The Frost Tolerance of Tobacco Plants Transformed With the Gene Encoding the Antifreeze Protein From Winter Flounder.

Jung-sook Lee

Louisiana State University and Agricultural & Mechanical College

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The frost tolerance of tobacco plants transformed with the gene encoding the antifreeze-protein from winter flounder

Lee, Jung-Sook, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
THE FROST TOLERANCE OF TOBACCO PLANTS
TRANSFORMED WITH THE GENE ENCODING
THE ANTIFREEZE-PROTEIN FROM WINTER FLOUNDER

A Dissertation
Submitted to the Graduate Faculty of the
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requirements for the degree of
Doctor of Philosophy
in
The Department of Biochemistry

by
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<td>Antifreeze-protein</td>
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<td>AFGP</td>
<td>Antifreeze-glycoprotein</td>
</tr>
<tr>
<td>CaMV 35S</td>
<td>Cauliflower mosaic virus 35 S promoter</td>
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<tr>
<td>Ti plasmid</td>
<td>Tumor inducing plasmid</td>
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<tr>
<td>LIH</td>
<td>Left inside homology</td>
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<td>NOS3'</td>
<td>Nopaline synthase 3' polyadenylation signal</td>
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<td>NPT II</td>
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<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indol-1 glucuronide</td>
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<td>BA</td>
<td>Benzyladenine</td>
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<td>NAA</td>
<td>Napthaleneacetic acid</td>
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<td>2,4 D</td>
<td>2,4 dichloro-phenoxyacetic acid</td>
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<td>CAP</td>
<td>Calf alkaline phosphatase</td>
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<td>MES</td>
<td>4-morpholine ethansulfonic acid</td>
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<td>MOPS</td>
<td>Morpholinopropane sulfonic acid</td>
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<td>PMSF</td>
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<td>AP</td>
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<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
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<tr>
<td>NBT</td>
<td>Nitro blue tetrazolin</td>
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Abstract

The winter flounder, *Pseudopleuronectes americanus*, can survive in seawater at temperatures below freezing by producing antifreeze-proteins (AFPs) which depress the freezing point of their cellular fluids. The antifreeze gene (IIA7 cDNA) encodes a 91 amino acid preproprotein which is processed to a mature protein of 53 amino acids. This gene encoding only the mature antifreeze-protein, including a start methionine, was cloned into a plasmid which allowed enhanced expression from a double CaMV 35S promoter. The AFP gene construct was subcloned into both the intermediate vector pMON200 and the binary vector pBI121. After triparental mating and infection of tobacco leaf discs with *Agrobacterium tumefaciens* containing either pBI121AF or pMON200AF, transgenic plantlets were obtained which were kanamycin resistant and GUS positive. Southern analysis confirmed the presence of single-copy gene integration. The transcription levels of the antifreeze gene were significantly higher than those of a single CaMV 35S promoter and a Western blot confirmed the synthesis of the immunoreactive 5.5 kd antifreeze-protein in the transgenic tobacco tissues. Several individual plant
seedlings which were kanamycin resistant were selected and tested for frost tolerance. At least 30% more transgenic plants survived than the control plants. These results confirmed the ability of this fish protein to confer increased frost-tolerance to plants.
Introduction

Plants may experience different environmental stresses such as freezing, drought and salt stress. Common to these stresses is a lowering of water potentials in the plant cells. The major stress caused by freezing is cellular dehydration in plants. When plants are exposed for a few hours or days to low nonfreezing temperatures, plants can acquire increased freezing tolerance. This process is known as cold acclimation or cold hardening. During cold acclimation, the plant cells modify several physiological parameters leading to an improved cold resistance and induce the expression of a new set of genes. Cold acclimation is the primary adaptation to freezing stress in the plant system. Cold acclimation has been extensively studied to elucidate the cold acclimation process as well as to reveal the mechanism of freezing injury and tolerance. However, the molecular basis for cold acclimation is not yet clear. Many biochemical alterations have been shown to occur during cold acclimation, including changes in free sugar concentration, soluble protein content, abscisic acid content, and membrane lipid composition, but cause and effect relationships remain to be established.
Another attempt to increase frost tolerance in plants will be the application of antifreeze-protein (AFP) originating from fishes. Because the AFP from winter flounder exerts a profound antifreeze effect by depressing the freezing point of fish's body fluids, the introduction of the AFP gene into a plant system may be a challenging area to increase the frost tolerance in plants.

Exposure of plant tissues in a solution of the winter flounder antifreeze-protein has confirmed the effectiveness of the AFP in decreasing the freezing temperature of plant tissues. In addition, one of the cold-induced proteins, the kin1 gene product expressed in cytoplasm, has a similar composition to that of winter flounder AFP. The similarity indicates that it may be a natural antifreeze-protein of the plant in response to cold stress. The above findings suggest that the expression of the AFP gene within a cytoplasmic location is likely to confer increased frost tolerance in plants.

Therefore, the purpose of this research is the introduction of the winter flounder AFP gene into tobacco plants to obtain frost tolerance. Because sudden temperature changes can occur in early spring or late fall that do not allow sufficient time for plants to become cold acclimated, plants incur severe cold damage. If plants
contained the AFP gene, which will be designed to be expressed constitutively, then this freezing injury will be diminished, and the plants will have time to achieve cold acclimation.

In this dissertation, we report the ability of AFP to confer frost tolerance in plants. The mature AFP gene, which was designed to contain its own initiation and termination codons, was placed under the control of the duplicated cauliflower mosaic virus (CaMV) 35S promoter for enhanced expression. The AFP gene construct was then introduced into tobacco plants by using an Agrobacterium-mediated plant transformation system. The stable integration and inheritance of the AFP gene into tobacco plant was determined. Finally, the AFP gene expression in these plants and their frost resistance was investigated.
Literature review

1. Antifreeze-Protein (AFP)

Proteins acting as a antifreeze substance were found in studies of fishes in the polar oceans and the near shore waters of the north temperate oceans. During the winter season, these oceans are at the freezing point of seawater (-1.9°C), a temperature well below the freezing point of a typical marine teleost (-0.8°C), but fishes in these oceans can survive in that seawater temperature. In most temperate marine fishes, sodium chloride is the principal electrolyte present in the blood, but in fishes inhabiting freezing environments, it is responsible for only 40-50% of the observed freezing point depression (DeVries,1984). The remaining antifreeze substances, responsible for the freezing point depression, were identified as a set of glycoproteins and proteins (DeVries,1988; Burcham et al.,1984; O'Grady et al.,1982; DeVries,1983).

Antifreeze-glycoproteins (AFGPs) have molecular masses ranging between 2.6 and 34 kd. They contain a tripeptide repeat (Ala-Ala-Thr)ₙ with a disaccharide moiety (-Naga-Gal) attached to the threonyl residues (DeVries et al.,1970).
Antifreeze-peptides (AFPs) ranging between 3.3 and 14 kd are of three types (Davies et al., 1988; Davies and Hew, 1990). Type 1 are the alanine-rich, amphiphilic, \( \alpha \)-helical AFP (Mr 3-5 kd) found in winter flounders and sculpins. Type 2 are the cystine-rich AFP (Mr 14 kd) found in sea raven. Type 3 are the AFP (Mr 6-7 kd) found in eel pouts, which lacks any distinctive features in its composition and sequence. The type 1 AFP of winter flounder is the most extensively characterized AFP in its structure and mechanism.

1) Mechanisms of AFP action

Many researchers have studied the antifreezing mechanism and the structural requirements of the AFP from winter flounder (\textit{Pseudopleuronectes americanus}) because of its effective action and its small size. There are some variations in the size and amino acid composition of AFPS depending on the separation method used to purify the AFP from fish's serum (Scott et al., 1987; Scott et al., 1985; Gourlie et al., 1984; Pickett et al., 1984; Davies et al., 1984).

The winter flounder AFPS have of molecular mass between 3300-5000 Da. The smallest antifreeze-protein sequences (37 amino acids) are shown in figure 1. The
Figure 1. Sequence of mature antifreeze-protein from winter flounder. The protein is displayed to emphasize the 11-amino acid repeats beginning with threonine. Residues on the right-hand side are numbered. Hydrophilic residues postulated to interact with the ice lattice are boxed. The upper sequence is the AFP component A from winter flounder serum (Scott et al., 1987), and residues in parentheses below positions 18, 22, and 26 are the substitutions that occur in winter flounder antifreeze peptide 3 (Lin and Gross 1981).
distinctive feature is the repeating 11-amino acid sequence of Thr-(X)2-polar amino acid-(X)7, where X is predominantly alanine. The AFP has an α-helical content over 80 % and shown to be a rod shaped molecules.

The study of chemically synthesized AFP analogs showed the structure-function relationships in a winter flounder AFP (Chakrabartty et al., 1989a). The N-terminal charged aspartic acid is important to stabilize the helix formation. The charged amino acids are ideally located to stabilize the α-helical conformation of this AFP. Helix dipole and hydrophobic interactions are also involved in stabilizing the AFP helix. Two repeated units of 11 amino acids showed stable helix formation but lacked antifreeze activity. Therefore, a minimum of three repeating units are required for the antifreeze action (Chakrabartty et al., 1989b).

AFPs depress the freezing point by preventing ice crystal growth without changing the melting point, the so-called thermal hysteresis effect. Thermal hysteresis values for most fish AFPs approach a plateau value of greater than 1°C. A high thermal hysteresis value indicates high antifreeze activity. According to the
adsorption-inhibition theory in antifreeze mechanisms (DeVries, 1984; DeVries, 1988), the 4.5 Å distance between threonine and the polar amino acids is important because it is the same as the distance between adjacent oxygen atoms in an ice crystal lattice (Figure 2). The helical form of AFP binds to the ice crystal lattice by the formation of hydrogen bonds between the side-chain hydroxyl groups of threonine and the carboxyl groups of aspartic acid with oxygen atoms.

The crystal structure studies also showed that the interaction of the AFP dipole and the water dipole is important to orient the AFP to the prism face (the normal growth direction of ice) (Yang et al., 1988; Yang and Hew, 1988). The AFP dipole may induce the arrangement of water molecules on the ice surface to align their dipoles to the AFP helix dipole. This ordering may direct further hydrogen bonding of AFP to the prism faces.

AFP has amphipathic character. Hydrophilic side chains, are placed on one side of the helix, which bind oxygen atoms by hydrogen bonding, and bulky hydrophobic groups (mostly alanines) are projected on the other side of the AFP helix.
Figure 2. Hydrogen bonding of the antifreeze-protein with ice lattice prevents ice crystal growth. Black circles indicate oxygen atoms in the ice lattice that participate in hydrogen bond formation (DeVries 1984).
AFP is bound to the prism face of the ice crystal, the hydrophobic groups repel the binding of water to the ice crystal, resulting in the inhibition of ice crystal growth along the a-axis direction (Figure 3).

Once the AFP is bound to the prism face, growth of the ice crystal in the a-direction is inhibited, but basal plane (c-axis) growth is not, resulting in the needle shaped structure of ice crystals (Raymond et al., 1989). However, at high concentrations, the AFP may interact with the basal plane by hydrogen bonding and inhibit the growth of ice crystals in the c-direction as well. Another report suggested that blocking of growth in the c-axis direction may occur by inhibiting surface nucleation on the basal plane (Harrison et al., 1987).

The mechanism for AFP action, therefore, can be summarized as: After alignment of the AFP helix and ice dipoles to the ice surface, the hydrogen bonding between the AFP and ice inhibits the growth of the ice crystal, in which the amphiphilicity of the AFP contributes to deter other water molecules from joining the ice lattice.

