# REGULATION OF ACCUMULATION OF ANTIFREEZE PROTEINS IN WINTER RYE (*Secale cereale* L.)

by

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## ABSTRACT

Winter rye acquires the ability to tolerate freezing when the plant is exposed to nonfreezing temperature. During cold acclimation, winter rye accumulates antifreeze proteins (AFPs) in the leaf apoplast where ice forms. The individual polypeptides with antifreeze activity were characterized under denaturing conditions to be similar to three classes of pathogenesis-related proteins: glucanase-like (GLPs), chitinase-like (CLPs), and thaumatin-like proteins (TLPs). The objectives of this study were two-fold: first, to characterize the winter rye antifreeze proteins in their native forms (Chapter Two), and second, to identify both hormonal and environmental regulators of AFP accumulation (Chapter Three and Four).

In characterizing the native state of these AFPs by native polyacrylamide gel electrophoresis, gel-filtration chromatography, immunoblotting, and immunoprecipitation (Chapter Two), my evidence suggests that the cold-induced rye AFPs form oligomeric complexes containing various combinations of GLP, CLP, and TLP, in addition to other unidentified proteins *in vivo*. The highest antifreeze activity was found in the apoplastic extract that is a mixture of all AFPs, followed by the complex containing GLP, CLP, and TLP, and then complexes consisting of GLP and TLP or GLP and CLP. By forming oligomeric complexes, antifreeze proteins may form larger surfaces to interact with ice or they may simply increase the mass of the protein bound to ice. In either case, the complexes of antifreeze proteins may inhibit ice growth and recrystallization more effectively than the individual polypeptides.

The cold-responsive AFPs are shown, for the first time to my knowledege, to be induced by drought (Chapter Three) and ethylene (Chapter Four) at a temperature

noninducive for cold acclimation, which suggests that drought and ethylene may regulate the accumulation of AFPs involved in freezing tolerance in winter rye. The accumulation of AFPs in response to low temperature is abscisic acid (ABA)-independent (Chapter Three) because exogenous ABA did not induce accumulation of AFPs at warm temperature and application of the ABA biosynthesis inhibitor fluridone failed to prevent the accumulation of AFPs in cold-acclimated plants. Although drought (Chapter Three), ethylene (Chapter Four), salicylic acid (Chapter Four), and the low temperature parasitic fungus snow mold all induce the accumulation of β-1,3-glucanase, chitinase, and thaumatin-like proteins immunologically similar to the low temperature-induced PR proteins in winter rye, the fact that only drought-, ethylene- and low temperature-induced PR proteins have antifreeze activity suggests that different genes encoding PR proteins may be expressed in response to different stimuli. One set of PR protein genes responds to pathogens and salicylic acid (SA), whereas the second set is induced by low temperature, drought, and/or ethylene.

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## CHAPTER ONE

# GENERAL INTRODUCTION

Winter rye (*Secale sereale*) is an overwintering plant that can survive freezing temperatures lower than -20 $^{0}$C by forming ice in the intercellular spaces (Pearce et al., 1988; Brush, et al., 1994). Winter rye acquires freezing tolerance when the plant is exposed to nonfreezing low temperature, a process known as cold acclimation. The biochemical, physiological and molecular changes associated with cold acclimation and freezing tolerance in winter rye have been the center of research and study in this laboratory.

## Extracellular Ice Formation

Extracellular ice forms in most plant tissues when ice nucleation occurs at a temperature just below 0 $^{0}$C and the tissues are allowed freeze slowly (Asahina, 1978). Ice first forms on the hydrated outer surfaces of the cell wall partly because the extracellular fluid has a lower solute concentration and a higher freezing point than the intracellular fluid. Because the free energy associated with ice is less than that of liquid water at a given temperature, the formation of extracellular ice leads to the movement of unfrozen water from inside of cell to the ice located in intercellular spaces, causing cellular dehydration. Lethal injury to unhardy plant cells can occur directly as a result of extracellular freezing, causing the breakage of the plasma membrane or simultaneous

freezing of intra- and extracellular water, and/or indirectly by freeze-induced dehydration and decrease in cell volume (Asahina, 1978; Levitt, 1980; Lindstrom et al., 1983). If tissues from cold-acclimated plants are frozen slowly, the cells form more extracellular ice and contract to a greater degree than cells from nonacclimated tissues. Therefore, important components of freezing tolerance must be the ability of the plants to restrict ice formation to extracellular spaces and to prevent physical damage to the cells from ice crystals.

**Antifreeze Proteins in Winter Rye**

One of the adaptive responses of winter rye plants to low temperature is the secretion and accumulation of proteins in the leaf apoplast where ice forms (Griffith et al., 1992). These apoplastic proteins accumulate to levels of about 0.3 mg protein $g^{-1}$ leaf fresh weight after 7 weeks of cold acclimation at $5^0$C and decrease quickly in concentration within a few days if the plants are returned to $20^0$C to deacclimate (Marentes et al., 1993). Little is known about the mechanism controlling the decrease in apoplastic proteins during deacclimation. Many of these apoplastic proteins are antifreeze proteins (AFPs) because they exhibit the ability to modify the growth of ice crystals (Griffith et al., 1992; Hon et al., 1994) and the ability to inhibit the recrystallization of ice, which is the growth of larger ice crystals at the expense of smaller ice crystals, at concentrations as low as 25 μg protein $L^{-1}$ (Griffith and Antikainen, 1996).

