



# The use of antifreeze proteins for frost protection in sensitive crop plants



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## ABSTRACT

Antifreeze proteins (AFPs), also known as ice binding proteins (IBPs), have evolved as an important adaptation in numerous organisms exposed to subzero temperatures. Plant AFPs have only been identified in freeze tolerant species (those able to survive extracellular freezing). Consequently, plant AFPs have very low specific activities as they have not evolved to completely prevent ice formation in the plant. In contrast, fish and most insect AFPs function to prevent freezing in species that have evolved freeze avoidance mechanisms. Therefore, the activity of these AFPs, especially those of insects (as they are generally exposed to considerably lower temperatures than fish), is much greater. The ability of AFPs to non-colligatively lower the freezing point of water (thermal hysteresis) has led to the idea that frost-sensitive crop plants could avoid damage resulting from common minor frost events in late spring and early autumn by expressing high activity AFPs that permit them to remain unfrozen to temperatures of approximately  $-5^{\circ}\text{C}$ . Over the past 20 years, the efficacy of this concept has been tested in a variety of studies that produced transgenic plants (including *Arabidopsis thaliana*, and several crop plants) expressing various AFPs. Initially, fish AFPs were employed in these studies but as insect AFPs, with higher levels of antifreeze activity, were discovered these have become the AFPs of choice in plant transformation studies. Some studies have produced transgenic plants that have exhibited improved cold tolerance of  $1-3^{\circ}\text{C}$  compared to the wild-type. None of the studies with transgenic plants, however, have yet attained a sufficient level of protection. Progress to this point indicates that more significant results are achievable. If so, the billions of dollars lost annually to frost damage of sensitive crops could be avoided. Geographic ranges and growing seasons could also be expanded. This review provides an overview of the studies of transgenic plants producing AFPs, and makes suggestions for future advancements in this field of study.

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## 1. Introduction

Despite overall increases in mean daily temperatures, it is expected that there will be an increase in the number of devastating spring frosts due to the erratic weather patterns associated with global climate change (Gu et al., 2008). In April, 2007 the midwest, central and southern plains, and southeast portions of the U.S. experienced a record breaking freezing event that cause unprecedented damage to many economically important crops in excess of 2 billion dollars (Gu et al., 2008). Since that time, several spring frost events have caused significant losses to the fruit industry. For example, 50–90% of fruit crops (grapes, peaches,

apples, cherries, etc.) were lost in the Northeast and Midwest in the spring of 2012. In 2013, Chile experienced a late spring frost that resulted in a 22% reduction in exportable fruit representing a loss of 800 million dollars. While efforts were made to provide frost protection during all these events, most were futile. Partially to blame for this failure is our continuing lack of knowledge about what makes plants freeze at a particular temperature and a lack of effective, economical, and environmentally-friendly methods of frost protection (Wisniewski et al., 2008). Lowering ice nucleation temperatures and inhibiting the propagation of ice from the outside to the interior of plants is an approach to frost protection whose potential has been noted by several authors (Ball et al., 2002; Hacker and Neuner, 2007; Lindow, 1995; Wisniewski et al., 2003, 2008, 2009). The use of highly active antifreeze proteins (AFPs), and possibly antifreeze glycolipids (AFGLs) (Walters et al., 2009, 2011), represents a logical approach to limiting frost damage by inhibiting inoculative freezing and enhancing supercooling in freeze-sensitive, annual plants and newly emerging plant parts on perennial plants. Transgenic plants expressing AFPs could also potentially extend the growing seasons

**Abbreviations:** AFP, antifreeze protein; DAFP, antifreeze protein from *Dendroica canadensis*; IBP, ice binding protein; kDa, kiloDalton; NMR, nuclear magnetic resonance; CAT, chloramphenicol acetyl transferase; LT<sub>50</sub>, temperature lethal to 50% of the individuals.

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and expand the geographic ranges of several crops and horticultural plants.

Plant phenology, such as the timing of budbreak and the onset of flowering and vegetative growth, is strongly controlled by climate and as such has become a strong bioindicator of ongoing climate change (Gordo and Sanz, 2010). An analysis of 29 years (1971–2000) of phenological data in Europe has indicated that 78% of all leafing, flowering and fruiting records have advanced and that the average advance of spring has been 2.5 days decade<sup>-1</sup> (Menzel et al., 2006). A report on Mediterranean ecosystems, which includes 29 perennial plant species monitored from 1943 to 2003, stated that spring phenological events are changing more than autumn events as the former events are more sensitive to climate conditions and are thus undergoing the greatest alterations (Gordo and Sanz, 2010). Khanduri et al. (2008) in a study of 650 temperate, globally-distributed plant species has reported that spring-related phenological events have advanced 1.9 days decade<sup>-1</sup> and autumn-related events an average of 1.4 days decade<sup>-1</sup>. Therefore, the impact of the predicted increase in episodes of spring frosts (Gu et al., 2008) will be exacerbated in many species due to the early onset of spring growth. Additionally, Ball and Hill (2009), in a review of several studies, indicated that elevated atmospheric CO<sub>2</sub> concentrations can have a negative impact on plant cold acclimation and as a result enhance vulnerability to frost damage. Consequently, in spite of anticipated global warming the need for protecting vulnerable plants from freeze damage will continue and become even more critical.