2) AFP gene structure

The AFP genes of the winter flounder exist as a multigene family of about 40 members (Davies et al., 1984;
AFP binds preferentially to prism faces, through dipolar and hydrogen bond interaction.

Continued ice growth on basal plane and continued binding of AFP to prism faces results in bipyramidal ice crystal.

Figure 3. Schematic representation of AFP interaction with ice. Ice crystal growth along the a-axis direction (prism face) is inhibited, but ice still grows on basal plane (c-direction).
Gourlie et al., 1984; Scott et al., 1985; Scott et al., 1988; Hew et al., 1988). Genomic Southern blots and restriction maps of genomic clones showed that the AFP genes of the winter flounder exist in 7- to 8- kb elements linked in direct tandem repeats. Five or more AFP gene elements (approximately 40 kb) are linked together, representing clusters of AFP gene elements in the genome. Although the repeated elements which contain a single AFP gene are highly homologous, they show some different restriction patterns.

Therefore, the cDNA clones characterized by different research groups showed some variations in their number, size and amino acid composition (Davies et al., 1982; Lin and Gross, 1981). Several nucleotide sequences of AFP cDNA clones were presented: CT5, determined by Davies et al (1982); IIC10, determined by Lin and Gross (1981); and IIA7, determined by Gourlie et al (1984).

The synthesis and degradation of antifreeze-protein is controlled by hormonal response to both temperature and photoperiod (Hew et al., 1986). The AFPs are exceedingly abundant during the winter season (9 mg/ml). The AFP mRNA levels are 0.5% of the total liver RNA in the winter, but <0.001% in the summer. The preproprotein, the precursor of AFP, is synthesized and processed in the fish liver,
secreted in the serum as a form of proprotein, and finally processed as mature antifreeze-protein (Figure 4). Among the post-translational modifications are the removal of the glycine residue and the amidation of the c-terminus.

The nucleotide sequence and the amino acid sequence of IIA7 cDNA clone which contains four repeating units of 11 amino acids are shown in Figure 5. High alanine content (72% alanine in the mature protein) of AFP contributes to high G.C content (79%) in the nucleotide sequence. The predicted protein is 91 amino acids, composed of a mature protein of 53 amino acids and a prepro-region of 38 amino acids. The pre-region consists of a hydrophobic-rich signal sequence (21 aa long), while the pro-region is proline-rich (17 aa long). Only the mature form of AFP is circulated in fish serum to depress the freezing point of the body fluid. The proprotein itself contains antifreeze activity and the reason for processing to the mature form is not known.

2. Freezing injury in plants.

Freezing of plant tissues and cell suspensions involves the redistribution of the liquid water.
Figure 4. Diagram of AFP synthesis in winter flounder. Approximate molecular weight of the proteins is in the parentheses.
Figure 5. The amino acid and nucleotide sequences of antifreeze protein cDNA clone IIA7. The hydrophobic-rich region, the proline-rich region, and the mature protein are indicated by PRE, PRO, and MATURE, respectively. The numbers correspond to the amino acid sequence of the mature protein.
The location of ice formation may be either extracellular or intracellular and is strongly influenced by the cooling rate. At relatively fast cooling rates, injury is caused by intracellular ice formation. The physical contact of the plant cell membranes with growing intracellular ice causes mechanical freezing damage.

During extracellular freezing, a water potential gradient is established between the extracellular ice crystal and the intracellular liquid water. The lower water potential of ice as compared to that of liquid cellular water will cause liquid water to move from the cell to the extracellular ice, resulting in cellular dehydration (Steponkus, 1984). This dehydration results in a concentration of intracellular and extracellular solutes which is called 'solution effects'. The solution effects include volumetric and area contraction, concentration of intra- and extracellular solutes, possible pH change because of different solubilities of buffering compounds, eutectic crystallization, and possibly the removal of water of hydration from macromolecules (Mazur, 1969).

Freezing damage to the plasma membrane is the major injury because it effects the plasma membrane's semipermeable characteristics. Whether the cell achieves equilibrium after continued dehydration or intracellular
ice formation is ultimately a consequence of the functioning of the plasma membrane (Steponkus, 1984; Hincha et al., 1987). The plant cell wall also influences the severity of freezing injury, because the cell wall provides a protective role against mechanical strains occurring during freeze-thaw stress (Bartolo et al., 1987).

1) Cold acclimation

When plants are exposed in subzero temperatures, they become tolerant to freezing temperature (Levitt, 1980). This phenomenon known as cold acclimation or cold hardening is a complex response involving a variety of physiological and biochemical changes. During cold acclimation, many changes occur in membrane lipid composition, carbohydrate content, osmotic concentration, hormonal balance and protein quality and quantity (Sakai and Larcher, 1987). These changes seem to adapt the plants to chilling or freezing temperatures and to resist any subsequent freezing temperatures.

Changes in membrane lipid composition can contribute directly to the freezing tolerance of plant cells (Steponkus et al. 1988). In addition, proline and many simple sugars, as well as certain soluble polypeptides, have cryoprotective effects (Van Swaaij et al., 1986; Chen
et al., 1983). The concentration of such cryoprotectants is increased during cold acclimation.

There are many reports that describe the changes in gene expression, including the synthesis of new transcripts and polypeptides, during cold acclimation in several plant species (Mohapatra et al., 1987; Kurkela et al., 1988; Gilmour et al., 1988; Meza-Basso et al., 1986; Guy and Haskell, 1987; Robertson et al., 1988). The changes in proteins during cold acclimation are regulated most probably at the transcriptional level. These cold-induced changes in protein synthesis and mRNA accumulation involve both the suppression as well as the induction of genes. For example, several cold-induced proteins are specifically increased in rice leaves after cold treatment. However, Rubisco synthesis and other chloroplast-encoded mRNA, and the nuclear encoded chlorophyll a/b binding protein are strongly repressed in the cold. This indicates that some chloroplast functions are disturbed during cold stress (Hahn and Walbot, 1989).

Another major change in cold acclimation is an increase in abscisic acid concentrations (Bornman and Jansson, 1980; Daie and Campbell, 1981; Chen et al., 1983; Chen and Gusta, 1983). Abscisic acid (ABA), a naturally occurring plant hormone, has been shown to play an
important role in plant water balance and in the adaptation of plants to stressful environments. Therefore, ABA has been proposed as a common mediator for plant responses to stresses. Cold acclimation-induced freezing tolerance is mediated by increased levels of endogeneous ABA which acts by activating the genetic system responsible for the freezing-tolerance response. ABA-induced gene expression is related to cold acclimation or freezing tolerance (Mundy and Chua, 1988; Mohapatra et al., 1988).

Several cDNAs related to cold- and/or ABA-inducible genes have been characterized (Kurkela and Franck, 1990; Lin et al., 1990; Close et al., 1989; Cattivelli and Bartels, 1990; Skriver and Mundy, 1990). The deduced amino acid sequence from cDNA clones showed two categories of protein involved in cold-induced gene expression. In one, the protein serves a regulatory function to control the gene expression related to cold acclimation. In another, the newly synthesized protein itself serves as a cryoprotectant.

In barley, the proteins deduced from cDNA clones of cold-regulated genes contain arginine rich basic domains. This suggests that these cold-induced protein may stabilize specific mRNA during cold stress because arginine-rich sequences are involved in protein-RNA interactions.
The dehydration-induced proteins (dehydrins) were recently characterized from several cDNAs related to an ABA-induced cDNA from barley and corn seedlings (Close et al., 1989). Each dehydrin is extremely hydrophilic, glycine rich, cystein- and tryptophan-free and contains repeated units in a conserved linear order. The function of these dehydrins are not yet known, but the existence of a highly conserved region with a consistent spacial arrangement may indicate that the dehydrins have a central role in gene regulation.

As an example of a cryoprotectants in cold-acclimating plants, Lin et al (1990) identified a number of the polypeptides as cold-regulated (cor) genes in both Arabidopsis thaliana and wheat. The distinctive features which they had in common were heat stability and a high degree of hydrophilicity. They suggested that these COR polypeptides have a fundamental role in plants acclimating to cold temperatures and these polypeptides may act as cryoprotectants.

The kin1 gene, characterized by Kurkela and Franck (1990), is induced at cold temperature as well as by water stress and by ABA in Arabidopsis thaliana. The level of kin1 mRNA is increased 20-fold in cold-treated plants.
The deduced 6.5 Kda polypeptide is quite hydrophilic and has an unusual amino acid composition, being rich in alanine, glycine and lysine. Due to the lack of a signal sequence, it is most probably located in the cytoplasm. The functions of Kin1 protein is unknown. However, sequence comparison of the deduced Kin1 protein with the AFP component B precursor from winter flounder revealed a certain similarity between them*. In addition to the small size, the amount of biochemically similar amino acids is 41%. Therefore, they suggest that Kin1 protein may serve as an antifreeze-protein in the plant's response to cold stress.

*Alignment of the deduced Kin1 protein with the AFP component B from winter flounder. Identical amino acids are shown by vertical lines, similar amino acids by double dots, and a low degree of similarity by single dots.

Kin1 27 DKAKDAAGA...GAGAQAGKSVDIAAGGGVNOVTDKTLNIK 66
  |||||..||.||.||.|..|.|..|.|..||.

AFPB 1 DTASDAAAAALTAANAAAAAKLTADNAAAA.....AATARG 38

In addition, a small cold protection protein, CS7.4, has recently been described in *E.coli* (Goldstein et al., 1990). CS7.4 represents a new group of AFPs with no
similarity to the small fish AFPs except for the size and high proportion of hydrophilic residues.

2) Attempts to increase frost tolerance in plants and application of the AFP.

Attempts to improve tolerance to freezing stress have been studied in several ways. Recently, freezing-tolerant spring wheat was regenerated from cryoselected calli following freezing in liquid nitrogen without the addition of cryoprotectants (Kendall et al., 1990). The surviving (cryoselected) calli exhibited freezing tolerance, and the seed progeny of regenerated plants also maintained freezing tolerance but to a lesser degree than the callus itself.

Frost tolerance in plants based on cold acclimation have been studied to elucidate the cold acclimation mechanism which involves osmotic adjustment, and changes in water potential and growth capability (Yelenosky and Guy, 1989; Van Swaaij et al., 1987; Kacperska and Kulesza, 1987). Under the most favorable hardening conditions, plants can increase tolerance to extracellular freezing by decreasing the osmotic potential and water potential of the cell.

Another attempt to increase frost tolerance may be the
application of the AFP from winter flounder exogenously or endogenously. Exposure of plant tissues in a 1mg/ml solution of the winter flounder antifreeze protein in vitro has revealed that AFP can function as an anti-nucleator in plant tissues, decreasing the freezing temperature by an average of 1.8°C. Moreover, AFP could act as a cryoprotectant to reduce the freezable water amount, and decrease the rate of ice crystal formation (Cutler et al., 1989). These results strongly demonstrate the possibility of improving the frost tolerance of plants by introduction of an AFP gene.

Certain bacteria promote ice formation in plants inter- and intracellularly, through ice nucleation activity, and cause frost damage. Two bacterial species, Pseudomonas syringae var Hall and Erwinia herbicola, are widely distributed on leaves of numerous plant species, in which they serve as the ice nucleators (Lindow et al., 1982). Prody-Morreale et al (1988) described that fish antifreeze glycoproteins inhibit the ice-nucleating activity of membrane vesicles from the bacterium Erwinia herbicola. These results also indicate that fish AFP/AFGP can reduce the ice formation in other systems besides the fish.