In characterizing the apoplastic proteins, Hon et al. (1994, 1995) extracted these proteins from winter rye leaves by first infiltrating leaves with extraction buffer and then

by centrifugation to recover the proteins. When the apoplastic proteins from cold-acclimated winter rye leaves are denatured and separated by SDS-PAGE, seven major polypeptides are present. Six of these apoplastic polypeptides with molecular masses ranging from 16 to 35 kD exhibit antifreeze activity when assayed individually *in vitro* (Hon et al., 1994). However, these six polypeptides are not unique proteins. Amino-terminal amino acid sequence analysis showed that the six polypeptides have high percentage identities to three classes of pathogenesis-related (PR) proteins: two of the apoplastic polypeptides correspond to β-1,3-endoglucanases, two are endochitinases, and two are thaumatin-like (TLP) proteins (Hon et al., 1994). Endoglucanases, endochitinases, and TL proteins are known as PR proteins because they can be induced to accumulate in plants by pathogens, and the presence of the PR proteins is positively correlated with disease resistance (Carr and Klessig, 1989; Stinzi et al., 1993). Carr and Klessig (1989) proposed that glucanases and chitinases may act either as lysozymes capable of destroying the cell walls of pathogens and/or as agents causing limited degradation of pathogen cell walls and consequent release of elicitor compounds which stimulate other defense systems. TL proteins that have high degree of sequence homology to the α-amylase and trypsin inhibitor of maize may protect plant from proteases released by pathogens (Carr and Klessig, 1989). The identities of these AFPs were confirmed by probing blots of the cold-induced apoplastic polypeptides with antisera produced against pathogen-induced tobacco endochitinase (PR-3; Legrand et al., 1987), β-1,3-endoglucanase (PR-2; Keefe et al., 1990), and TL protein (PR-5; Kauffmann et al., 1990; Pierpoint et al., 1992). Although β-1,3-endoglucanase and endochitinase activities are present in crude apoplastic extracts of both nonacclimated

and cold-acclimated leaves, only apoplastic extracts obtained from cold-acclimated leaves exhibit antifreeze activity (Hon et al., 1995). In addition, a native chitinase purified from cold-acclimated leaves exhibits both antifreeze activity and endochitinase activity (Hon et al., 1995). Because the apoplastic proteins that accumulate at cold temperature in winter rye have both antifreeze activity and enzymatic activities, these proteins may play dual roles in freezing tolerance and resistance to low-temperature diseases. More studies have yet to be done to test this hypothesis.

**Possible Roles of Antifreeze Proteins**

Three lines of evidence suggest that AFPs have roles in the mechanism of cold acclimation and freezing tolerance in winter rye. First of all, AFPs specifically accumulate in the apoplast during cold acclimation because extractable levels of apoplastic proteins decrease dramatically within a few days if the plants are returned to $20^0$C to deacclimate (Marentes et al., 1993). Secondly, the accumulation of AFPs is positively associated with increased freezing tolerance in winter rye (Marentes et al., 1993) and with greater winter survival in winter wheat (Chun et al., 1998). Moreover, leaves from cold-acclimated winter rye plants become less freezing-tolerant after the concentration of apoplastic proteins is reduced by extraction (Marentes et al., 1993). Thirdly, AFPs have the ability to modify the growth of ice crystals and inhibit the recrystallization of ice *in vitro* (Griffith et al., 1992; Griffith and Antikainen 1996).

How might the AFPs enhance freezing tolerance? Two possible functions of AFPs have been proposed (Griffith and Antikainen, 1997; Antikainen et al. 1996). One

potential role of AFPs is to prevent the formation of large ice crystals. Physical damage caused by ice can occur in frozen tissues when small ice crystals condense into larger ones, a process known as recrystallization (Knight and Duman, 1986). Although recrystallization occurs slowly during prolonged freezing at very low temperatures, it can happen quickly at temperatures near the melting point of ice. In nature, the inhibition of ice recrystallization may be the primary role of AFPs in freezing-tolerance organisms (Knight and Duman, 1986). In the field, winter rye leaves form extracellular ice during winter (Pearce, 1988) and are exposed to fluctuating subzero temperatures that promote the recrystallization of ice. Winter rye AFPs inhibit ice recrystallization effectively at very low concentrations (Griffith and Antikainen, 1997). Therefore, even the presence of low concentrations of rye AFPs in the apoplast may be effective in maintaining the small size of extracellular ice crystals when the plants are exposed to conditions promoting recrystallization. This enhances freezing tolerance by preventing physical damage caused by larger ice crystals. AFPs may also function as a barrier to inhibit ice propagation. Immunolocalization studies have shown that GLPs, CLPs, and TLPs with antifreeze activity all accumulate in the epidermis and cell walls lining intercellular spaces of CA rye leaves (Antikainen et al., 1996; Pihakaski-Maunsbach et al., 1996). Given the capabilities of AFPs to bind to ice and modify its growth, epidermal AFPs, coupled with cold-induced cutinized cell walls and increased epicuticular waxes, may reduce the likelihood of secondary ice nucleation of plant tissues arising from ice formed on the surfaces of the leaves. In addition, AFPs associated with the cell walls of xylem vessels and the cell wall junctions of the mestome sheath (Pihakaski-Maunsbach et al.,

1996) may slow the propagation of ice through the xylem and outward from the xylem into surrounding tissues.

**Other Cold-Responsive Proteins**

Besides AFPs, a number of proteins have been shown to accumulate during cold acclimation. Many of these proteins have no known enzymatic activity but their presence is correlated with the development of freezing tolerance. Guy et al. (1992) reported that spinach seedlings accumulate 160- and 85-kD (CAP160 and CAP85, respectively) proteins in the cytoplasm at low temperature. To determine whether these two proteins have roles in freezing tolerance, transgenic tobacco plants that constitutively express the spinach CAP85 and CAP160 proteins have been made and show slower rate of freeze-induced cellular damage than the wild type (Kaye et al., 1998). In addition, the amount of electrolyte leakage with time of freezing at $-2^0$C is less in the transgenic plants (Kaye et al., 1998). These results suggest that CAP85 and CAP160 proteins have a small but detectable effect on freezing tolerance. Cold-acclimated spinach and cabbage synthesize polypeptides that may be effective in protecting isolated thylakoid membranes against freeze-thaw damage *in vitro* (Volger and Heber, 1975). Using a partially purified protein preparation Hincha and co-workers (1990) showed that these polypeptides may function by reducing membrane permeability during freezing and increasing membrane expandability during thawing. One of the cryoprotective proteins with a molecular mass of 7 kD was partially purified recently from cold-acclimated cabbage and protected isolated thylakoids against freeze-thaw damage in vitro (Sieg et al., 1996). A peach

