Introduction of Insect Antifreeze Protein Genes into the Model Plant *Arabidopsis thaliana*.

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ABSTRACT

Injury inflicted by frost and freezing is found in all plants exposed to such damaging temperatures. Antifreeze proteins protect against these injurious temperatures by providing freeze resistance to organisms in which they occur. The expression of antifreeze proteins in a plant is a possible means of increasing the frost resistance and freeze tolerance of plants. Freeze tolerance curves of non-cold acclimated and cold acclimated *A. thaliana* were generated in untransformed *A. thaliana*. Research was conducted to obtain organisms with highly efficient antifreeze proteins. The *Tenebrio molitor* and *Choristoneura fumiferana* cDNA clones were acquired from A/F Proteins Inc. through a material transfer agreement. The antifreeze protein-encoding cDNA fragments will be cloned into an expression vector and transformed into *Arabidopsis thaliana* to determine if expression of antifreeze proteins will increase the freeze tolerance of *A. thaliana*.

INTRODUCTION

Freezing temperatures are responsible for more crop losses worldwide than any other single cause. Annual losses total several billion dollars in the United States alone. For example, a single freeze in California in December of 1998 resulted in an estimated $591 million in damages to citrus crops (CDFA, 1998).

The process of freezing in plants and the resulting damage to plant cells has been studied intensively (Steponkus and Webb, 1992; Wallis et al., 1997). As the temperature decreases ice formation begins on the epidermal surface. This is followed by ice formation within the plant tissue. At about -1°C, the extracytoplasmic space, where solute concentration is the lowest, begins to freeze and as a result withdraws water from the cytoplasm, dehydrating the cells and withering the cell membranes (Steponkus and Webb, 1992). The plant cells have defenses against freezing-induced dehydration, but if the temperature decreases in the tissues to about -10°C, ice crystals penetrate the cytoplasm, resulting in the destruction of the cell membrane and disorganization of the sub-cellular space (Steponkus and Weist, 1988).

Plants have developed a variety of defenses against low temperatures. Frost-hardy plants produce colligative cryoprotectants such as sucrose and proline to reduce the dehydration of the cell (Wallis et al., 1997). However, some cold-hardy plants produce specific antifreeze proteins (Griffith et al., 1994).

Antifreeze proteins (AFPs) share a common function in providing freeze resistance to organisms in which they occur. These proteins reduce freezing injury by thermal hysteresis and inhibition of ice recrystallization. Thermal hysteresis is the process of lowering the freezing point of a liquid below the melting point. Antifreeze proteins bring about thermal

hysteresis by inhibiting ice crystal growth through adsorption to the surface of the crystal (Davies and Sykes, 1997; Yeh and Feeney, 1996). Once the ice crystals melt, ice recrystallization (growth of larger ice crystals at the expense of smaller ones) is inhibited by the adsorption of AFPs to the crystal surface.

Thermal hysteresis has been detected in at least 26 species of higher plants and some candidate proteins have been purified. The values of these extracted thermal hysteresis proteins, however, are low, from 0.2 to 0.6ºC when purified from plants encountering low environmental temperatures (Worrall et al., 1998). Most freeze inhibitors found in plants are polysaccharides, not proteins. Polysaccharides modify ice structure after ice nucleation has occurred. They apparently do not inhibit the initiation of freezing (Urrutia et al., 1992).

Antifreeze proteins have also been discovered in certain marine teleosts (fish) that survive in seawater temperatures below the freezing point of their blood sera by producing antifreeze proteins (Kenward et al., 1993). The maximum level of thermal hysteresis shown by fish antifreeze proteins is approximately 1.5ºC. Recently, the isolation, cloning, and expression of a thermal hysteresis protein from an insect has demonstrated that some insect antifreeze proteins are 10 to 30 times more active than any known fish antifreeze protein (Tyshenko et al., 1997), or more than 60 times more active than any known plant protein.

The discovery of AFPs in two insects represents a major advance in cryobiology. Genes encoding AFPs have recently been cloned from the spruce budworm (*Choristoneura fumiferana*) and the common mealworm beetle (*Tenebrio molitor*). During the winter months, the spruce budworm can be exposed to temperatures approaching -30ºC, but it still resists freezing (Hew, 1997). The production of antifreeze proteins is an important part of the spruce budworm's survival strategy. Taking into account the conditions of its environment, it is not startling that this insect has evolved an AFP that is the most active known. The vastly superior insect antifreeze proteins may find wider applications in the cryoprotection of cells and tissues.

Antifreeze proteins have exceptional properties, and when applied these properties could have beneficial results. The expression of insect antifreeze protein genes in plants is a possible means of increasing the frost resistance and freeze tolerance of plants. The introduction of such insect antifreeze proteins through gene transfer into a model plant may confer increased freeze resistance to the plant. Likewise, increased freezing tolerance may result when such technology is applied to other plants (e.g. citrus trees). The model plant *Arabidopsis thaliana* was used herein because of its short generation period, size, ease of transformation, ease of regulating pollinations, and the vast amount of available information regarding this plant.