Besides the in vitro application of AFP, the AFP gene
was successfully transferred to Atlantic salmon (Fletcher et al. 1988) and *Drosophila melanogaster* (Rancourt et al., 1987). The winter flounder AFP genomic DNA, under the control of the heat shock promoter, was incorporated into a Drosophila genome. The transgenic *D. melanogaster* expressed the AFP gene, including splicing and processing of the primary transcripts. The transcripts were translated and the AFP was secreted into the hemolymph as a form of proprotein of the winter flounder AFP. Those reports provide the possibility that limited freeze resistance can be conferred to plants through the transfer and expression of the AFP gene.

3. *Agrobacterium*-mediated plant transformation

*Agrobacterium tumefaciens* induces crown gall tumors by transferring oncogenes from the tumor-inducing (Ti) plasmid to plant cells. The oncogenes encode enzymes involved in biosynthesis of auxin and cytokinin, which cause unorganized proliferation of plant cells. A specific segment of the Ti plasmid which is called T-DNA is transmitted to plant cells and stably integrated into the plant nuclear DNA (Ream, 1989; Binns, 1988). The stable integration of foreign T-DNA into the plant nuclear genome
provides a useful tool for Agrobacterium-mediated plant transformation. The development of such a plant transformation system provides great potential in plant genetics to improve resistance to herbicides, insect attack, environmental stress, and bacterial and viral infections.

The interaction between Agrobacterium and plants begins with the attachment of the bacteria to the wounded plant cell surface. Three genetic loci encoded by the Agrobacterium chromosome have been defined as having roles in A. tumefaciens attachment to plant cells: chvA, chvB, and pscA (exoC) (Douglas et al., 1985). chvA and chvB are responsible for the synthesis of β-1,2-D-glucan which has a role in attachment. The pscA locus also has a role in the production of surface polysaccharides: cellulose fibrils, extracellular polysaccharide succinylglycan, and β-1,2-D-glucan (Thomashow et al., 1987).

Wounded plants produce numerous substances, including phenolic compounds, that presumably help the plant defend itself against further damage. Among several phenolic compounds acting as plant signal molecules are acetosyringone and hydroxyacetosyringone, which induce expression of specific vir genes in the Ti plasmid (Stachel
et al., 1985). The vir gene products are required for the excision of the T-DNA from the Ti plasmid, and for its transfer and integration into the plant genome.

The vir region is approximately 35 Kb and encodes six transcriptional loci: virA, virB, virC, virD, virE, and virG (Stachel and Nester, 1986). A constitutively expressed VirA protein interacts with vir inducing compounds. This interaction results in activation of the VirG protein by phosphorylation. Activated VirG protein increases transcription of its own gene and induces transcription of the virB,C,D,and E operons (Stachel and Zambryski, 1986). T-DNA transmission involves the action of vir gene products and the cis-acting border sequence elements, 25 bp direct repeats that flank all T-DNA regions from Ti plasmids. The virD operon encodes an endonuclease activity that generates specific single-strand nicks and double-strand cuts within the border repeats. The nick within the right border repeat initiates production of full length single-stranded copies of the bottom strand of the T region, and a similar nick in the left border repeat releases this single-stranded DNA (T-strand) from the Ti plasmid (Stachel et al., 1987).

The overdrive (ode) enhancer sequence, a 15bp element with a highly conserved 6bp core, lies near the right
border and stimulates T-strand production and T-DNA transfer. T-strand formation and right-border nicking are also stimulated by the presence of overdrive (Peralta et al., 1986; Veluthambi et al., 1988). A nonspecific single-stranded DNA binding protein encoded by virE2 binds and protects the single-stranded T-strands. Subsequently, T-DNA is transferred into plant cells by a process involving the virB operon.

T-DNA transfer is similar to the bacterial conjugation mechanism (Buchanan-Wollaston et al., 1987). However, the integration of T-DNA into the plant genome likely differs from the conjugation mechanism. T-DNA integrates at a variety of locations in the plant genome, as single copies or as short tandem arrays (direct or inverted repeats). Agrobacterium-mediated insertion into the plant genome appears to be random at the chromosomal level (Chyi et al., 1986).

The resultant transformed plant cells produce novel sugar and amino acid conjugates, termed opines, in addition to the plant hormones auxin and cytokinin. Opines are used by the inciting bacteria as a source of carbon and nitrogen and as an inducer of Ti plasmid transfer between bacteria.

Efficient transmission (transfer and integration) of T-DNA to a plant genome does not require tumorigenesis, but
requires, in cis, the right 25bp border repeat and, in trans, vir genes located outside of the T region of the Ti plasmid. Therefore, the Ti plasmid-based vector system has been developed to adapt these two essential features for the introduction of new DNAs into plant cells (Rogers et al., 1987; Bevan, 1984; Fraley et al., 1985; Barton et al., 1983). The altered Ti plasmids that have had the oncogene and opine synthase genes deleted or replaced by selectable antibiotic markers are referred to as 'disarmed' (Shahin and Simpson, 1986).

As the great size of the disarmed Ti plasmid and the lack of unique restriction endonuclease sites prohibit direct cloning into the T-DNA, intermediate vectors have been developed for introducing genes into the Ti plasmid. The intermediate vectors commonly contain the selectable antibiotic markers which function in plants and bacteria, the right border sequence and the multilinker site.

There are two kinds of intermediate vectors, cointegrating (cis) vectors and binary (trans) vectors. Cointegrating intermediate vectors such as pMON200 must carry DNA segments homologous to the disarmed T-DNA called the left inside homology (LIH) fragment. The LIH sequence provides the sites to be cointegrated into the disarmed Ti plasmid by homologous recombination. The cointegrated
intermediate vector is replicated by the Ti plasmid origin of replication. Binary vectors are developed by finding that the T-DNA did not have to be physically joined to the Ti plasmid (Hoekema et al., 1983). Because a binary vector such as pBI121 has an origin of replication that functions in both \textit{E.coli} and \textit{A.tumefaciens}, it is able to replicate independently of the Ti plasmid (An et al., 1988).

To introduce the intermediate vectors into \textit{A.tumefaciens}, there are two methods, conjugation and transfection. The conjugation method (triparental mating) needs three bacteria: \textit{A.tumefaciens} cells carrying the disarmed Ti plasmid, \textit{E.coli} cells carrying a intermediate vector and \textit{E.coli} cells carrying the pRK2013 helper plasmid. The mobilization plasmid pRK2013 provides the RK2 transfer functions and the ColE1 mob protein, allowing the intermediate vector to be mobilized into \textit{A.tumefaciens} (Rogers et al., 1988). The transformation frequency by direct \textit{A.tumefaciens} is low (approximately \(10^3\) transformants per ug DNA) compared to the conjugation method. However, the technique is reliable and very rapid, and also eliminates much of the plasmid rearrangement that often occurs during the conjugation method (An et al., 1988).
Materials and Methods

Materials

1. Bacterial Strains

*E. coli* JM101, JM107, HB101 and DH5α' were used for the transformation of the DNA constructs (Table 1). *Agrobacterium tumefaciens* LBA4404 and GV3111SE were used for the transformation of pBI121 and pMON200 derivatives, respectively (Table 2).

2. Vectors

Table 3 provides a list of the plasmids used in these experiments, as well as the purpose of each plasmid.

3. Radioactive Compounds

[α-32P] and [γ-32P] dNTPs (3000 Ci/mmole) were purchased from ICN Biomedicals, Inc. (Irvine, CA) and New England Nuclear (NEN)/Du Pont Co. (Wilmington, DE).

4. Enzymes, DNA, RNA and Proteins

All restriction endonucleases, DNA ligase, DNA kinase, T4 DNA polymerase, and the Klenow fragment were purchased
Table 1. *E. coli* strains

<table>
<thead>
<tr>
<th><strong>E. coli</strong></th>
<th><strong>Genotype</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>JM101</td>
<td>(lac proAB) supE, thi/F', tra D36, pro AB, lac Iqz M15</td>
<td>Messing et al., (1981)</td>
</tr>
<tr>
<td>JM107</td>
<td>(lac proAB) supE 44/F', tra D36, proAB, hsdR17 (rk-mk+) thi, gyrA96, endA1, lac Iqz M15</td>
<td>Yanisch-Perron et al., (1985)</td>
</tr>
<tr>
<td>HB101</td>
<td>F-, hsdS20(rb-mb-), recA13, leuB6, ara14, proA2, lacY1, galK2, rpsL20(str), xyl-5, mtl-1, supE44-</td>
<td>Boyer et al., (1969)</td>
</tr>
<tr>
<td>DH5α'</td>
<td>F', endA1, recA1, lacZ M15, hsd R(rk-mk-), supE44, thi-, gyrA96, relA1, (lac ZYA-argF), U169</td>
<td>BRL Focus (1986) 8:2,9</td>
</tr>
</tbody>
</table>
Table 2. *Agrobacterium tumefaciens* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA4404</td>
<td>a non-tumor-forming derivative of LBA4401, it contains a Ti plasmid, PAL4404, with deleted T-region but intact vir-region; streptomycin resistance</td>
<td>Oomas et al., (1982)</td>
</tr>
<tr>
<td>GV3111SE</td>
<td>disarmed strain which carries pTi B6 S3-SE, it contains only the TL DNA left border sequence and a region of homologous Ti DNA which allows for cointegrate formation; chloramphenicol and kanamycin resistance</td>
<td>Fraley et al. (1985)</td>
</tr>
</tbody>
</table>
Table 3. Plasmids used for cloning

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>4.3</td>
<td>initial cloning of mature AFP gene, DNA amplification</td>
<td>Boliver (1978)</td>
</tr>
<tr>
<td>pMON530</td>
<td>12.0</td>
<td>intermediate vector for further cloning</td>
<td>Rogers et al., (1987)</td>
</tr>
<tr>
<td>pIBI76</td>
<td>4.2</td>
<td>cloning vector for use in the poly I linker region</td>
<td>Dente et al., (1983)</td>
</tr>
<tr>
<td>pCa2</td>
<td>3.3</td>
<td>source of the double cauliflower mosaic virus promoter</td>
<td>Kay et al., (1987)</td>
</tr>
<tr>
<td>pMON200</td>
<td>9.5</td>
<td>cointegrating vector compatible with Agrobacterium GV3111SE</td>
<td>Rogers et al., (1987)</td>
</tr>
<tr>
<td>pBI121</td>
<td>13.0</td>
<td>binary vector compatible with Agrobacterium LBA4404</td>
<td>Bevan (1984)</td>
</tr>
<tr>
<td>pRK2013</td>
<td></td>
<td>contains mob genes to mobilize intermediate vectors into A. tumefaciens during triparental mating</td>
<td>Fraley et al., (1985)</td>
</tr>
</tbody>
</table>
from Bethesda Research Laboratories [BRL] (Bethesda, MD), New England Biolabs, Inc. [NEB] (Beverly, MA), Promega Biotech Corp. (Madison, WI), Pharmacia (Piscataway, NJ), Boehringer- Mannheim Biochemicals [BMB] (Indianapolis, IN) and United States Biochemicals [USB] (Cleveland, OH). DNase, RNase, proteinase, and DNA, RNA, and protein size markers were purchased from BRL or USB.

5. Chemicals

Bacto-tryptone, bacto-yeast, bacto-peptone and bacto-agar were obtained from Difco Laboratories (Detroit, MI). Agarose, dithiothreitol(DTT), urea and phenol were purchased from BRL or American Research Products Company [AMERESCO] (Solon, OH). Acrylamide, N,N'-methylene bisacrylamide, TEMED and ammonium persulfate were purchased from Biorad (Richmond, CA).