The process by which plants actively undergo changes in gene expression and biochemistry resulting in an enhanced ability to withstand freezing temperatures and desiccation stress is referred to as cold acclimation (Weiser, 1970; Wisniewski et al., 2003). Mechanisms associated with cold hardiness are generally divided into two categories: freeze avoidance and freeze tolerance (Sakai and Larcher, 1987). Plants that are cold acclimated and can withstand exposure to subzero temperatures are principally freeze tolerant or exhibit combined mechanisms of freeze tolerance in some tissues and freeze avoidance (deep supercooling) in other tissues (Kasuga et al., 2007, 2008). In contrast, many animals completely avoid freezing in winter, and antifreeze proteins are often involved in this ability. For example, freeze-avoiding larvae of the Alaskan beetle *Cucujus clavipes* routinely supercool to -40 °C in winter, and when exposed to especially cold temperatures further adapt to avoid freezing to as low as -100 °C while vitrifying at temperatures near -70 °C by means of cryoprotective dehydration, antifreeze proteins and glycolipids, and high molar concentrations of glycerol (Sformo et al., 2010, 2011; Walters et al., 2009), as well as numerous other mechanisms (Carrasco et al., 2011, 2012).

Cold acclimation is a multigenic, quantitative trait that involves biochemical and structural changes that have a dramatic effect on the physiology of a plant (Levitt, 1980; Weiser, 1970). There is no consensus on the number and identity of genes causally related to cold acclimation. Various reports have estimated that from <100 to 1000 genes are up-regulated and a similar number are down-regulated (Bassett and Wisniewski, 2009; Fowler and Thomashow, 2002). Cold acclimation is an inducible process requiring both low temperature (<10 °C) and moderate to high light (generally above 400 μmol m<sup>-2</sup> s<sup>-1</sup>) to achieve maximum hardiness. The specific conditions required to achieve maximum cold hardiness are species specific. It is a dynamic process that may require days or weeks.

Because of the complex changes in physiology, metabolism, structure, and water content associated with cold acclimation, plants that are actively growing, flowering, or breaking dormancy typically have little to no frost tolerance (Sakai and Larcher, 1987; Wisniewski et al., 2003) and thus are very susceptible to frost damage. Therefore, current approaches to improving frost tolerance typically involve the overexpression of a specific transcription

factor (e.g. CBF) controlling a cold regulon (defined as a suite of cold-inducible genes) or overexpression of a specific “cryoprotective” gene. The former approach, however, requires the use of a cold-inducible promoter to avoid the adverse effects of overexpression of the transcription factor on growth (Wisniewski et al., 2011). While true cold acclimation requires numerous adaptations, it is still possible that less encompassing short-term approaches can be identified that are capable of protecting sensitive plants, or plant parts such as blossoms, from spring frosts, where temperatures reach only a few degrees below freezing. The use of antifreeze proteins obtained from freeze avoiding animals such as insects, are potential candidates.

Antifreeze proteins (AFPs) are an important component of the repertoire of adaptations to subzero temperatures in many organisms, including numerous plants. AFPs are thought to function by adsorbing onto the surface of ice crystals, blocking the addition of water molecules to growth sites and thereby lowering the temperature at which the crystal will grow (Jia and Davies, 2002; Knight et al., 1991; Raymond and DeVries, 1977; Raymond et al., 1989). Recent evidence has demonstrated that some AFPs can also affect water structure at some distance from the actual surface of the AFP and this may also be important in the antifreeze capabilities of these proteins (Ebbinghaus et al., 2010, 2012; Meister et al., 2013). By conventional definition, the freezing and melting points of an aqueous sample are identical. The temperature at which a small crystal will melt completely if the temperature is raised slightly is the melting point and the temperature at which the crystal will begin to grow as the temperature is lowered slightly is the freezing point. However, this is not the case if antifreeze proteins are present. AFPs have only a small effect on the normal melting point of water, raising it slightly (Celik et al., 2010; Knight and DeVries, 1989). AFPs, however, decrease the temperature at which an ice crystal grows, defined as the hysteretic freezing point, by an average of 1–2 °C below the melting point in fishes and 2–5 °C in insects, although the difference can be as much as 13 °C in the Alaskan beetle *Cucujus clavipes* in winter when the insect is dehydrated and the AFPs concentrated (Duman et al., 2010). This difference between the melting and hysteretic freezing points is termed thermal hysteresis (TH) and is characteristic of AFPs (DeVries, 1971, 1986). The usual technique for determining the presence of AFPs in a sample is to assay for this unique thermal hysteresis activity (DeVries, 1986). The magnitude of the measured thermal hysteresis is often, depending on the AFP, inversely correlated with the size of the crystal(s) present in the sample being tested (Husby and Zachariassen, 1980; Nicodemus et al., 2006). Consequently, the level of freezing protection provided by AFPs in insects to combat inoculative freezing across the cuticle is generally greater than the measured thermal hysteresis, because the size of the cuticular water pores through which external ice might propagate are much smaller than the size of the crystals used in the thermal hysteresis measurements (Duman et al., 2010). AFPs can also inhibit ice nucleators, thereby lowering the nucleation temperature and promoting supercooling (Duman, 2001, 2002). Therefore, AFPs can promote freeze avoidance by inhibition of (1) inoculative freezing across the body surface and (2) ice nucleators (Duman et al., 2010).

Although AFPs were first found in Antarctic fish (DeVries, 1971), thermal hysteresis has been identified in numerous diverse organisms including many insects (Duman, 1977, 1979a, 2001; Duman et al., 2010), collembola (Graham and Davies, 2005; Lin et al., 2007; Zettel, 1984), spiders (Duman, 1979b; Zachariassen and Husby, 1982), mites (Block and Duman, 1989), nematodes (Wharton et al., 2005), plants (Duman, 1994; Duman and Olsen, 1993; Griffith and Yaish, 2004; Griffith et al., 1992; Huang and Duman, 2002; Simpson et al., 2005; Smallwood et al., 1999; Urrutia et al., 1992; Worrall et al., 1998), and fungi and bacteria (Duman and Olsen, 1993; Hoshino et al., 2003; Sun et al., 1995). AFPs have been purified from

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