dehydrin protein (PCA60) that is induced in response to low temperature and other environmental stimuli was purified and has detectable cryoprotective and antifreeze activity (Wisniewski et al., 1999). In Arabidopsis plants, a 9.4 kD polypeptide (COR (cold-regulated)15am) encoded by a *COR15a* gene is synthesized in the chloroplast stroma in response to low temperature, drought, and ABA (Thomashow, 1994). This polypeptide has been recently confirmed to have a direct role in freezing tolerance. Artus et al. (1996) demonstrated that transgenic nonacclimated Arabidopsis plants that constitutively express the COR15am polypeptide exhibited increased freezing tolerance of both chloroplasts frozen *in situ* and isolated leaf protoplasts frozen *in vitro* by 1 to $2^0$C over the temperature range of -4 to $-8^0$C when compared with nonacclimated wild-type plants that do not contain COR15am polypeptide. However, this effect is below $LT_{50}$. The specific mechanism of how constitutively expressed COR15am enhances the freezing tolerance in the transgenic plants has yet to be determined.

Many proteins that are encoded by cold-regulated genes and that are predicted to have known enzymatic activity from sequence homology with known functional proteins may have a role in freezing tolerance. For instance, a fatty acid desaturase and a putative lipid transfer protein encoded by the Arabidopsis *FAD*8 gene might contribute to freezing tolerance by altering lipid composition (Gibson et al., 1994). Heat-shock proteins (HSPs) encoded by a spinach *HSP*70 gene (Anderson et al., 1994) and a Brassica napus *HSP*90 gene (Krishna et al., 1995) may function as molecular chaperones to stabilize proteins against freeze-induced denaturation. Also, various signal transduction and regulatory proteins encoded by cold-responsive genes have been identified, including a mitogen-activated protein (MAP) kinase (Mizoguchi et al., 1993), a MAP kinase kinase

(Mizoguchi et al., 1996), calmodulin-related proteins (Polisensky et al., 1996), and 14-3-3 proteins (Jarillo et al., 1994). These proteins might contribute to freezing tolerance by controlling the expression of freezing tolerance genes or by regulating the activity of proteins involved in freezing tolerance.

## Regulation of Cold-Induced Genes and Their Products

The ability of plants to develop freezing tolerance is generally believed to be under genetic control (Guy et al., 1985). Many genes and their products that are induced during cold acclimation and may contribute to freezing tolerance have been identified (Thomashow 1999; Hughes and Dunn, 1995)

### *Regulation of Arabidopsis COR Genes by CBF Transcriptional Activators*

Arabidopsis *COR* genes are highly expressed in response to low temperature, drought, and/or ABA. The *COR* genes consist of four gene families, each of which is composed of two genes that are physically linked in tandem array. The *COR78* and *COR15* gene pair encodes newly discovered polypeptides (Nordin et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1994; Lin and Thomashow, 1992). The *COR6.6* gene pair encodes polypeptides that have high homology to an AFP of winter flounder (Hajela et al., 1990; Kurkela and Franck 1990; Kurkela and Borg-Franck, 1992). The *COR47* gene pair encodes homologs of LEA II (late embryogenesis abundant group II) proteins (also known as dehydrins and LEA D11 proteins) (Lin et al., 1990). Recent

studies have shown that the multiple *COR* genes act in concert to increase freezing tolerance. Artus et al. (1996) found that the expression of the *COR15a* gene and accumulation of its product, the COR15am polypeptide do not increase freezing tolerance at the whole-plant level in nonacclimated transgenic Arabidopsis plants. Instead, the expression of the entire battery of *COR* genes increases freezing tolerance at whole-plant level more than expressing the *COR15a* gene alone (Jaglo-Ottosen et al., 1998). Expression of the suite of *COR* genes was achieved by overexpressing the gene encoding the Arabidopsis transcriptional activator CBF1 (CRT/DRE binding factor1) (Stockinger et al., 1997). CBF1 binds to a DNA regulatory element, the DRE (dehydration responsive element) or CRT (C-repeat), which is present in the promoters of *COR15a*, *COR78*, *COR6.6*, and *COR47* genes and stimulates transcription in response to low temperature and drought (Yamaguchi-Shinozaki and Shinozaki, 1993). CBF activators are composed of three closely related proteins, CBF1, CBF2, and CBF3, that are encoded by *CBF* genes (Gilmour et al., 1998). Based on the finding that the transcript levels of the three *CBF* genes increase within 15 min after Arabidopsis plants are exposed to low temperature, followed by accumulation of *COR* gene transcripts at about 2 h, Gilmour et al. (1998) proposed that induction of *COR* gene expression in response to low temperature involves a two-step cascade of transcriptional activators. The first step is *CBF* induction that involves an unknown activator or inducer of *CBF* expression (ICE) that is activated by low temperature. Activated ICE binds to a cold-regulatory element present in the promoters of each *CBF* gene, resulting in *CBF* gene expression. The accumulation of CBF proteins, in turn, acts at the CRT/DRE element to induce *COR* gene expression and lead to cold acclimation responses in plants. The CBF regulatory pathway may be

conserved in plants. For example, CRT-containing LTREs (low-temperature-responsive-elements) are present in the promoter region of the cold-regulated *Brassica napus BN115* gene (Jiang et al., 1996). Vazquez-Tello et al. (1998) reported that the cold-regulated wheat *WCS120* gene has a cold-inducible promoter containing two putative CRT/LTREs that also function at low temperature in the monocotyledonous plants barley, rye and rice, as well as in the dicotyledonous plants alfalfa, *Brassica napus* and cucumber.