Antibiotics ampicillin, tetracycline, kanamycin, spectinomycin, streptomycin and chloramphenicol were obtained from Sigma Chemical Company (St. Louis, MO). Most of the chemicals were purchased from Sigma, including 2-morpholine ethanesulfonic acid (MES), hexamine cobalt chloride, Tris- hydroxymethyl aminoethane (Tris), polyvinyl pyrrolidone (PVP), ethylenediamine tetraacetic acid (EDTA), tetramethyl ammonium chloride, sodium dodecyl sulfate
(SDS), polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), and Sephadex G-100. Rubidium chloride was obtained from Morton Thiokol Inc. (Danvers, MA).

Nitrocellulose filters were purchased from Schleicher and Schuell (Keene, NH). Duralon UV membranes were obtained from Stratagene (La Jolla, CA). Immobilon membranes were purchased from Millipore Corp. (Bradford, MA). Prepacked Sephadex G-50 columns came from 5Prime-3Prime, Inc. (Paoli, PA).

Tissue culture agar, phytoagar and premixed Murashige and Skoog salts were purchased from Hazleton Biologies, Inc. (Lenexa, KS). The phytohormones benzyladenine (BA), napthaleneacetic acid (NAA), 2,4 dichloro-phenoxyacetic acid (2,4 D), and the vitamins myoinositol, thiamine HCl, nicotinic acid, pyridoxine were purchased from Sigma. Cefotaxime sodium salt was purchased from Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ).

4-methylumbelliferone (MU), 4-methyl umbelliferyl glucuronide (MUG) and 5-bromo-4-chloro-3-indol-1 glucuronide (X-gluc) were purchased from Clontech and Research Organics, Inc. (Cleveland, OH).
6. Kits

Nick translation and random priming kits were purchased from BMB, BRL and USB. The GUS assay kit was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). The Western blot "Protoblot" kit was purchased from Promega. The Protein assay kit and Immunopure Ig-G purification kit were purchased from Pierce (Rockford, IL).

7. Others

The tobacco seeds (*Nicotiana tabacum* var *xanthii* and var *samsung*) were obtained from Dr. Hector Flores (Department of Plant Pathology, Pennsylvania State University). Pro-Mix soil, Jiffy pots, Temik pesticide and Miracle Grow fertilizer were purchased from BWI, Inc. (Jackson, MI). The antibody raised against winter flounder AFP was a gift from Dr. DeVries (Department of Physiology and Biophysics, University of Illinois).
Methods

1. Plasmid construction

1) Cloning of the mature AFP gene into plasmid pBR322

The winter flounder antifreeze-protein (AFP) cDNA clone, IIA7, which was provided by Dr. DeVries (Department of Physiology and Biophysics, University of Illinois), was digested with HpaII restriction enzyme. The 441-bp HpaII fragment was isolated and purified by agarose gel electroelution. The purified 441-bp DNA fragment was further digested with SfaN1 restriction enzyme to remove the 5' site of the poly(dG) tail and the pre-pro region of the AFP gene. SfaN1 recognizes the sequence of GCATC (5/9) with the reaction buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM MgCl2, 100 ug/ml BSA) at 37°C. The 258-bp of the larger fragment from the SfaN1 digestion was electroeluted from the agarose gel.

The synthetic oligonucleotide linker (GATCC ACC ATG GAC), producing the BamH1 site, the start codon, and the first amino acid codon of the mature AFP gene, was kinased and annealed with the 258-bp DNA fragment by heating the DNA to 90 °C for 10 minutes and then cooling to room temperature. The linker ligated DNA was fully digested
with Sau3A restriction enzyme to remove the 3' site of poly(dC) tail from the AFP gene, producing the complete coding sequence of the mature AFP gene. Figure 6 shows the scheme for the cloning of the mature AFP gene into plasmid pBR322.

The 174-bp Sau3A-digested AFP gene sequence was cloned into plasmid pBR322. pBR322 was cut with BamH1 and treated with calf alkaline phosphatase (CAP) to prevent its religation. The mature AFP DNA and CAP treated pBR322 were ligated and transformed into E.coli HB101. Transformants were selected by ampicillin resistance and tetracycline sensitivity. The presence of the 174-bp insert was confirmed by plasmid miniscreens of transformants. The resulting plasmid was called pBR322AF. The plasmid was prepared in large scale quantities and purified using cesium chloride banding.

2) Construction of the expression vectors.

The plant transformation vectors were constructed in several steps outlined in Figure 7. The 174-bp BamH1 fragment was excised from pBR322AF and cloned into the BglII site of plasmid pMON530. The polylinker site of pMON530 is located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase
Figure 6. Cloning of the mature region of antifreeze gene into pBR322.
Figure 7. Cloning of the antifreeze gene into pBI121 and pMON200.
polyadenylation site (NOS 3' terminator). Since the insertion of the 174-bp BamHI fragment into the BglII site of pMON530 destroyed both BamHI and BglII sites in the resulting plasmid pMON530AF (Figure 8), the plasmid miniscreen was accomplished by EcoR1 and EcoRV digestion.

The orientation of pMON530AF was determined by restriction enzyme mapping (Figure 9). The 324-bp EcoRV-EcoR1 fragment of pMON530AF was purified and digested with SfaN1. If the 174-bp insertion has a correct orientation, the digestion with SfaN1 generates 134-bp and 190-bp fragments. Otherwise, it produces 280-bp and 44-bp fragments.

The CaMV 35S promoter is a strong, constitutively expressed plant transcriptional promoter, but duplication of the CaMV 35S promoter sequence creates much stronger enhancement of plant genes with the transcriptional activity almost ten-fold higher than that of the natural promoter (Kay et al., 1987). Therefore, cloning of the mature AFP gene was further carried out to produce the duplicated CaMV 35S promoter sequence.

The EcoRV and HindIII fragment from pMON530AF was cloned into the plasmid pIBI76, which contains a polyI multilinker region just behind the HindIII site, hence it is useful for further cloning (Figure 10). The EcoRV and
Figure 8. Cloning of the mature AFP gene into pMON530. The resulting plasmid pMON530AF was identified by the presence of the 324-bp EcoRV-EcoRI fragment.
Figure 9. Determination of pMON530AF orientation.
Figure 10. Maps of the pIBI76AF and pCa2AF for further cloning into plant transformation vectors.
SacI fragment from the resulting pIBI76AF was cloned into the plasmid pCa2, which has the CaMV 35S promoter with a duplicated upstream region in pUC 18, creating plasmid pCa2AF (Figure 10).

The 1111-bp HindIII fragment from pCa2AF containing the duplicated CaMV 35S promoter, the mature AFP coding sequence and NOS 3' terminator was finally cloned into a binary vector pBI121 and a cointegrating vector, pMON200 (Figure 11). Plasmid pBI121 contains a β-glucuronidase (GUS) gene cassette under the CaMV 35S promoter and NOS terminator. This GUS gene construct expresses at high levels in transformed plant cells. Both pBI121 and pMON200 contain the neomycin phosphotransferase (NPT II) gene which confers resistance to the antibiotic kanamycin, so it is useful for selection of the transformed plants.

To determine the orientation of the pBI121AF and pMON200AF, pBI121AF and pMON200AF were digested with EcoRV, and with SstI and EcoR1, respectively.

The digestion of plasmid pBI121 with EcoRV produces 7 fragments, 4.5-, 2.6-, 2.5-, 1.8-, 1.7-, 0.7-, and 0.25-kb. The 1.1-kb AFP gene cassette is inserted into the site of 1.7-kb region, and the pBI121AF clone contains one EcoRV site between the CaMV 35S promoter and the AFP coding sequence. Therefore, if the insertion has a correct
Figure 11. Maps of the final clones, pBI 121AF (A) and pMON 200AF (B).
orientation, the EcoRV digestion of pBI121AF produces 1.5-kb and 1.3-kb fragments instead of 1.7-kb of pBI121 (Figure 12). If the 1.1-kb insertion has an opposite direction, the EcoRV digestion generates 1.6-kb and 1.2-kb fragments in the site of 1.7-kb of pBI121.

The digestion of pMON200AF clones with SstI and EcoRI generate different digestion patterns according to their orientations. If the orientation is correct, the digestion produces 820-bp and 1090-bp fragments, otherwise, it should be 290-bp and 1620-bp fragments (Figure 13).

2. Plant transformation and regeneration

1) *Agrobacterium* transformation

The *Agrobacterium* strains LBA4404 and GV3111SE were used for the transformation of pBI121AF and pMON200AF, respectively. Mating was followed by the triparental conjugation system, using the helper plasmid pRK2013 developed by Rogers et al (1988).

*Agrobacterium* LBA4404 was grown in AB-Bio medium at 28°C for two days. *Agrobacterium* GV3111SE was grown in LB medium at 28 °C. AB-Bio medium was prepared as follow:
Figure 12. Determination of pBI121AF orientation. The two possible orientations are shown here. Orientation I is the proper type to be expressed in the plant system. The arrows indicate the EcoRV restriction site.
Figure 13. Determination of pMON200AF orientation. Orientation II is the correct type for expression in the plant.
AB-Bio medium; 5g glucose
1ml CaCl₂.2H₂O (13 mg/ml)
0.3g MgSO₄.7H₂O
0.1ml FeSO₄.7H₂O (25 mg/ml)
100 ml 10X AB salt
1ml biotin (0.2 mg/ml)
100mg kanamycin per liter

10X AB salt; 30.0 g K₂HPO₄
10.0 g NaH₂PO₄
10.0 g NH₄Cl
1.5 g KCl per liter

The E.coli containing either pBI121AF or pMON200AF, and the E.coli containing pRK2013 were grown in LB medium plus antibiotics overnight. The cells were diluted back and grown to log phase. Then 1 ml from each of three cultures were mixed together, spun down and resuspended in 2 ml of 10 mM MgSO₄. The mixture was transferred onto sterile filter discs on a fresh, non-dried LB agar plate. The plate was incubated at 28 °C overnight. The filters were removed and placed into sterile tubes containing 2 ml of 10 mM MgSO₄. The solution was vortexed to remove the
cells from the filter discs. 100 ul of the cells were then plated onto an LB selection plate. Transconjugants were selected on an LB plate containing 50 ug/ml kanamycin, and streptomycin for LBA4404 containing pBI121AF, and 25ug/ml chloramphenicol, 50ug/ml kanamycin, 100ug/ml streptomycin and spectinomycin for GV3111SE containing pMON200AF, respectively.

*Agrobacterium* transformation was further confirmed by a Southern blot of the isolated *Agrobacterium* DNA. The *Agrobacterium* plasmid was isolated by the method of An et al (1988). The conjugated *Agrobacterium* was grown in AB-Bio or LB medium containing an appropriate concentration of antibiotics at 28 °C. 1ml of the overnight culture was pelleted in an Eppendorf centrifuge for 30 seconds. The cells were resuspended in 0.1 ml of ice-cold solution 1 (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8.0, 4mg/ml lysozyme). After 10 minutes incubation at room temperature, 0.2ml of solution II (1% SDS, 0.2N NaOH) was added and incubated for 10 minutes at room temperature. 30ul phenol and 150ul of 3M sodium acetate pH4.8 were added and centrifuged. The supernatant was precipitated with ice-cold 95% ethanol and centrifuged. After centrifugation, the DNA pellet was washed with ice-cold 70%
ethanol and resuspended in 50μl of TE buffer (20 mM Tris-HCl, 1 mM EDTA).

