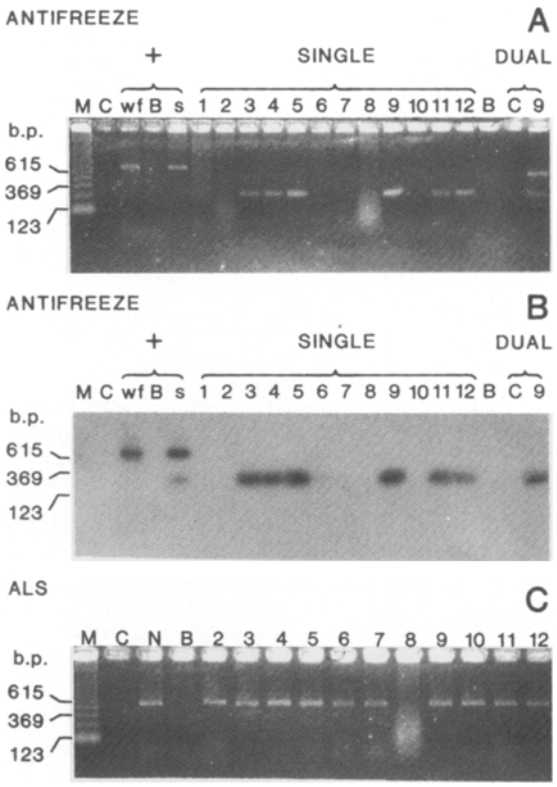

Fig. 2. PCR amplification of the Type I AFP transgene to identify transgenic plants. A. Ethidium bromide-stained gel of amplification products derived from the AFP primers (single), or both the AFP and ALS primers (dual). M refers to the 123 bp DNA ladder size markers, C is a control reaction in which template DNA was omitted, and B is a blank. The larger PCR products from winter flounder (wf) and transgenic salmon containing the winter flounder AFP gene (s) correspond to the genomic PCR product, and contain intron sequences. Samples 1–12 were derived from progeny plants that were potentially transgenic. B. Southern blot analysis of the gel in (A) showing hybridization to ^{32}P-labelled winter flounder AFP cDNA. C. Ethidium bromide-stained gel of the amplification products derived from the ALS primers. Other details are as described in (A) except that N represents a sample derived from a non-transgenic plant.

and, in comparison to other sequences has been difficult to amplify by PCR (unpublished results). High denaturation (96 °C) and annealing (65 °C) temperatures were required to achieve amplification. To distinguish between non-amplifiable and non-transgenic samples an internal control reaction was performed using primers that would amplify a fragment of the tobacco ALS gene. This

enzyme is essential for branch chain amino acid synthesis and is present in low copy number [26]. Primers, 5′-TCCCGTTTTGCTCCTGACGAACCCAGAA-3′ (sense) and 5′-TAAAAGCATCTCATTGGGCAATTAGGCAA-3′ (antisense) amplified a 602 bp fragment at the 5′ end of the tobacco ALS mature protein-coding region from nucleotides 430 to 1032 [26] (Fig. 2C). Reaction conditions were compatible for amplification of ALS and AFP gene sequences as single or dual amplifications (Fig. 2A). The identity of amplification products was initially confirmed by blot hybridization to a winter flounder AFP cDNA probe (Fig. 2B), then by size in subsequent screens. The ALS control reaction was used as the discriminator in samples which lack the AFP gene PCR product (lanes 2, 6, 7, 8 and 10) between a non-amplifiable DNA sample (lane 8) and non-transgenic DNA (lanes 2, 6, 7 and 10). Identified transgenic plants were self-crossed through several generations to establish a line of transgenic plants.

AFP expression in transgenic plants

Transgenic tobacco plants transformed with sense (pMON 237 15^+) and antisense (pMON 237 20^-) versions of Type I AFP cDNA both produced AFP mRNA transcripts of the expected size (500–700 nt) that were detected on northern blots probed with a nick-translated, double-stranded AFP cDNA (not shown). There was no such hybridization with RNA from a control plant that had been transformed with pMON 237 lacking an AFP gene insert. The sense and antisense AFP mRNA transcripts were at least as abundant as actin mRNA used as an internal control. Despite the evidence of AFP gene expression at the mRNA level, western blot analysis of either total protein or TCA-soluble extracts failed to detect the presence of AFP in parent or progeny transgenic plants.

These initial protein analyses were done on plants that were maintained in an incubator under optimal growth conditions. One explanation for the failure to detect AFP under these conditions is that the protein could be degraded as quickly as it is made. Type I AFP is a single α-helix devoid of tertiary structure [40]. Consequently, the helix content is very temperature-dependent and the protein is half unfolded at 25 °C [1]. On the premise that Type I AFP is more susceptible to proteolytic degradation under these conditions, the western blotting study was repeated with plants from the sense orientation transgenic line incubated for different periods at 4 °C. This resulted in the dramatic appearance of an AFP product. Protein extracts of plants incubated for 24 h at 4 °C were found to contain a protein that co-migrated with winter flounder proAFP and cross-reacted with the Type I AFP antisera (Fig. 3). Two other minor bands of slightly higher M_r also cross-reacted with the antisera. All three proteins were unique to the transgenic plant protein extracts and were absent in extracts of plants taken prior to cold exposure. AFP levels increased over the first 48 h of cold incubation then remained stable.