*Role of Drought*

Evidence suggests that drought plays a role in the development of freezing tolerance in plants. First of all, drought can induce freezing tolerance in many plants at warm temperature. Chen and Li (1978) demonstrated that water deficit induces freezing tolerance in red osier dogwood stems. A drought stress imposed on young seedlings grown at 25$^0$C also led to the induction of maximal freezing tolerance in spinach plants (Guy et al., 1992). Moreover, winter rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) plants exposed to a mild drought stress (40% relative humidity) at ambient temperature for 24 h also became as freezing-tolerant as plants subjected to cold acclimation for 4 weeks at 4$^0$C (Cloutier and Andrews, 1984; Cloutier and Siminovitch, 1982; Siminovitch and Cloutier, 1982). Secondly, some of the cold-regulated proteins exhibit both common biochemical properties and sequence homology with the drought-responsive proteins known as late embryogenesis abundant (LEA) proteins. For example, cold-regulated spinach CAP85 (Neven et al., 1993), wheat WCS120 (Houde et al., 1992), Arabidopsis COR47 (Gilmour et al., 1992; Lin et al., 1990) and COR15a (Thomashow,

1994), alfalfa CAS15 (Monroy et al., 1993), and barley HVA1 (Hong et al., 1982; Hong et al., 1992) are all similar proteins that are highly hydrophilic, heat-stable, are rich in glycine, histidine or lysine and contain a lysine-rich repeated sequence that is present in LEA proteins (Close, 1997). Thirdly, transcription of many of the genes encoding those drought-responsive proteins is also regulated by both low temperature and drought and their products have important roles in cold acclimation and freezing tolerance (Thomashow, 1999). Although freezing tolerance can be increased in overwintering plants by exposure to either cold temperature or drought, the regulatory pathway(s) by which environmental changes are sensed is(are) not well characterized. As mentioned above, a conserved DNA regulatory sequence known as the dehydration-responsive element (DRE) or C-repeat (CRT) is present in the promoters of Arabidopsis *COR15a*, *COR78*, *COR6.6*, and *COR47* genes (Thomashow, 1999) and is responsive to both low temperature and drought (Yamaguchi-Shinozaki and Shinozaki, 1994). Therefore, the role of drought in freezing tolerance may involve transcriptional regulation of cold-responsive genes and their products.

*Role of ABA*

ABA (abscisic acid) is implicated in the plant response to low temperature by a number of observations. (1) Endogenous ABA levels increase, at least transiently, in plants exposed to low temperature (Chen et al., 1983; Lang et al., 1994). (2) Application of ABA can substitute for low temperature in inducing freezing tolerance and the synthesis of cold-induced proteins in several plant species (Bray, 1988; Chen et al., 1983;

Chen and Gusta, 1983; Lång et al., 1989; Tseng and Li, 1990). (3) ABA-null mutants of Arabidopsis (*aba1*) that are impaired in their ability to synthesize ABA are less freezing tolerant than wild-type plants (Gilmour and Thomashow, 1991; Heino et al., 1990; Mantyla et al., 1995). In addition, a number of cold-responsive genes, such as *kin*1 (Hajela et al., 1990) and *kin*2 (Kurkela and Franck, 1990), *cor*15A (Lin and Thomashow, 1992), and *rab*18 (Lång and Palva, 1992) genes of Arabidopsis, *A*13 (Zhu et al., 1993) of *Solanum commersonii*, and *blt*4 of barley (White et al, 1994), are regulated by ABA and/or drought (Hughes and Dunn, 1996). Chen et al. (1983) and Lee and Chen (1993) hypothesized that ABA may function as a trigger in cold acclimation, that is, low temperature activates ABA biosynthesis, resulting in increased endogenous ABA concentration, which, in turn, initiates the cold acclimation response, possibly through activating transcription of genes required for freezing tolerance. This has been shown to be the case for the Arabidopsis *RAB18* and *LTI65* genes. Mutant plants carrying either ABA-deficient (*aba1*) or ABA-insensitive (*abi*) mutations severely decrease cold-induced accumulation of transcripts for these genes (Lång and Palva, 1992; Nordin et al., 1993, Warren et al., 1996). In addition, ABA-responsive elements (ABREs) are found in the promoter regions of these genes (Lång and Palva, 1992; Nordin et al., 1993, Yamaguchi-Shinozaki and Shinozaki, 1994), which suggests that ABA-regulated expression of these genes is mediated through the action of ABREs. However, although many cold-regulated genes can also be induced by both cold and ABA (Hajela et al., 1990; Kurkela and Borg-Frank, 1992; Nordin et al., 1993), the cold induction of these genes may not necessarily depend on endogenous ABA. For example, the ABA-deficient mutant *aba1* and ABA-insensitive mutant *abi1* of Arabidopsis plants do not reduce cold

induction of *COR* gene expression (Gilmour and Thomashow, 1991) and *lti140* gene expression (Nordin et al., 1991). Moreover, the DNA regulatory element DRE/CRT mediates *COR* gene expression by low temperature and drought, but not ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). These results suggest that an ABA-independent pathway exists in the regulation of some cold-responsive genes and their products.

*Role of Ethylene*

The simple gas ethylene plays an important role in regulating growth and development in higher plants (Abeles et al., 1992). Ethylene is also a signal for plants to adapt to a changing environment. For instance, ethylene mediates stress responses such as the wound response and pathogen response (Ecker,1995; O'Donnell et al., 1996).

Considerable progress has been made in the genetic and molecular dissection of the ethylene-response pathway based on the isolation of the ethylene-response mutants in Arabidopsis plants. Briefly, ethylene is perceived by a family of membrane-associated ethylene receptor (ETR) homodimers, ETR1, ERS1, ETR2, EIN4, and ERS2 (Schaller et al., 1995). In the absence of ethylene, the receptors repress ethylene responses, possibly through direct activation of the downstream negative regulator CTR1. CTR1 is thought to be function as a MAPKKK in a MAP kinase module ( Kieber, 1997). Binding of ethylene inhibits receptor activation of CTR1, possibly by inhibition of histidine autophosphorylation, followed by phosphotransfer to an unidentified receiver (Gamble et al., 1998). The inactivated CTR1 induces the expression of the *EIN2* (ethylene-

insensitive 2) gene encoding a novel protein with a domain similar to an ion transporter (Alonso et al., 1999). The carboxy-terminal portion of EIN2 protein activates the nuclear protein EIN3, which is a member of the EIN3 family of transcription factors and acts downstream of EIN2 (Chao et al., 1997). EIN3 is a positive regulator that induces expression of the *ERF/EREBP* (ethylene-responsive factor/ethylene-responsive element binding protein) gene (Solano et al., 1998). The accumulated ERF/EREBPs will, in turn, bind to the AGCCGCC sequence (GCC box) present in the promoter regions of ethylene-regulated genes (Solano et al., 1998), leading to ethylene responses (Chang and Shockey, 1999).