2) Tobacco leaf disc transformation

The basic tobacco leaf disc transformation system was followed (Horsch et al., 1988). Nurse cultures of tobacco cells (Nicotiana tabaccum var Xanthii) were used in the tobacco leaf disc transformation to improve the transformation efficiency.

Tobacco cell suspension cultures were maintained in 50 ml of suspension culture medium (4.3g/l MS salts (Gibco), 30g/l sucrose, 5ml/l B5 vitamins stock and 1mg/l 2,4 dichlorophenoxyacetic acid (2,4D) pH5.8) with a constant agitation of 150 rpm at room temperature.

**B5 Vitamins stock solution;**

<table>
<thead>
<tr>
<th>Amount</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mg</td>
<td>myo-inositol</td>
</tr>
<tr>
<td>10mg</td>
<td>thiamine-HCl</td>
</tr>
<tr>
<td>1mg</td>
<td>nicotinic acid</td>
</tr>
<tr>
<td>1mg</td>
<td>pyridoxine-HCl per ml</td>
</tr>
</tbody>
</table>

The two-week old tobacco suspension culture was filtered through a 540um sieve mesh filter to remove cell clumps. The filtrate was then centrifuged at 4,000 rpm
for 20 minutes. The cells were resuspended in MSO medium (4.3 g/l MS salts, 1 ml/l B5 vitamins stock, 30 g/l sucrose pH5.7). The resulting suspension was again centrifuged and the cells resuspended in MSO to wash out 2,4 D which interferes with the hormone balance required for shoot regeneration from explants. The final cell suspension could then be used for subculturing and leaf disc transformation.

To prepare nurse culture plates, 1 ml of the final cell suspension was dispensed on each MS 104 medium plates (MSO with 7 g/l phytagar, 1 mg/ml bencyladenine (BA) and 0.1 mg/ml napthalene acetic acid (NAA)) and covered with a piece of Watman 3mm filter paper.

Leaf discs (1cm diameter) were obtained from tobacco plants asceptically grown in magenta boxes. Young leaves were punched with a sterile cork borer to produce leaf discs. The leaf discs were precultured for 1 or 2 days upside down on MS104 medium plates to allow initial growth. 10 ml of an overnight culture of \textit{A.tumefaciens} were centrifuged for 10 minutes at 4,500 rpm. The bacterial pellets were resuspended in MSO to a final concentration of $10^8$/ml. Explants were soaked for few seconds in a resuspended culture of \textit{A.tumefaciens}, blotted dry, and placed upside down on MS104 nurse culture plates.
After 3 days of co-culture, the explants were transferred to MS selection medium (MS104 with 500 ug/ml carbenicillin or cefatoxime, 300 ug/ml kanamycin) for selection of transformed callus. When defined stems were visible, the shoots were excised and placed upright in MS rooting medium (MSO with 0.6% phytagar, 500 ug/ml carbenicillin, 100 ug/ml kanamycin). The plantlets which developed roots after about two weeks in rooting medium were then transferred to Magenta boxes containing MS rooting medium.

When the plants developed extensive roots, the lid was opened for 3 days to acclimatize the plants to the environment. The plants were then transferred to Jiffy pots containing Pro-Mix soil, and then to 10 inch pots and grown in a greenhouse under standard conditions.

3) Determination of the segregation ratio of kanamycin resistance gene

Transgenic tobacco plants grown in the greenhouse were watered every other day and supplemented with a Miracle Grow fertilizer biweekly. When the plants reached at an early floral stage, the tobacco flower buds were covered with brown paper bags to insure self-pollination. After
six weeks, ripe capsules were removed and further dried at 37 °C.

Seeds were wrapped in cheesecloth and sterilized by incubation for 20 minutes in 10% commercial bleach with 0.1% Tween-20 with gentle agitation. The seeds were washed several times with sterile water. The seeds were then sown in MS minimal plate (4.3 g/l MS salts, 10 g/l sucrose, 0.8% agar) supplemented with 100 mg/l kanamycin to select for kanamycin gene resistance. The seeds were grown in environmental growth chambers at 25 °C, 3000 lux. Seeds which did not carry the kanamycin resistance gene had white leaves and died after four to eight weeks.

3. β-glucuronidase (GUS) assay.

The presence of β-glucuronidase activity in the transgenic plants was assayed by the flurometric method described by Jefferson (1987). Leaf tissue (15mm diameter) punched with a cork borer was ground in 500 ul of GUS extraction buffer (50mM NaPO₄ pH7.0, 10mM β-mercaptoethanol, 10mM Na₂ EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). 50 ul of extract was incubated at 37 °C in 500 ul of assay buffer containing 1mM
4-methyl umbelliferyl β-D-glucuronide (MUG). At regular intervals, 100 ul of the reaction mixture was added to 0.9 ml stop buffer (0.2M Na₂CO₃). The fluorescence of liberated 4-methylumbelliferone (MU) was measured with excitation at 365 nm, and emission at 455 nm using a TKO 100 spectrofluorometer (Hoefer Scientific Inst., CA). Total protein concentration in leaf tissue was determined by the method of Bradford (1976) using the Pierce BCA protein assay reagent.

4. DNA analyses

1) Total plant DNA isolation.

To check for stable integration of AFP gene into the tobacco plant genome, tobacco genomic DNA was isolated by the procedure of Chen (1986). Two g fresh tissue was ground with liquid nitrogen in a mortar and pestle to a fine powder. The powder was transferred into a 15 ml Falcon propylene tube which contained 6 ml of urea extraction buffer (168 g urea, 25 ml 5M NaCl, 20 ml 1M Tris-HCl pH8.0, 16 ml 0.5 M EDTA pH8.0, 20 ml 20% sarkosine, 190 ml water). Phenol/chloroform (1:1) was added up to 12 ml. The tube was shaken hard to mix the
two phases well to a thick paste and incubated at room temperature for 15 minutes.

The mixture was centrifuged at 8K for 10 minutes at 4 °C. The supernatant was filtered through a small piece of miracloth to get rid of floating particles. One ml 4.4M ammonium acetate, pH5.2, and isopropanol to 13 ml were added to precipitate the DNA. The DNA was spun down in a table top centrifuge at the highest speed for 3 minutes. The pellet was transferred into an Eppendorf tube and dissolved with 500 ul of TE. The DNA was precipitated by adding 100 ul 4.4M ammonium acetate and 0.7 ml isopropanol. The DNA pellet was collected by microfuge for 5 minutes and washed with 75% ethanol. The DNA pellet was dried and dissolved in 200 ul TE buffer.

2) Preparation of hybridization probes.

Hybridization probes were prepared by using a random primed DNA labelling kit supplied by Boehringer Mannheim Laboratories (IN). The 174-bp mature AFP DNA and the 1111-bp HindIII fragment of pBI121AF were used for probe preparation.

The DNA fragment (25ug) was denatured by heating for 10 minutes at 95 °C and subsequent cooling on ice. The denatured DNA was mixed with 3 ul of dNTP mixture (dATP,
dGTP, dTTP), 2 ul of reaction buffer, and 5ul of [α-32P]dCTP (3000 Ci/mmole). The reaction mixture was brought up to 19 ul with water and then 1 ul of Klenow enzyme was added. The reaction was carried out at 37 °C for 30 minutes and stopped by adding 2 ul of 0.2M EDTA to the reaction mixture. The labeled DNA was separated on a prepacked Sephadex G-50 column (5 prime-3 prime, INC., PN) as described in the manufacturer's manual.

3) Southern blot

Southern analyses were performed to check the stable incorporation of the AFP gene into the A. tumefaciens and tobacco plant genomes. The DNA samples were cut with HindIII. For Agrobacterium DNA, 10 units of HindIII were used for every 4 ug DNA and for plant genomic DNA, 10 units of HindIII were used for every 2 ug of DNA. RNase was incubated with the digestion reaction. After HindIII digestion, the DNA was run on 1% agarose gel.

After staining the gel with ethidium bromide, the gel was denatured in 1.5M NaCl, 0.5M NaOH for 1 hour, and neutralized in 1.5M NaCl, 0.5M Tris-Cl, pH 7.0, for 1 hour. Transfer of DNA to a nitrocellulose filter was done by the traditional capillary blotting procedure using the 10X SSC
(87.5 g/l NaCl, 44.1 g/l Sodium citrate pH 7.0) described by Maniatis et al (1982). After transfer, the filter was washed with 6X SSC for 5 minutes at room temperature, air dried on a sheet of 3MM paper, and baked under vacuum at 80°C for 2 hours.

The nitrocellulose filter was prehybridized for 2 hours at 68 °C in prehybridization solution (6X SSC, 0.5% SDS, 5X Denhardt's solution and 100 ug/ml denatured salmon sperm DNA). The amount of prehybridization solution was 0.2ml/cm2 of nitrocellulose filter. After the prehybridization solution was poured off, the filter was incubated with hybridization solution (10mM EDTA, $^{32}$P-labeled denatured probe DNA in prehybridization) at 68°C for 12-16 hours.

After hybridization, the filter was washed with a solution of 2X SSC and 0.5% SDS at room temperature for 5 minutes, and then with a solution of 2X SSC and 0.1% SDS for 15 minutes with occasional gentle agitation. The filter was incubated in a solution of 0.1X SSC and 0.5% SDS at 68°C for 2 hours with gentle agitation. The filter was dried at room temperature on a sheet of Whatman 3MM paper, wrapped in Saran Wrap, and applied to X-ray film to obtain an autoradiographic image.
5. RNA analyses

1) Total tobacco RNA isolation.

To determine the expression of the AFP gene in transgenic tobacco plants, total RNA was isolated from tobacco leaf tissues using the protocol of Logemann et al (1987). Leaf tissues (2g) were frozen in liquid nitrogen and ground, using a mortar and pestle. The tissue powder was homogenized by the addition of 2 volumes of guanidine buffer (8M guanidine hydrochloride, 20 mM 4-morpholine ethansulfonic acid (MES), 20mM EDTA, 50mM β-mercaptoethanol pH7.0). The guanidine hydrochloride extract was centrifuged in a precooled centrifuge for 10 minutes at 10,000 rpm.

The supernatant was mixed vigorously with 0.2-1.0 volume of phenol/chloroform/isoamylation to remove proteins. The mixture was centrifuged for 45 minutes at 10,000 rpm at room temperature to separate the phase. The RNA-containing aqueous phase was collected and precipitated with precooled 0.7 volume of ethanol and 0.2 volume of 1 M acetate at -20°C overnight. The precipitated RNA was pelleted at 10,000 rpm for 10 minutes and washed with 70% ethanol. The RNA was dried and dissolved in sterile water.
2) Northern blot.

The formaldehyde RNA gel electrophoresis procedure as described by Maniatis et al (1982) was followed. To prepare the formaldehyde gel, melting agarose in water (3.0%) was cooled to 60°C, and 5X gel buffer (0.2M morpholinopropanesulfonic acid (MOPS) pH7.0, 50mM sodium acetate, 5mM EDTA pH8.0) and formaldehyde were added to give 1X and 2.2M final concentrations, respectively.

4.5ul of the RNA sample (up to 20 ug) was mixed with 2ul of 5X gel-running buffer, 3.5ul formaldehyde and 10ul formamide, and incubated at 55°C for 15 minutes. Then, 2 ul of sterile loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% Xylene cyanol) was added to the RNA sample mixture.