MATERIALS AND METHODS

Plant Material. Wild type RLD *Arabidopsis thaliana* seeds were planted four to a pot and designated with sterilized toothpicks. Fifteen pots were placed into a flat, watered with 2 L of H_2O and 200 ml of fertilizer. The flat was covered with plastic wrap and placed in a 4ºC cold chamber for 48 h. Plants were grown at 20ºC, 50% RH and 100 microEinsteins of light.

Freeze tolerance. The ability of *A. thaliana* to survive freezing of the cellular fluids (freeze tolerance) was determined by a freeze tolerance assay (adapted from Sukumaran and Weiser (1972)) performed on non-cold acclimated *A. thaliana*. Two to four leaves were excised from Arabidopsis plants and placed in capped culture tubes maintained in a -2ºC

water bath for one hour. Ice crystals were added to each culture tube to nucleate freezing by rubbing a frost-covered pipe cleaner (stored at -80ºC) across the test tube. The culture tubes were then maintained at -2ºC for an additional hour. Samples were cooled at a rate of 1ºC/45 min by the addition of salt to an ice water bath. Three samples were subjected to testing temperatures ranging from -2 to -7ºC. After an hour at each testing temperature, three culture tubes were removed and thawed on ice. A control set of culture tubes was kept at 0ºC.

Electrolyte leakage was measured by a protocol adapted from P.L. Steponkus (personal communication). Three ml of distilled water was added to each culture tube. Each tube was then sealed and vortexed for 1 min. The culture tubes were then incubated at room temperature for 3 h. with vigorous horizontal shaking. The distilled water was decanted to a clean test tube for electrolyte measurement. The conductivity of the solution was measured by an ohmmeter (VWR Scientific, Model 604). This represented the level of electrolyte leakage due to freezing damage (EL sample). The plant samples were then frozen at -80ºC for1 h. and thawed for 45 min. The original water solution that was decanted was returned to the culture tubes, the tubes were resealed and vortexed for 1 min. The samples were then incubated at room temperature while vigorously shaking for 3 h. The conductivity of the final solution was measured and represented the conductivity due to 100% electrolyte leakage (EL LN). The percentage electrolyte leakage was then determined as the ratio of EL sample to EL LN.

The entire assay was also conducted for cold acclimated *A. thaliana*. The *Arabidopsis* plants were grown at 20ºC up to 48 h prior to the freeze tolerance assay. At this time the growth chamber temperature was reduced to 4ºC and held for a minimum of 48 h. The samples were cooled at a rate of 2ºC/45 min and reduced to a maximum of -10ºC.

cDNA clones. To obtain the cDNA clones (gene constructs for AFPs) of the spruce budworm and the yellow mealworm A/F Proteins Inc. was contacted. Permission to use the clones was obtained through a material transfer agreement, which is constructed to ensure the rights to products and patents to A/F Proteins Inc. The plasmid vectors pBluescript and pET-20b containing the spruce budworm and yellow mealworm cDNA clones, respectively, were received. The 1400bp spruce budworm AFP cDNA was excised from the plasmid pBluescript by digestion with *Kpn* I and *Xba* I. The 250bp yellow mealworm AFP cDNA was excised from the plasmid pET-20b by digestion with *Xho* I and *Xba* I.

Plasmid Isolation. The plasmid pBI121 was chosen as the plant transformation vector. *Escherichia coli* strain DH5α containing pBI121 was grown in LB broth and selected with kanamycin (100µg/ml). *E. coli* plasmid DNA was isolated essentially as described (Sambrook et al., 1989). An RNase protocol adapted from Green (1996) was conducted to purify the DNA. Plasmids containing the antifreeze protein cDNAs were isolated in the same manner.

Electroporation. Competent bacterial cells were prepared as described (A. van Hoof, personal communication). *E. coli* was grown in LB broth to an optical density of 0.71 as measured at 595nm on a spectrophotometer. *Agrobacterium tumefaciens* C58pmp90 was grown in LB broth inoculated with the antibiotics gentamycin and rifampicin, to an optical density of 1.5.

Electro-transformation (Eppendorf Model 2510, Eppendorf Inc., Madison, WI) was conducted on *E. coli* (DH5α and JM109) and *A. tumefaciens* to determine the optimal charge (1.8 or 2.5 kV) at 25µF and 200 Ω. The electro-transformation protocol was also used to introduce the plasmid vector pBI121 into *E. coli* and *A. tumefaciens*.