Although there is no report, to my knowledge, that ethylene is involved in regulation of cold-regulated genes and proteins associated with freezing tolerance in plants, three lines of evidence suggest that ethylene may play a role in plants in response to low temperature. (1) Exposure to low temperature (0-10$^0$C) increases ethylene synthesis in the leaves and fruits of several plant species (Field, 1981; 1990). (2) Application of the ethylene-releasing agent ethephon results in both increased levels of ethylene and frost tolerance in tomato plants (Liptay et al., 1982). (3) The ethylene-insensitive tomato mutant (*Nr*) synthesizes more ethylene than the ethylene-sensitive wild-type plant in response to low temperature, but fails to develop chilling tolerance. In contrast, the wild-type plants develop chilling tolerance in association with increased levels of ethylene (Ciardi et al., 1997), indicating that ethylene may regulate the development of chilling tolerance.

Ethylene has been found to induce the expression of many PR genes and their products, including β-1,3-glucanase and chitinase (Felix and Meins, 1987; Mauch et al.,

1992; Xu et al., 1994;). Promoter deletion analysis revealed that an ethylene-responsive element (ERE) or GCC box is present in the promoter regions of the β-1,3-glucanase gene (Ohme-Takagi and Shishi, 1995), basic chitinase gene (Zhou et al., 1997), and *PR-5d* gene encoding an isoform of the thaumatin-like protein (Sato et al., 1996) of tobacco plant. In addition, the accumulation of EREBPs is required for the expression of PR genes in pathogen-infected tobacco leaves and germinated seeds (Ohme-Takagi and Shishi.1995; Leubner-Metzger et al., 1998). It is thought, therefore, that ethylene-mediated expression of PR genes and their products is through the activation of ethylene signaling pathway, acting via EREBPs with the ERE as the target. Because winter rye AFPs are PR proteins composed of β-1,3-glucanase, chitinase and thaumatin-like proteins (Hon et al., 1995), they may be regulated by ethylene.

**Objectives**

Antifreeze proteins have been detected in more than twenty plant species, including both dicotyledonous and monocotyledonous plants (Antikainen and Griffith, 1997; Chun et al., 1998). These proteins gradually accumulate mainly in the leaf apoplast in response to low temperature and have been characterized in their denatured form in winter rye (Hon et al., 1994; 1995). However, the regulation of accumulation of antifreeze proteins has not been studied in plants. The objectives of this work were two-fold: first, to characterize the winter rye antifreeze proteins in their native form (Chapter Two), and, second, to identify both hormonal and environmental regulators of AFP accumulation (Chapters Two and Three). The results and conclusions summarized below

will help to further elucidate the function of multiple antifreeze proteins in freezing tolerance of winter rye.

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CHAPTER TWO

# Antifreeze Proteins in Winter Rye Leaves Form Oligomeric Complexes[1]

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Abbreviations: AFP, antifreeze protein; BCIP, 5-bromo-4-chloro-3-indolylphosphate-toluidine salt; CA, cold-acclimated; CLP, chitinase-like protein; GLP, ß-1,3-glucanase-like protein; NA, nonacclimated; NBT, nitro blue tetrazolium; NP, native protein; PR, pathogenesis-related; TLP, thaumatin-like protein

## ABSTRACT

Antifreeze proteins similar to three pathogenesis-related proteins, glucanase, chitinase and thaumatin-like protein, accumulate during cold acclimation in winter rye (*Secale cereale*) leaves, where they are thought to modify the growth of intercellular ice during freezing. The objective of this study was to characterize the rye antifreeze proteins in their native forms, and our results show that these proteins form oligomeric complexes *in vivo*. Nine proteins were separated by native PAGE from apoplastic extracts of cold-acclimated winter rye leaves. Seven of these proteins exhibited multiple polypeptides when denatured and separated by SDS-PAGE. After isolation of the individual proteins, six were shown by immunoblotting to contain various combinations of glucanase-, chitinase-, and thaumatin-like proteins in addition to other unidentified proteins. Antisera produced against individual cold-induced winter rye glucanase-, chitinase-, and thaumatin-like antifreeze proteins all dramatically inhibited glucanase activity in apoplastic extracts from cold-acclimated winter rye leaves and each antiserum precipitated all three proteins. These results indicate that each of the polypeptides may be exposed on the surface of the protein complexes. By forming oligomeric complexes, antifreeze proteins may form larger surfaces to interact with ice or they may simply increase the mass of the protein bound to ice. In either case, the complexes of antifreeze proteins may inhibit ice growth and recrystallization more effectively than the individual polypeptides.

# INTRODUCTION

Winter rye is an overwintering plant that survives freezing to temperatures below -20°C by forming ice in intercellular spaces (Brush et al., 1994; Pearce, 1988). As winter rye plants acclimate to low temperatures, they secrete proteins into the leaf apoplast where ice forms. These apoplastic proteins accumulate to levels of about 0.3 mg protein $g^{-1}$ leaf fresh weight after seven weeks of cold acclimation and decrease dramatically in concentration within a few days if the plants are returned to 20°C to deacclimate (Marentes et al., 1993). Many of these apoplastic proteins are antifreeze proteins (AFPs) because they modify the growth of ice crystals and inhibit the recrystallization of ice when assayed *in vitro* (Griffith et al., 1992; Hon et al., 1994; Griffith and Antikainen, 1996). Although the AFPs are an important component of winter survival in winter cereals (Chun et al., 1997), we know little about how these proteins may function *in vivo*. The objective of this study was to characterize the rye AFPs in their native forms.