The RNA samples were loaded onto the gel. After gel electrophoresis, the RNA was transferred onto Duralon-UV membrane (Stratagene, CA) according to the manufacturer's manual. The gel was rinsed twice with water, for 5 minutes each time. The gel was soaked for 30 minutes in a solution of 0.15M NaCl, 0.05M NaOH, and then for 30 minutes in a solution of 0.15M NaCl, 0.1M Tris-HCl, pH8.0. RNA was transfered by the traditional capillary method using the 10X SSC as the transfer buffer. After transfer, the membrane was blotted of excess buffer and baked under vacuum for 2 hours at 80°C.
The membrane was prehybridized in a solution containing 50% deionized formamide, 10% dextran sulfate, 1% SDS, 1M NaCl and 100 ug/ml denatured sonicated salmon sperm DNA for 1 hour at 42°C with constant agitation in a heat sealable bag.

After prehybridization, the probe was added in the bag and the bag was incubated overnight at 42°C. The probe specific activity was 1 to 5 x 10⁵ cpm/ml. The membrane was washed one time at room temperature for 15 minutes with a solution of 2X SSC, 0.1% SDS to remove any unbound probe and the hybridization solution. The membrane was then washed with a solution of 0.1X SSC, 0.1% SDS at 65°C for 15 minutes. At this time, the membrane was monitored with a Geiger counter to decide about further washing steps. The membrane was blotted off with filter paper and exposed to X-ray film.

6. Protein analyses

1) Silver staining of total tobacco protein.

The total protein of tobacco plants was extracted from leaf tissues with extraction buffer (50mM Tris-HCl pH7.5, 5mM DTT, 0.05% Triton X-100, 50mM EDTA, 0.19 mg/ml PMSF). Tobacco leaf tissues were ground in liquid nitrogen and
centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was collected and the total protein concentration was determined using the Biorad protein assay reagent. 5 ug of protein sample were boiled for 5 minutes in a loading solution (4% SDS, 12% glycerol, 50mM Tris, 2% mercaptoethanol, 0.1% bromophenol blue pH6.8). The Mini-PROTEAN II Dual Slab Cell from Bio-Rad was used to run the 16.5%T, 3%C Tricine-SDS polyacrylamide gel to separate the proteins (Herman et al., 1987). The electrophoresis was run at 150 V for 60 minutes.

The silver staining of the polyacrylamide gel was performed with the Bio-rad silver stain kit. After electrophoresis, the gel was soaked in 200 ml of fixative solution (40% methanol, 10% acetic acid) for 30 minutes. The gel was further fixed two times in 200 ml of a solution containing 10% ethanol/5% acetic acid for 15 minutes each.

After fixing the gel, the gel was oxidized for 5 minutes, followed by washing with deionized water until all the yellow color was removed from the gel. The gel was stained with 100 ml silver reagent (1:10 dilution) for 20 minutes. After washing the gel with deionized water, the gel was developed with developing solution until the bands appeared dark brown. Development was stopped by incubating in 5% acetic acid solution for 5 minutes.
2) Western blot

After electrophoresis as described above, the proteins were transferred to Immobilon PVDF membrane using a Hoefer's Semiphor Semi-Dry Transfer Unit. The membrane and Whatman 3MM filter paper were equilibrated in blotting buffer (192 mM glycine, 25mM Tris pH 8.3, 20% methanol) for 10 minutes, after wetting in 100% methanol. The transphor sandwich was constructed according to Hoefer's instructions. The Hoefer semiphor unit was run at a constant current of 100 mAmp for 30 minutes. The membrane was air dried and stored at 4°C until ready to be immunostained.

After re-wetting in methanol for 1-2 seconds, the membrane was briefly rinsed in water to remove the excess solvent and then placed in a protein blocking solution containing 5% BSA in TBS (0.9% NaCl in 20 mM Tris-HCl pH 7.4) for 1 hour at 37°C with gentle agitation. The membrane was washed three times for 5 minutes each in the wash solution (0.1% BSA in TBS). The membrane was then incubated with the purified anti-IgG raised against a winter flounder antifreeze peptide in a volume of 10 ml of antibody incubation buffer (1% BSA, 0.05% Tween-20 in TBS) for 2 hours at room temperature with gentle agitation. The membrane was washed as described above, and then
incubated with the secondary antibody, anti-IgG-Alkaline phosphatase (AP) conjugate (1:7500 dilution), for 1 hour at room temperature in 10 ml of antibody incubation buffer. The membrane was washed again as described above.

The AP color reaction was performed using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as the substrate. AP color development solution was prepared by mixing 66 ul of nitro blue tetrazolim (NBT 50 mg/ml) and 33 ul of BCIP in 10 ml AP buffer (100mM Tris-HCl pH 9.5, 100 mM NaCl, 5mM MgCl2). The membrane was incubated in the AP color development solution until the reactive areas turned purple. The reaction was stopped by washing the membrane with distilled water.

3) Purification of IgG

The winter flounder antifreeze-protein antiserum was provided by Dr. DeVries (Department of Physiology and Biophysics, University of Illinois). IgG-antifreeze-protein (IgG-AF) was purified by using the Pierce immunopure IgG purification kit. A Protein A Affinity Pak column was equilibrated with 5 ml of Immunopure IgG binding buffer. Antiserum was diluted 1:1 with Immunopure IgG binding buffer and then applied to the column. The column
was washed with 15 ml of Immunopure binding buffer, followed by eluting the bound protein with 5 ml of elution buffer.

The 5 ml eluent was precipitated with an equal volume of saturated ammonium sulfate solution at 4 C overnight. The precipitate was centrifuged at 3,000 g for 30 minutes and the pellet was resuspended in 1ml PBS (20mM sodium phosphate, 100mM NaCl pH7.4). The precipitated antibody solution was desalted in an Excellulose column equilibrated with 10 ml PBS. A 1 ml sample of the precipitated IgG-AF was applied to the Excellulose column and 1 ml fractions were collected. The IgG-AF concentration was monitored by absorbance at 280 nm.

7. Frost test.

The measurement of frost tolerance of tobacco plants was followed by the procedure of Lindow et al (1982) with a little modification. Kanamycin selected seedlings (about the 4 leaf stage) were transferred into Magenta boxes, each containing a jiffy tablet. The plants were grown in an environmental growth chamber until the plants reached the top of the lid. Tobacco seeds do not germinate synchronously, so the developmental stage of all the
seedlings was not precisely the same. The plants were placed randomly at 0°C in the temperature controlled incubator. After 30 minutes equilibrium at 0°C, the temperature was lowered rapidly to -4°C, then slowly cooled to -7°C at a rate of -1°C/20 min, then increased to 30°C and kept overnight to recover. The surviving plants were counted visually.
Results and Discussion

1. Construction of recombinant DNA

The IIA7 cDNA clone contains the whole winter flounder antifreeze-protein (AFP) gene sequence: pre-, pro-, and mature regions and poly d(G) and poly d(C) tails. The signal sequences governed by fish hormones would not be necessary for expression in the plant system. Therefore, the 174-bp mature AFP gene was designed to contain its own initiation and termination codons and inserted into plasmid pBR322 digested with BamH1. Figure 14 shows the presence of a correctly sized band of 174-bp derived from the resultant plasmid pBR322AF after BamH1 digestion. The pBR322AF was amplified in large quantities for further cloning into suitable plant transformation vectors.

The 174-bp mature AFP gene from the pBR322AF was cloned into plasmid pMON530. Twelve colonies were miniscreened by digestion with EcoRV and EcoR1, and four clones showed bands of the correct size of 324-bp (Figure 15). Clone 12 showed the correct orientation of (CaMV)35S/AFP/NOS 3' gene cassette (Figure 16). The resulting plasmid was named pMON530AF.
Figure 14. Agarose gel showing the presence of 174-bp BamH1 fragment of AFP gene from pBR322AF. Lanes 1-4: pBR322AF digested with BamH1, lane 5: pBR322 alone, lane 6: 123-bp ladder.
Figure 15. Agarose gel showing the presence of 324-bp EcoRV-EcoR1 fragment of pMON530AF clones. Lanes 1, 2, 4, 5: pMON530AF clones digested with EcoRV and EcoR1, lane 3: 123-bp ladder.
Figure 16. Agarose gel showing the orientation of pMON530AF. The 324-bp EcoRV-EcoR1 fragments from pMON530AF clones were isolated and digested with SfaN1. The digestion with SfaN1 generates 134-bp and 190-bp fragments for a correct orientation, and 280-bp and 44-bp fragments for an incorrect orientation. Lane 1: clone 5, lane 2: clone 5 without digestion with SfaN1, lane 3: clone 6, lane 4: clone 7, lane 5: clone 12, lane 6: 123-bp ladder.
The final clones, pBI121AF (binary vector) and pMON200AF (cointegrating vector), were confirmed by restriction enzyme digestion and Southern blotting. The HindIII digestion of pBI121AF and pMON200AF (Figure 17) excised an approximately 1.1kb band which corresponded to the AFP gene cassette; that is, the duplicated (CaMV) 35S promoter, the mature AFP gene coding sequence, and the NOS 3' terminator.

The correct orientation of pBI121AF and pMON200AF was determined according to figures 12 and 13, respectively, which are described in Materials and Methods. For pBI121AF, the clones either showed correct orientation (lanes 2,3,5), or else showed the opposite orientation (lanes 1,4) (Figure 18). Clone 6 was used for Agrobacterium transformation. For pMON200AF, all the clones showed correct orientation (Figure 19). Southern hybridization using the labeled 174-bp AFP DNA further confirmed the presence of the AFP gene in the final clones, pBI121AF and pMON200AF (Figure 20).

2. Transformation of Agrobacterium tumefaciens

Using the triparental mating procedure, pBI121AF and pMON200AF were transformed into the disarmed A. tumefaciens
Figure 17. Agarose gel showing the presence of 1.1-kb HindIII fragment of pBI121AF and pMON200AF. The plasmids were digested with HindIII and run on the 0.9% agarose gel. A) lane 1: 1-kb size marker, lanes 2-6: pBI121AF, lane 7: pBI121 only. B) lanes 1-4: pMON200AF, lane 5: pMON200 only, lane 6: 1-kb size marker.
Figure 18. Agarose gel showing the orientation of pBI121AF clones. The plasmids were digested with EcoRV. The EcoRV digestion of pBI121AF generates 1.5-kb and 1.3-kb fragments for a correct orientation, and 1.6-kb and 1.2-kb fragments for an incorrect orientation. Lane 1: pBI121AF clone 7, lane 2: pBI121AF clone 6, lane 3: pBI121AF clone 5, lane 4: pBI121AF clone 3, lane 5: pBI121AF clone 1, lane 6: pBI121 only, lane 7: 1-kb size marker.
Figure 19. Agarose gel showing the orientation of pMON200AF clones. The plasmids were digested with SstI and EcoR1 and run on the 0.9% agarose gel. The digestion produces 1620-bp and 290-bp fragments for a correct orientation, and 1090-bp and 820-bp fragments for an incorrect orientation. The pMON200 produces an approximately 800-bp band with digestion of SstI and EcoR1. Lanes 1-4: pMON200AF, lane 5: pMON200 only, lane 6: 1-kb size marker.
Figure 20. Southern hybridization analysis of plasmid DNAs. The plasmid DNAs were digested with HindIII and hybridized with the labeled 174-bp AFP DNA. Lane 1: pBI121, lane 2: pBI121AF, lane 3: pMON200, lane 4: pMON200AF.
strains LBA4404 and GV3111SE, respectively. The colonies resistant to kanamycin and streptomycin for LBA4404-containing pBI121AF, and to chloramphenicol, kanamycin, streptomycin, and spectinomycin for GV3111SE-containing pMON200AF, were selected and total Agrobacterium DNA was isolated. Stable transformation was confirmed by Southern hybridization with the labeled 174-bp AFP DNA probe. The results are shown in figures 21 and 22. HindIII digestion of total Agrobacterium DNA excised a 1.1-kb band corresponding to the sequence of the double-(CaMV)35S/AFP/NOS 3' gene construct.