The AFP gene construct present in these plants is under the control of the constitutive CaMV 19S promoter and transcription of the transgene would not be expected to vary much under the conditions of the experiment. Nevertheless, northern blot analysis was conducted on RNA extracts of cold-exposed plants to determine if the observed AFP accumulation could be the result of a change in AFP mRNA levels (Fig. 4). Two sizes of RNA transcript were detected using AFP cDNA as a probe. The smaller of these AFP mRNA from transgenic tobacco is larger than the standard AFP mRNA from flounder liver because it has a 5′ extension of 43 nucleotides derived from the 19S RNA leader sequence. Other mobility differences might stem from a difference in the length of the poly(A) tract. The larger transcript (ca. 780 bp) matched the predicted size of an AFP mRNA that used the downstream NOS polyadenylation signal. When a similar blot was probed with NOS DNA only the larger transcript hybridized (not shown). These AFP mRNAs were observed in plants at 25 °C prior to exposure to low temperatures. A gradual increase in the steady state level of AFP transcripts was

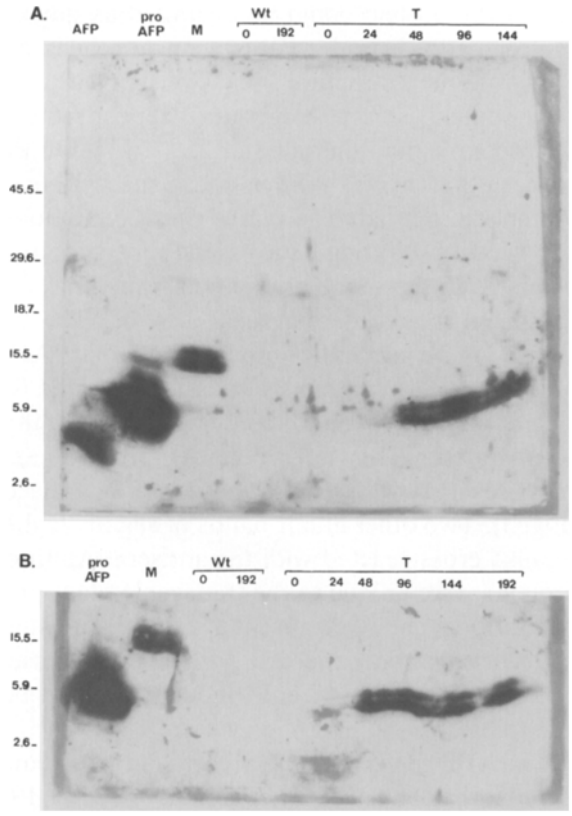

Fig. 3. AFP accumulation in Type I AFP transgenic plants exposed to cold conditions. Protein extracts from Type I AFP transgenic plants TI-39$_2$ K (A) and TI-39$_2$ J (B) were analyzed by western blotting. Transgenic (T) and wild type (Wt) plants were incubated at 4 °C for up to 192 h. The time of incubation at which protein was extracted are as marked. Positive controls include winter flounder sera (AFP) containing approximately 100 ng mature Type I AFP, and 10 ng of purified proAFP (proAFP) produced in transformed *E. coli* [29]. Molecular weight standards (M) were also run on the gels and the sized of these standards (in kDa) have been marked to the left of the autoradiographs.

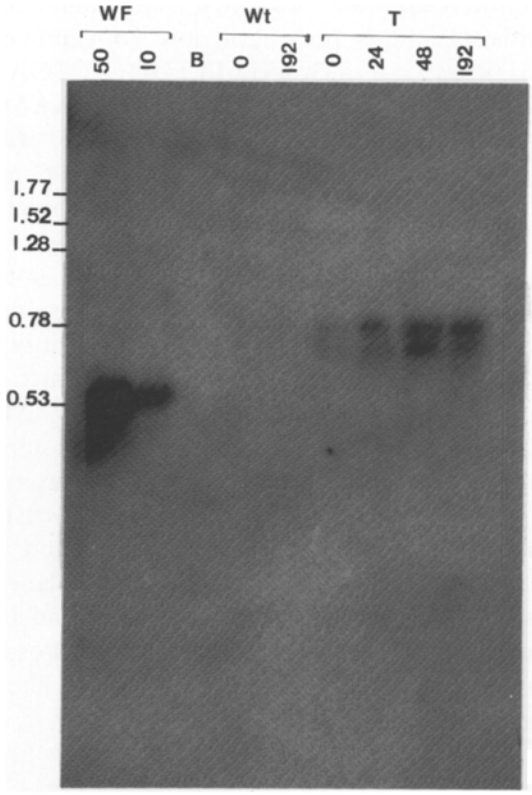

Fig. 4. Northern blot analysis of RNA extracts of cold-exposed plants. Transgenic (T) and wild-type (Wt) plants were incubated at 4 °C for up to 192 h. The time of incubation at which RNA was extracted is marked. Total RNA from winter flounder (Wf) containing the equivalent of 50 ng and 10 ng of AFP mRNA, and 10 μg of total plant RNA extracts were included in the analysis. B indicates an empty lane. The blot was probed with a genomic Type I AFP DNA probe [9]. RNA size standards were also run on the gel and are indicated on the left of the autoradiograph.

observed following incubation in the cold ((Fig. 4), but the increase was not as dramatic as that seen in proAFP levels.