When the apoplastic proteins from cold-acclimated (CA) winter rye leaves are denatured and separated by SDS-PAGE, six major polypeptides with molecular masses ranging from 16 to 35 kD are present. All six polypeptides exhibit antifreeze activity when assayed individually (Hon et al., 1994). However, these six polypeptides are not unique proteins, as shown by amino-terminal amino acid sequencing, immunoblotting and assays of enzymatic activity (Hon et al., 1995). Two of the apoplastic polypeptides were identified as ß-1,3-endoglucanases (GLPs), two are similar to endochitinases (CLPs) and two are similar to thaumatin-like proteins (TLPs). Endoglucanases, endochitinases and thaumatin-like proteins are also known as pathogenesis-related (PR) proteins

because they can be induced to accumulate in plants by many plant pathogens and the presence of PR proteins is positively correlated with disease resistance (Carr and Klessig, 1989; Stintzi et al., 1993). Although ß-1,3-endoglucanase and endochitinase activities are present in crude apoplastic extracts of both nonacclimated (NA) and CA leaves, only apoplastic extracts obtained from CA leaves have antifreeze activity (Hon et al., 1995). In fact, a native chitinase purified from CA leaves exhibits both antifreeze activity and endochitinase activity (Hon *et al.*, 1995). Because the apoplastic proteins that accumulate at cold temperature in winter rye have both antifreeze and enzymatic activities, these proteins may play dual roles in freezing tolerance and resistance to low temperature diseases.

Our first attempts at isolating the native AFPs from apoplastic extracts of CA winter rye leaves showed that the antifreeze polypeptides co-eluted during gel filtration chromatography (Griffith et al., 1992), which suggested that they may be associated in complexes. In order to elucidate the possible synergistic role of AFPs in the mechanism of freezing tolerance of winter rye plant, we characterized winter rye AFPs accumulated during cold acclimation in their native forms using three different approaches: (1) separation of cold-induced apoplastic proteins by native-PAGE and by chitin-affinity chromatography, (2) examination of each native protein by SDS-PAGE and immunoblotting, and (3) immunoinhibition of glucanase activity and immunoprecipitation of AFPs in cold-acclimated rye apoplastic extracts. Our results indicate that the winter rye AFPs form oligomeric complexes *in vivo*.

# MATERIALS AND METHODS

## Plant Materials and Growth Conditions

Winter rye (*Secale cereale* L. cv Musketeer) seeds were surface-sterilized in a 0.3% sodium hypochlorite solution for 5 min, rinsed with distilled water several times, planted in 15-cm pots of coarse vermiculite and germinated at 20/16°C (day/night) with a 16-h daylength and a PFD of 300 μmol $m^{-2}s^{-1}$ for a week. NA plants were grown under these conditions for an additional two weeks. CA plants were transferred to 5/2°C (day/night) with an 8-h daylength and a PPFD of 300 μmol $m^{-2}s^{-1}$ for an additional seven weeks. NA plants grown at 20/16°C for 3 weeks have similar physiological age to the CA plants grown at 5/2°C for 7 weeks (Krol et al., 1984; Griffith and McIntyre, 1993). Plants were watered as needed with modified Hoagland solution (Huner and Mcdowall, 1976).

## Apoplastic Protein Extraction

Apoplastic proteins were extracted by vacuum-infiltrating the leaves with extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$, followed by centrifugation at 900 g to recover the proteins (Hon et al., 1994). Total protein was measured using the Bradford (1976) method, as modified by BioRad (Mississauga, ON, Canada), with BSA as the standard protein. Diluted crude apoplastic extracts were concentrated about two-fold for CA samples and ten-fold for NA samples, as needed, by ultrafiltration with Centriprep-10 concentrators (Amicon, Beverly, MA, USA).

**Protein Electrophoresis and Purification**

Apoplastic proteins extracted from rye leaves were separated by an 8% (w/v) continuous native-polyacrylamide gel using the Mini-Protein II Cell and a single-well preparative comb according to the manufacturer's instructions (BioRad). The gel buffer was 30 mM ß-alanine and 20 mM lactic acid, pH 3.8. In order to locate the position of each protein band, an 0.5-cm wide gel strip was cut from each of the two longitudinal edges of the gel immediately after electrophoresis, stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid (for 10 min), destained with 40% (v/v) methanol and 10% (v/v) acetic acid (for 20 min), and then carefully matched to the remaining gel. Gel pieces corresponding to the individual proteins shown on the two Coomassie blue-stained gel strips were cut from the remaining gel. The gel pieces of each native protein were placed in five-fold diluted gel buffer and homogenized using a Gel Nebulizer (Amicon, Beverly, MA, USA). The homogenized gel slurries were sonicated overnight to allow the proteins to diffuse out of the gel. After centrifugation at 14,500 g for 10 min, the native proteins were recovered from the supernatant and concentrated in one step with Micropure separators and Microcon microconcentrators (Amicon, Beverly, MA, USA). The above procedures were carried out at 4°C. Each of the native proteins from the apoplastic extracts was denatured and the component polypeptides were separated on SDS-PAGE (15% w/v acrylamide) and stained with Coomassie brilliant blue R-250 according to Laemmli (1970).

**Immunoblotting**

Isolated native proteins and polypeptides were transferred onto 0.45-mm nitrocellulose membranes (BioRad) using the Mini Trans-Blot cell (BioRad) according to the manufacturer's instructions. A solution of 0.7% (v/v) acetic acid, pH 3.8, was used to transfer native proteins, and a buffer composed of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3, was used to transfer polypeptides. After the blots were blocked in a buffer of 25 mM Tris-HCl (ph 7.6), 140 mM NaCl, and 1 % (w/v) milk powder over night at room temperature, the blots were probed with the anti-GLP antiserum (dilution 1:2000), or anti-CLP antiserum (dilution 1:2000), or anti-TLP antiserum (dilution 1:10000) produced against isolated winter rye AFPs similar to GLPs, CLPs, and TLPs, respectively (Antikainen et al., 1996). After incubation with each antiserum for 2 hr at room temperature, the blots were incubated with alkaline phosphatase conjugated to goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) for 1 hr at room temperature. The coloured immunoreactions were detected with BCIP (BioShop, Burlington, ON, Canada) and NBT (Sigma) as substrates within 5 min at room temperature.