RESULTS

The ability of *Arabidopsis thaliana* to survive freezing was assessed by the freeze toler-
ance assays performed on both non-cold acclimated and cold acclimated plants. The
electrolyte leakage percentage was recorded at decreasing temperatures and plotted graphi-
cally. Cellular death was defined as 50% electrolyte leakage (Sukumaran and Weiser, 1972).
Exposure of the non-cold acclimated plant leaves to decreasing temperatures resulted in
increased electrolyte leakage, ending in cellular death at -3.5ºC (Figure 1).
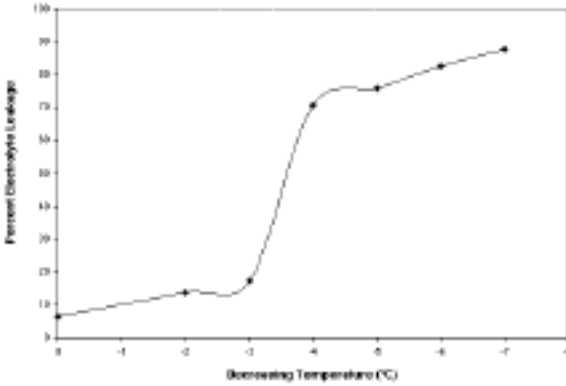

**Figure 1. Effects of low temper-
ature treatment on the freezing
tolerance of non-cold acclimat-
ed *Arabidopsis thaliana*.**

The cold acclimated plants retained their electrolytes for a longer period of time and did
not reach 50% electrolyte leakage until -7ºC (Figure 2); more than three full degrees lower
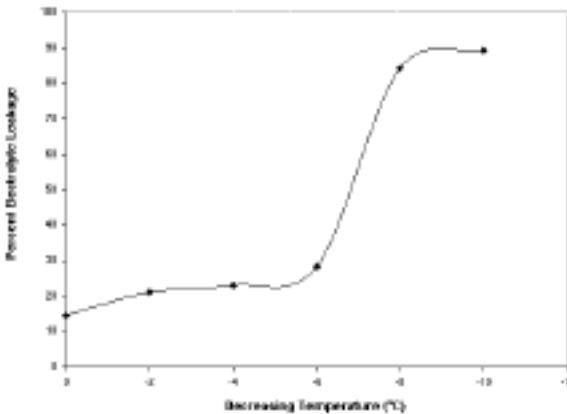than the non-cold acclimated plants.

**Figure 2. Effects of low temper-
ature treatment on the freezing
tolerance of cold acclimated
Arabidopsis thaliana.**

The plasmids from the *E. coli* samples containing the AFP cDNA sequences were success-
fully isolated. AFP-encoding cDNA sequences were excised from their respective plasmids
and separated from the remaining plasmid by gel electrophoresis. The spruce budworm
cDNA was successfully excised from the plasmid as indicated by the presence of a 1400bp
band on the agarose gel (data not shown). The yellow mealworm cDNA, however, was not as

easily distinguishable due to its small fragment size of 250bp. Further research must be done to isolate the 250bp AFP cDNA region from the pET-20b plasmid.

Electro-transformation of DH5α with pBI121 was achieved. The appropriate charge of 1.8 kV was determined to be most efficient for electroporation. Electro-transformation of *A. tumefaciens* was not as easily executed. The LB+amp media had extensive growth. It also was not clear if the bacterial cells were in fact electroporated due to the ampicillin resistance expressed in both the desired DNA (pGEM) and the bacterial host (*A. tumefaciens*). Another problem arose with the incubation temperature. *A. tumefaciens* was incubated at 37°C when its preferred growth temperature is 27°C.

DISCUSSION

The data obtained in the freeze tolerance assays (Figures 1 and 2) support data from other studies. Gilmour et al. (1988) found that for *Arabidopsis thaliana* ecotype Landsberg (ecotype RLD was used in the current study) the non-cold acclimated freezing temperature was -3°C and the cold acclimated freezing temperature was -6°C to -8°C. The corresponding values obtained in the current investigation were -3.5°C for the non-cold acclimated tissue and -7°C for the cold acclimated tissue. Similarly, non-cold acclimated excised leaves from different varieties of potato showed freezing points of -2°C to -5.5°C (Sukumaran and Weiser, 1972).

The research conducted to this point has been successful; however, much work remains to be done. The results obtained thus far laid the basic groundwork for transformation of *A. thaliana*. Freeze tolerance curves of untransformed *A. thaliana* were created to determine the freezing point for the plant before the addition of the insect AFP cDNA sequence. The plant transformation vector pBI121 has been electroporated into *E.coli* but not *A. tumefaciens*. The isolation of the spruce budworm cDNA from pBluescript has been successful and purification of the cDNA is underway. The yellow mealworm AFP cDNA has not been isolated, but this is also underway. The preparation of competent cells was performed to determine the parameters for further electroporation. A charge of 1.8 kV provided the most transformed electroporated cells.

From the results obtained all the necessary pieces to create a plant transformation vector complex with the AFP cDNA sequence can be completed. The pBI121 vector-AFP gene complex will then be electroporated into *E. coli* and subsequently transferred into *A. tumefaciens*. *A. tumefaciens* will be used due to its specific function of DNA transfer between kingdoms; it has the ability to transfer the desired gene from a plasmid into the host plant chromosome. *A. tumefaciens* will be vacuum infiltrated into *A. thaliana* by the protocol adapted from Green (1996). Following vacuum infiltration, the transformed *A. thaliana* will be tested using the freeze tolerance assay, protein and RNA blots, protein expression assays, and extracellular washes of the plant tissues.

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