3. Transformation of tobacco plants with A. tumefaciens

After infection of leaf discs with LBA4404-containing pBI121AF (LBA4404/pBI121AF) and GV3111SE-containing pMON200AF (GV3111SE/pMON200AF), calli started to form from most of the leaf discs. The leaf discs were transferred to shoot-inducing media containing kanamycin, and three to eight shoots per disc were thereby developed (Figures 23 and 24). These kanamycin selected shoots were regenerated to become healthy plants (Figure 25). Kanamycin resistance
Figure 21. Southern hybridization of transformed *A. tumefaciens* LBA4404/pBI121AF. To confirm the integration of AFP DNA into *A. tumefaciens* LBA4404, the agrobacterium DNA was digested with HindIII and hybridized with the labeled 174-bp AFP DNA. Lane 1: negative control (LBA4404/pBI121), lanes 2-7: *A. tumefaciens* LBA4404 transformed with pBI121AF, lane 8: positive control (pBI121AF).
Figure 22. Southern hybridization analysis of *A. tumefaciens* GV3111SE transformed with pMON200AF. To confirm the integration of AFP DNA into *A. tumefaciens* GV3111SE, the agrobacterium DNA was digested with HindIII and subjected to Southern hybridization analysis with the 174-bp AFP DNA probe. Lane 1: GV3111SE/pMON200, lanes 2-5: GV3111SE/pMON200AF.
Figure 23. Leaf disc transformation. A) Tobacco leaf discs transformed with *A.tumefaciens* LBA4404/pBI121AF were grown in shoot-inducing medium. B) Individual shoots were removed from the leaf discs and transferred to rooting medium.
Figure 24. Tobacco leaf disc transformation with *A. tumefaciens* GV3111SE/pMON200AF. Transformed tobacco leaf discs on shooting medium (A), and transformed shoots on rooting selection medium (B).
Figure 25. Transgenic tobacco plants.
was the selection marker of the transformed plants, because the introduced plasmids, pBI121AF and pMON200AF, contain the neomycin phosphotransferase (NPT II) gene which confers resistance to the antibiotic kanamycin.

Researchers have reported that a transformed callus does not necessarily regenerate transformed shoots (Jordan and McHughen, 1988a). And indeed, the kanamycin-selected plants developed from the transformed calli are not actually transformed themselves. Escapes from the screening, based on kanamycin resistance, might have occurred in the nontransformed plants. Escapes most likely arise from non-transformed cells cross-protected from the selective agent by transformed cells in the callus.

Therefore, as another marker for plant transformation, β-glucuronidase (GUS) activity was measured in the regenerated plants with LBA4404/pBI121AF. Eight out of 33 plants transformed with LBA4404/pBI121AF showed negative GUS activity even though all the plants were kanamycin resistant. Table 4 shows the expression level of the GUS gene in the transformed plants. The GUS activity was varied in each of the plants. The difference in GUS activity may be attributed to several factors, including the chromosomal integration site of the introduced gene, gene copy numbers, DNA methylation, and inherent variation
Table 4. GUS activities of transgenic tobacco plants. The GUS activity was measured as a nmole 4-methylumbelliferone (MU) produced per minute in the 1cm² leaf disc with an excess 4-methylumbelliferyl glucuronide (MUG).

<table>
<thead>
<tr>
<th>Plants</th>
<th>nM Mu/cm² min</th>
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<tbody>
<tr>
<td>control</td>
<td>ND</td>
</tr>
<tr>
<td>L1</td>
<td>70.9</td>
</tr>
<tr>
<td>L3</td>
<td>388.9</td>
</tr>
<tr>
<td>L5</td>
<td>76.0</td>
</tr>
<tr>
<td>L12</td>
<td>ND</td>
</tr>
<tr>
<td>L13</td>
<td>291.1</td>
</tr>
<tr>
<td>L17</td>
<td>26.4</td>
</tr>
<tr>
<td>L24</td>
<td>ND</td>
</tr>
<tr>
<td>L25</td>
<td>39.3</td>
</tr>
<tr>
<td>L26</td>
<td>93.8</td>
</tr>
<tr>
<td>L29</td>
<td>35.3</td>
</tr>
<tr>
<td>L30</td>
<td>86.3</td>
</tr>
<tr>
<td>L32</td>
<td>389.6</td>
</tr>
</tbody>
</table>
in leaf sampling.

The most likely cause of quantitative variation in gene expression is the existence of different sites of integration in the chromosome, a phenomenon known as the position effect. The position effect is well known to influence gene expression in Agrobacterium-mediated plant transformation. The position effect may be due to insertion near cis-elements (positive or negative) which can influence expression from the transgene. If the foreign gene is integrated near the plant enhancer sequence, the introduced gene may be highly transcribed by the near-by enhancing factor. The interaction between trans-factors and cis-elements of the introduced DNA may also be influenced by the site of integration.

DNA methylation in plants is shown to be inversely correlated to gene expression. The degree of methylation varies between actively transcribed regions of the genome and inactive regions. Highly methylated DNA can inactivate the gene expression. If the regions in the GUS coding sequence are methylated, low GUS activity will be expected.
4. Integration of the mature AFP gene into tobacco plants

Southern blot analysis unambiguously proves the integration of the mature AFP gene into the plant genome. All transgenic plants transformed with LBA4404/pBI121AF had a complete HindIII fragment approximately 1.1-kb in size corresponding to the gene construct, double-(CaMV)35S/mature AFP/NOS 3' (Figure 26). Those which had negative GUS activity, L12 and L24 plants, did not excise the 1.1-kb HindIII band (lanes 5 and 8). This may indicate that plants L12 and L24 are not transformed, or contain only the NPTII gene by gene rearrangement or deletion because of their kanamycin resistance. As the probe used was the 1.1-kb HindIII fragment of pBI121AF, the plant genomic DNA may be hybridized with the NOS3' sequence of the labeled 1.1-kb DNA probe. This would explain the presence of the high molecular size band in the blots.

Ten out of 13 plants transformed with GV3111SE/pMON200AF showed a distinct band corresponding to the 1.1-kb antifreeze gene construct (Figure 27). The control plants transformed with GV3111SE/pMON200 did not excise the 1.1-kb band.
Figure 26. Southern hybridization analysis of tobacco plants transformed with LBA4404/pBI121AF. To demonstrate the integration of AFP DNA into the tobacco genome, plant DNAs were isolated from transgenic tobacco leaf tissues, digested with HindIII and hybridized with the 174-bp AFP DNA probe. Lanes 1 and 2: negative control plants transformed with LBA4404/pBI121, lanes 3-13: transformed plants with LBA4404/pBI121AF. The arrow shows the distinct band corresponding to the 1.1 kb HindIII fragment containing the double (CaMV)35S/AFP/NOS3' gene construct. The letters on the bottom of the blot indicate the specific plants used.
Figure 27. Southern hybridization analysis of tobacco plants transformed with GV3111SE/pMON200AF. The tobacco genomic DNAs were isolated from leaves and digested with HindIII. The probe used was the 174-bp AFP DNA. Lanes 1,2: control plants transformed with GV3111SE/pMON200, lanes 3-15: plants transformed with GV3111SE/pMON200AF. The arrow shows the distinct band corresponding to the 1.1-kb antifreeze gene insert. The letters on the bottom of the blot indicate the specific plants used.
The GUS assay and Southern blot analysis indicate that the efficiency of plant transformation was almost the same between plants infected with LBA4404/pB1I21AF (binary vector system) and GV3111SE/pMON200AF. In addition, no spurious rearrangements occurred during the integration process into the plant chromosome. However, GV3111SE/pMON200AF infected plants showed higher copy numbers of the AFP gene than that of the plants infected with LBA4404/pMON200AF.

As each of these transgenic plants is a product of an independent transformation, the integration site of T-DNA may differ in each plant. Since the introduced DNA is integrated into the plant genome by nonhomologous recombination, the integration site will influence the gene expression (position effect). Recently, gene targeting in plants by homologous recombination has been attempted to control the integration site (Paszkowski et al., 1988; Baur et al., 1990; Lee et al., 1990). This would be useful in modifying endogenous plant genes at their natural position in the genome or in delivering foreign DNA into a predicted genomic location to eliminate the possibility of the position effect in the transgene.

Gene copy number can also alter the gene expression. Copy number of the introduced T-DNA varies among
transformants. It has often been shown that there is no positive correlation between increased copies and increased expression of the genes in the T-DNA (Shirsat et al., 1989). Recently, it has been reported that single T-DNA insertion tends to result in increased expression of the introduced gene (Hobbs et al., 1990). Addition of extra copies to the genome reduces the level of gene expression (van der krol et al., 1990; Napoli et al., 1990). Truncation, rearrangement or repetition of the introduced T-DNA may also affect gene expression.

To demonstrate stable inheritance of the AFP gene in tobacco first generation progeny plants, the progeny plant DNAs were subjected to Southern blot analysis (Figure 28). The progeny plants show a distinct 1.1-kb HindIII fragment hybridizing with the 174-bp AFP probe.

5. Kanamycin gene segregation test

Kanamycin gene segregation was observed in the progeny plants (Table 5). 100 or 150 seeds from each of the transgenic plants were germinated on selective medium and seedlings were scored for susceptibility (bleached) and resistance (green) to kanamycin after 4 weeks. It has been known that the integrated Ti plasmid is inherited as a
Figure 28. Southern hybridization analysis of tobacco first generation progeny plant DNAs. To confirm stable inheritance of the AFP gene, the progeny plant DNAs were isolated from leaves and digested with HindIII. The 174-bp AFP DNA was used as a probe. Lane 1: control progeny plant, lanes 2-4: progeny plants from transgenic tobacco plants.
Table 5. Kanamycin Gene Segregation

<table>
<thead>
<tr>
<th>Plants</th>
<th>number of seedlings</th>
<th>Ratio (KmR/KmS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KmR</td>
<td>KmS</td>
</tr>
<tr>
<td>Control(C2)</td>
<td>101</td>
<td>44</td>
</tr>
<tr>
<td>L1</td>
<td>142</td>
<td>8</td>
</tr>
<tr>
<td>L5</td>
<td>102</td>
<td>48</td>
</tr>
<tr>
<td>L25</td>
<td>114</td>
<td>36</td>
</tr>
<tr>
<td>L26</td>
<td>138</td>
<td>10</td>
</tr>
<tr>
<td>L32</td>
<td>118</td>
<td>32</td>
</tr>
<tr>
<td>G11</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>G13</td>
<td>94</td>
<td>6</td>
</tr>
</tbody>
</table>
For plants L5, L25, and L32, the segregation data fit the 3:1 ratio expected for a single dominant Mendelian gene, indicating a single copy kanamycin resistance gene. Some progeny plants showed a 15:1 kanamycin resistance gene segregation ratio, indicating the presence of two copies of the kanamycin gene segregating in a dominant fashion.

Jordan and McHughen (1988b) reported that when A. tumefaciens carrying a disarmed Ti-plasmid vector containing both a chimeric NPTII gene and a glyphosate resistance gene was transformed to flax hypocotyl tissue, co-segregation was observed in the progeny for kanamycin and glyphosate resistance. As the NPTII gene and the AFP gene are located in the same T-DNA region, these two genes are most likely to be co-segregated in the plant genome. Therefore, it may suggest that plants containing a single copy of the kanamycin gene also have a single copy of the AFP gene.