**Glucanase Activity Assay and Immunoinibition of Glucanase Activity**

Total ß-1,3-glucanase (EC 3.2.16) activity was assayed colorimetrically using laminarin (Sigma) as a substrate and dinitrosalicylic reagent to detect the reducing sugars produced according to Abeles and Forrence (1970) with some modifications. Crude apoplastic extract (50 µL) was added

to 50 μL of 1% (w/v) laminarin in the extraction buffer and then incubated at 37°C for 10 min. The reaction was stopped by adding 300 μL of dinitrosalicylic reagent and heating at 95°C for 5 min. The resulting colored solution was cooled to room temperature, diluted 1:10 with distilled, deionized water, and the absorbance at 500 nm was read using a Microplate reader (Model EL308, Bio-Tek, Burlington, VT, USA). The blank was a mixture of 50 μL of crude extract, 50 μL of 1% laminarin and 300 μL of dinitrosalicylic reagent with zero time incubation. The specific enzyme activity was defined as the amount of enzyme that produced reducing sugar at a rate of 1 nmole of glucose equivalent per second per mg protein.

For the experiment involving immunoinhibition of ß-1,3-glucanase activity, crude apoplastic extracts from cold acclimated plant were incubated (apoplastic extract:antiserum, 2:1 v/v) with either the preimmune serum, or anti-GLP antiserum, or anti-CLP antiserum, or anti-TLP antiserum at 25°C for 20 min, and then total ß-1,3-glucanase activity was assayed as described above.

**Chitinase Assay**

Endochitinase activity was assayed colorimetrically as the release of N-acetyl-glucosamine (GlcNAc) from colloidal chitin (Legrand et al., 1987), which was generated from chitosan for use as a substrate (Molana et al., 1977). Briefly, apoplastic extract (500 μL) was incubated with 0.5 mg of colloidal chitin at 37°C for 1 hr. After centrifugation at 10,000 g for 10 min, 300 μL of supernatant was mixed with 10 μL of 3 % (w/v) snail gut enzyme and incubated at 37°C for 1 hr. The enzymatic reaction was stopped by adding 100 μL of 0.6 M potassium tetraborate and heating at 90°C for 2 min.

After rapid cooling, the mixture was mixed with1 mL of 5% (w/v) 4-(methylamino)benzaldehyde in glacial acetic acid and 5.7 M HCl and incubated at 37°C for 20 min. The amound of released GlcNAc was determined spectrophotometrically at 585 nm. The specific enzyme activity was defined as the amount of enzyme that produced GlcNAc at a rate of 1 nmole of GlcNAc equivalent per second per mg protein.

**Immunoprecipitation of AFPs**

Crude apoplastic extract from CA rye leaves (500 μL) was mixed with 200 μL of pre-swollen protein A-sepharose CL-4B (Sigma) in extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$ to remove apoplastic proteins that bind nonspecifically to the beads. After gently shaking at room temperature for 4 hours, followed by centrifugation at 17,300 g for 10 min, the beads were discarded. The supernatant was mixed with either preimmune serum or anti-GLP antiserum or anti-CLP antiserum or anti-TLP antiserum (10 μL ) and gently shaken at room temperature for 2 hours. Then 50 μL of protein A-sepharose CL-4B (Sigma) pre-swollen in extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$ were added to the extract-antiserum mixture. After 2 hours of shaking at room temperature, followed by centrifugation at 17,300 g for 5 min, the supernatant was discarded and the pellet was washed five times with extraction buffer to remove unbound proteins. The washed pellet was denatured with SDS reducing buffer (Laemmli, 1970) at 90°C for 5 min and centrifuged at 17,300 g for 10 min. The supernatant was analyzed by SDS-PAGE and immunoblotting.

**Purification of Chitinase**

The native rye chitinase was purified from apoplastic extracts of CA winter rye leaves by affinity chromatography using colloidal chitin as the column substrate (Huynh et al., 1992; Hon et al., 1995). The purity of chitinase was examined by 8% native-PAGE and 15% SDS-PAGE stained with Coomassie blue R-250, followed by immunoblotting in which antisera produced against cold-induced rye CLP, GLP, and TLP were used as probes.

**Antifreeze Activity Assay**

Antifreeze activity was assayed qualitatively by examining the morphology of ice crystals (DeVries, 1986) grown in apoplastic extracts of CA rye leaves, in solutions of individual proteins eluted from native gels and in chitinase fractions purified by chitin-affinity chromatography. The growth of ice crystals in each sample was controlled by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) and the morphology of ice crystals was examined using a phase-contrast photomicroscope (Olympus BHT). In order to quantify and compare the effects of different apoplastic extracts on ice crystal growth, a rating system was developed (Chun et al., 1997). Briefly, the round disc-like crystals grown in water were rated 0, because they have no antifreeze activity. Hexagonal discs grown in very low concentrations of AFPs (nM) or in solution of AFPs with low specific activity were rated 1. Hexagonal columns grown in dilute solutions of AFPs (μM) or in solution of AFPs with moderate specific activity were rated 3. Crystals forming complex hexagonal

bipyramids in high concentrations of AFPs (100 μM) or in solution of AFPs with high specific activity were rated 5.