Discussion

Prior to this report Type I AFP had only been produced in transgenic plants as a fusion protein. We have shown that the proAFP form of the AFP will accumulate if the plant is exposed to low temperatures. This result suggests that in previous analyses, where plants were kept at 25 °C, AFP was expressed in the transgenic host but did not accumulate to a detectable level. Since the 19S CaMV promoter is not known to be temperature sensitive, the accumulation of AFP at 4 °C appears to be a post-transcriptional phenomenon. We attribute the accumulation to a decrease in AFP mRNA turnover and enhanced stability of the AFP at low temperatures. CD spectra have shown that the α-helix content of Type I AFP is temperature-dependent. At −1 °C the helix content of the 37 amino acid Type I AFP

is 85% or greater, but falls to 47% at 25 °C, and is close to zero at temperatures above 70 °C as the structure becomes a random coil [1]. All previous analyses of Type I AFP expression in transgenic plants were conducted on plants maintained at optimal growing temperatures between 20 and 25 °C. It is probable that the decreased α-helix content of the AFP at the higher temperatures resulted in an increased susceptibility to degradation by proteolysis. The presence of C-terminal CAT and N-terminal protein A domains in AFP fusion proteins may have decreased degradation of the AFP component and allowed AFP fusion proteins to be detected. Such protection has been observed in *E. coli* following the attachment of an N-terminal β-galactosidase fragment to the proAFP [29].

Little is known about Type I AFP stability *in vivo*. AFP production in winter flounder, the natural host, is seasonal [37] and negatively regulated by growth hormone [14]. After the gene is down-regulated in the spring, the rate of AFP disappearance from the fish is insignificant until the seawater temperature increases [6]. Destabilization of the AFP structure at higher temperatures might increase its susceptibility to proteolysis and help explain the accelerated clearance of the antifreeze in late spring and early summer.

Most of the AFP produced by our transgenic tobacco plants co-migrated with proAFP. This suggests that the plant was able to recognize and process the fish transit peptide to some extent. This result is consistent with the finding that a small amount of preAFP-CAT fusion protein expressed in corn protoplasts was exported into the extracellular space. However, in this instance substantial changes were made to the signal sequence on either side of the signal peptidase cleavage point and only the N-terminus to hydrophobic core was conserved. Similarly, transgenic potatoes expressing the human serum albumin (HSA) gene recognized and cleaved th mammalian signal sequence to accumulate HSA in the extracellular space [36]. It has been postulated that plants act by a default mechanism and target proteins to the extracellular space if a transit peptide is recognized sufficiently for secretion into the endoplasmic reticulum [10]. It is therefore likely that the proAFP expressed by our transgenic plants will also be present in the extracellular space.

The fact that the plants produce Type I AFP in the pro-form and do not process it to mature protein is consistent with results found in other transgenic systems. A genomic Type I AFP gene has been expressed in transgenic salmon [35[and *Drosophila melanogaster* [33], where proAFP was found to accumulate in the fish sera and insect hemolymph, respectively. A mechanism proposed for the removal of the pro peptide is stepwise cleavage by a dipeptidyl aminopeptidase IV [23]. This process must be very efficient in winter flounder where circulating concentrations of mature AFP reach 10 mg/ml. Liver, the main organ for AFP export, contains only proAFP, whereas flounder serum has insignificant amounts of this precursor present [18]. It is surprising, therefore, that Atlantic salmon appear not to process the proAFP [35]. However, Edman degradation of proAFP recovered from the hemolymph of transgenic *Drosophila* demonstrated two partially processed forms, one shortened at the N-terminus by one dipeptide unit and the other by two dipeptide units [30]. Inexplicably the slightly truncated proAFP in *Drosophila* hemolymph migrates more slowly on SDS PAGE than authentic proAFP [33]. This might be one explanation for the minor bands on the western blot (Fig. 3) that migrate more slowly than the proAFP standard.

Since proAFP has 70% of the activity of the mature Type I AFP, it could be very effective as an antifreeze and cryoprotectant in the extracellular space of the plant cell. Vacuum infiltration of the mature protein into this area has already been shown to decrease the spontaneous freezing temperature of the plant tissue [5]. We have demonstrated that it is possible to express a form of Type I AFP in transgenic plants without the addition of a fusion protein component. The amount of AFP present in the plant is temperature-dependent. Quite by chance there is an element of regulation built into the system, whereby AFP is apparently recycled in warm weather when not required, but should accumulate as the tem-

perature falls prior to the onset of frost conditions. In the laboratory, we have found that 48 h at 4 °C are required for AFP to build up to a steady-state level. It would be interesting to test these plants under field conditions to see if AFP can accumulate at intermediate temperatures or after a series of cold nights and warm days.

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