6. Expression of the AFP gene in tobacco plants

The total RNA that was isolated from the transgenic
tobacco tissues was hybridized with the 174-bp AFP DNA probe. The expected mRNA length is about 400-bp, which includes 30 nucleotides upstream of the AFP gene, the 174-bp of the AFP DNA, and about 190 bases downstream of the AFP gene. The results of the Northern blot analysis are given in figures 29 and 30 for the plants transformed with LBA4404/pB1121AF and GV3111SE/pMON200AF, respectively. The abundance of these putative AFP RNAs hybridized with the AFP DNA probe was a significantly high amount in the total RNA extract.

The duplicated CaMV35S promoter may indeed increase the transcriptional activity of the AFP gene. The duplicated CaMV 35S upstream sequences act as transcriptional enhancers for obtaining high levels of expression of foreign genes in transgenic plants (Kay et al., 1987).

The CaMV 35S promoter activity has been described as 'constitutive' (Harpster et al., 1988; Sanders et al., 1987), but recent studies suggest that the expression of genes fused to the CaMV 35S promoter may not be constitutive (Williamson et al., 1989; Benfey et al., 1989). They found that younger, actively dividing leaf, stem, root, and flower tissues contained higher steady state levels of the RNA than did old tissues.
Figure 29. Northern hybridization analysis of total RNA from tobacco plants transformed with LBA4404/pBI121AF. To demonstrate the expression of the AFP gene at transcriptional level, total RNAs from the transgenic tobacco leaf tissues were hybridized with the 174-bp AFP DNA probe. Lane 1: control plants transformed with LBA4404/pBI121, lanes 2-6: tobacco plants transformed with LBA4404/pBI121AF.
Figure 30. Northern hybridization analysis of total RNA from tobacco plants transformed with GV3111SE/pMON200AF. Total RNAs from the transgenic tobacco leaf tissues were hybridized with the 174-bp AFP DNA probe. Lane 1: control plants transformed with GV3111SE/pMON200, lanes 2-5: tobacco plants transformed with GV3111SE/pMON200AF.
In plants L1 (lane 2 in figure 29) and L26 (lane 5 in figure 29), the high molecular size of RNA hybridized with AFP DNA probe was also detected in longer exposure time. The amounts were significantly lower than the correct gene size transcribed. Since L1 and L26 showed double NPTII loci in the kanamycin segregation test, this possibly indicates that the multiple insertion or rearrangement of the AFP gene into the tobacco genome may cause the high molecular size RNA bands.

The transcripts of the AFP gene were accumulated to a similar extent in each plant, except L13 (lane 3 in figure 29) which showed one of the highest GUS activity. The level of the AFP transcript of L13 was much lower than that of the other transformed plants. It seems that in this case the expression of the GUS gene and the AFP gene does not correlate well. There are reports to show that the expression of physically linked genes cotransferred in a plant genome can vary independently (Jones et al., 1985; Nagy et al., 1985).

The transcription levels of the AFP gene in the transformed plants with GV3111SE/pMON200AF differed significantly. Such differences in the expression level could be explained by positional effects, DNA methylation and gene copy numbers inserted.
The mature antifreeze-protein expression in the transformed leaf tissues was detected by protein blotting. Partial purified AFP from the winter flounder serum was compared as control. The antiserum was raised against the winter flounder AFP peptide 3 (Figure 1), which has almost an identical sequence and conformation with IIA7 except it contains three-11 amino acids repeating units. The AFP used in this experiment should be of 5.5 kd molecular weight because of addition of one more 11 amino acid repeating unit than the smallest AFP sequence. One more addition of an 11- amino acid repeating unit probably exhibits more antifreeze function because of more binding sites with water molecules in ice lattice.

Figure 31 shows the results of Western blotting. The control AFP from winter flounder serum produced a immunoreactive band corresponding to mature antifreeze -protein (about 4kd). The immunoreactive 5.5kd AFP bands were identified in samples of L1, L25, and L26. The occurence of the doublet in immunoreactive peptides may indicate a specific cleavage or degradation of AFP in the transformed tobacco plants. Since the intensities of the doublet were almost equal to each other, the indication may be that a specific cleavage in AFP occured and the AFP stably remained in plants.
Figure 31. Western blot analysis of total protein from transgenic plants. To demonstrate the expression of antifreeze protein in transgenic tobacco plants, total proteins were isolated from transformed tobacco leaf and run on the polyacrylamide gel. The antibody raised against the AFP peptide 3 was used for immunoblotting. Lane 1: molecular weight size marker, lane 2: AFP from winter flounder serum, lane 3: control plant transformed with LBA4404/pBI121, lanes 4-8: transgenic plants with LBA4404/pBI121AF. The arrow indicates the immunoreactive 5.5 kd antifreeze-peptide.
There is some non-specific binding in the high molecular weight bands as a background. The possible explanations for the non-specific binding are 1) the antibody against AFP recognizes epitopes shared by other plant protein antigens, or 2) the antiserum contains a mixture of antibodies with multiple specificities. To distinguish these two possibilities, the antiserum was primarily absorbed with the wild type tobacco protein extract before doing the Western blot. It somewhat reduced the background signal but the problem still existed. The cold-induced proteins in plants might have a similar conformation to AFP, for example, the Kinl protein (Kurkela and Franck, 1990). Therefore, the transformed plants might express proteins containing the shared epitopes with the antibody against AFP.

The molecular weight difference between the heterogenous control AFP and AFP construct used in this experiment, as well as the non-specific binding, hampered the exact calculation of the level of AFP accumulation in transformed tobacco plants. However, the amount of AFP expression was highly significant, because the intensity of the immunoreactive 5.5kd band was much stronger than the control AFP (about 5 ug).

Silver staining of the total protein showed almost
identical expression patterns in the transformed plants (Figure 32). Moreover, accumulation of 6kd proteins was identical in the transgenic plants and the control plants, but the immunoreactive 5.5kd peptides were identified only in the transformed plants, and not in the control plants. This undoubtedly eliminates the possibility of the non specific binding of the 5.5 kd peptide with the antibody against AFP.

7. Frost tolerance of seedlings in the transgenic plants

The frost tolerance of tobacco seedlings from control and transgenic plants containing the AFP gene was determined. The seedlings which were kanamycin resistance were selected, transferred to Jiffy soil, and grown in an environmental growth chamber.

The plants were placed randomly at 0°C to eliminate the positioning effect of plants in the temperature controlled incubator. Four thermocouples from a multisensor temperature recorder, distributed into the freezing chamber, indicated a less than 0.5°C spread in the air temperature at the time of minimum temperature.
Figure 32. Silver staining of total protein from transgenic plants. Total proteins isolated from transformed tobacco leaf were run on the polyacrylamide gel. Lane 1: molecular weight size marker, lane 2: control plant transformed with LBA4404/pBI121, lanes 3-5: transgenic plants with LBA4404/pBI121AF.
The results are shown in Table 6 and figure 33. The control is the transformed plants without the AFP gene coding sequence.

An increase in frost tolerance compared to the control plants was observed in plants L25 and L26, which coincides well with the result of the Western blot. In the preliminary test, the tobacco plants had no damage at -5°C, which has been reported by Lindow et al (1982), and this indicates that the damage the plants sustained at -7°C was not the result of a chilling injury. All plants, both transgenic and control, were completely killed at -8°C. Only some of the small plants survived at -8°C. The killing temperature varied depending on the plant developmental stage. The plants of 5-6 leaf stage (the largest leaf size was about 15 mm and height was about 2 cm) showed more freezing tolerance than the bigger plants (height ~8 cm).

As described previously, the kin1 gene product which is induced at cold stress, has similar composition to the AFP (Kurkela and Franck, 1990). In addition, the in vitro application of the AFP in plant leaves does indeed increase the frost tolerance (Cutler et al., 1989). Therefore, the high expression of the AFP in cytoplasmic location may attribute the frost tolerance in plants L25
Table 6. Enhancement of frost tolerance of seedlings. The plants which were kanamycin resistance were tested for frost tolerance in the freezing chamber. Survival (%) indicates the mean value of three replicates.

<table>
<thead>
<tr>
<th>Plants</th>
<th>n\textsuperscript{a}</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>L1</td>
<td>21</td>
<td>48</td>
</tr>
<tr>
<td>L5</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>L25</td>
<td>22</td>
<td>55</td>
</tr>
<tr>
<td>L26</td>
<td>20</td>
<td>56</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: Number of plants tested
Figure 33. Comparison of freezing tested tobacco plants. C2 indicates control plants without the AFP gene coding sequence and the front row indicates half freezing damaged plants. The picture was taken two weeks after freezing at -7°C.
and L26.

However, the plant L5 did not show any frost tolerance in the latest freezing test (Table 6) even though it showed some frost tolerance in the preliminary test. In the later freezing test, L5 germinated very poorly in the kanamycin selection plates (about 30% germination). This indicates that L5 may suffer some changes in the seed germination process causing the alteration of the gene expression. The integration site of AFP gene in L5 may cause some deleterious effects in seed germination. Therefore, L5 may have lost the AFP gene during seed germination or during the period of seed storage. In the previous preliminary tests, L5 germinated very well, over 95% like that of other plants. During the time passed, L5 lost some of its germination ability, and may have lost the AFP gene. This could explain why L5 has same level of survival as the control plants.
Summary

In this dissertation, I describe the plasmid construction and transformation of the mature antifreeze-protein gene from winter flounder into tobacco plants, and the frost tolerance of these transgenic tobacco seedlings. To maximize the expression of the mature AFP gene, a duplicated CaMV 35S promoter was used instead of a single CaMV 35S promoter. The level of AFP transcription using the double CaMV 35S promoter plasmid construct was significantly higher than the single CaMV 35S promoter. Since the AFP gene has a high GC content (~80%), it may have also affect AFP gene expression by serving as DNA methylation sites or stabilizing the mRNA.

The preliminary freezing tests of transgenic seedlings and analysis of AFP expression by Western blots of transgenic plants were performed. This was because physically linked genes can be expressed independently in the transgenic plants, a high level of GUS activity does not have to correlate with a high expression of the AFP gene. Plants L1, L5, L25, and L26 were chosen for further freezing tests.

The expression of the antifreeze-protein in tobacco plants does confer frost tolerance. At least 30% more transgenic plants survived freezing conditions than the
control plants. These results indicate that even though the AFP gene was designed to be expressed in a cytoplasmic location, a high-label of AFP expression can prevent freezing injury.

Since ice formation occurs first at the extracellular regions of the plant cells, one hypothesis would require the antifreeze-protein to be expressed extracellularly to prevent freezing injury caused by cellular dehydration. However, this rational is somewhat controversial. Cellular dehydration caused by extracellular ice formation is not lethal at the time of freezing. Otherwise, intracellular ice formation which occurs at rapid cooling rates is lethal in plants. Therefore, cytoplasmic expression of an antifreeze-protein may reduce cell damage by preventing intracellular ice formation during rapid cooling. Plants may already have such a protein. The cold-induced protein, the kinl gene product, is a good example.

Targeting of an antifreeze-protein into a cell vacuole may improve the frost tolerance, because the vacuole contains most of water in the plant cell. However, the vacuole also contains varieties of hydrolytic enzymes to degrade the metabolites and abnormal proteins produced by plants. Therefore, vacuole targeting may face several problems in designing the expression of the antifreeze-protein without degradation in that organelle.
References


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Title of Dissertation: The frost tolerance of tobacco plants transformed with the gene encoding the antifreeze-protein from winter flounder

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Date of Examination:

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