# RESULTS

## Separation of Apoplastic Proteins

Proteins present in the apoplastic extracts from the leaves of NA plants and of plants cold-acclimated for seven weeks were quantified and examined under nondenaturing conditions. The concentration of extractable apoplastic proteins was about ten-fold higher in CA apoplastic extracts ($0.43 \pm 0.05$ mg protein $mL^{-1}$, mean ± SE, n=5) compared with NA apoplastic extracts ($0.04 \pm 0.01$ mg $mL^{-1}$, n=4). We first tried to separate the native apoplastic proteins on a sizing column (BioRad BioGel P-100, 1 x 50 cm column, eluted with 30 mM b-alanine and 20 mM lactic acid, pH 3.8, at a rate of 70 mL $min^{-1}$). Although BSA (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD) and cytochrome C (12.4 kD) were easily separated on the column, the native apoplastic proteins from CA rye leaves yielded only one peak with a trailing shoulder (data not shown). According to the standard curve, these proteins had molecular masses well below 12 kD, which indicated that the proteins were eluted from the column at a slower rate than predicted by their apparent sizes on SDS-PAGE. A similar result had been reported earlier using a sizing column packed with Sephacryl 200 (Griffith et al., 1992).

In contrast to the open columns, good resolution and consistent separations were obtained using a continuous native polyacrylamide (8% w/v) gel system at pH 3.8. As shown in Figure 1, nine native proteins (labeled 1 to 9 and abbreviated as NP1 to NP9) were identified in apoplastic extracts from CA rye leaves, while only six native proteins were present in apoplastic extracts from NA leaves

when equal amounts of apoplastic proteins were separated by native PAGE (Fig. 1, lanes CA and NA2). Although NP2, NP4, NP5, NP6, NP8 and NP9 were present in both CA and NA leaves, three native proteins (NP1, NP3 and NP7) were detected only in CA leaves. Moreover, NP4, NP5, NP6, NP8 and NP9 all accumulated to higher levels in CA leaves, as indicated by the intensity of the Coomassie-blue stain, when equal volumes of apoplastic extracts from both CA and NA rye leaves were loaded on the native polyacrylamide gel (Fig. 1, lanes CA and NA1). Only NP2 was found to accumulate to high levels in the apoplast of NA leaves.

**Comparison of Apoplastic Polypeptides**

When CA and NA apoplastic proteins were denatured and separated by SDS-PAGE, there were 13 polypeptides present in NA apoplastic extracts with apparent molecular masses of 144, 97, 36, 35, 34, 33, 32, 28, 26, 25, 16 (doublet), and 14 kD (Fig. 2A, lane 3), while only seven polypeptides were found in CA apoplastic extracts with molecular masses of 144, 35, 32, 28, 25, 16, and 14 kD (Fig. 2A, lane 1) when equal amounts of protein were loaded on the SDS-polyacrylamide gel. Interestingly, both CA and NA apoplastic proteins contained 32 and 35 kD GLPs, a 35 kD CLP and a 25 kD TLP as detected positively on immunoblots probed with anti-GLP (Fig. 2B), anti-CLP (Fig. 2C) and anti-TLP antisera (Fig. 2D). No GLPs, CLPs and TLPs were detected in NA apoplastic extracts when equal volumes of unconcentrated CA and NA extracts were loaded onto the gels and immunoblots (Figs. 2B, 2C, and 2D, lanes 1 and 2).

## Analysis of the Composition of Native Apoplastic Proteins

The individual proteins shown in Figure 1 were eluted from the native gels, concentrated by ultrafiltration and then denatured and examined by SDS-PAGE. Interestingly, all the native proteins except NP8 and NP9 contained multiple polypeptides when electrophoresed by denaturing SDS-PAGE (Fig. 3A). NP1 was composed of two polypeptides (12 and 97 kD). NP2 contained two polypeptides, which migrated as a doublet with a molecular mass of 35 kD. NP3 showed four polypeptides with molecular masses of 10, 12, and a doublet at 35 kD. NP4 showed seven polypeptides of 12, 14, 24, 25, 30, 32, and 35 kD. NP5 was composed of eight polypeptides with molecular masses of 12, 14, 22, 24, 25, 30, 32, and 35 kD. NP6 showed five polypeptides (13, 15, 22 23, and 25 kD). NP7 showed four polypeptides (15, 22, 23, and 25 kD), whereas NP8 and NP9 exhibited only one 15 kD polypeptide.

## Analysis of Native Proteins by Immunoblotting and Immunoprecipitation

The composition of each native protein was further examined by immunoblotting. The apoplastic proteins initially separated by native-PAGE were denatured and separated by SDS-PAGE (Fig. 3A), then blotted and probed with antisera against cold-induced winter rye AFPs similar to glucanase (Fig. 3B), chitinase (Fig. 3C), and thaumatin-like protein (Fig. 3D). The specificities of these antisera have been described previously (Antikainen et al., 1996). Briefly, antiserum raised against the denatured 32-kD GLP, used in a dilution of 1:2000, recognizes two polypeptides with

molecular masses of 32 kD and 35 kD, which were both identified as GLPs by amino-terminal amino acid sequencing (Hon et al., 1995). Anti-CLP antiserum recognizes only one 35-kD polypeptide at a dilution of 1:2000, although the 35-kD and 28-kD polypeptides were both identified as CLPs by Hon et al. (1995). The anti-CLP antiserum was raised against the native 35-kD CLP, which has a chitin-binding domain that is lacking in the 28-kD CLP. Antiserum raised against the denatured 25-kD TLP detects only one polypeptide with molecular mass of 25 kD at a dilution of 1:10000. At this dilution, the 16-kD polypeptide also identified as a TLP by Hon et al. (1995) is not detected. Each antiserum is specific to one type of AFP and does not cross-react with other apoplastic proteins. Within each type of AFP, the antiserum is more reactive with the protein against which it was raised. As shown in Figure 3, polypeptides associated with NP2 and some of the polypeptides associated with NP3 were positively detected by anti-GLP and anti-CLP antisera. Polypeptides associated with NP4 were detected by anti-GLP, anti-CLP and anti-TLP antisera. Polypeptides associated with NP5 were detected by anti-GLP and anti-TLP antisera, whereas those associated with NP6 and NP7 were detected by anti-TLP antiserum. In addition, native proteins separated by nondenaturing gel electrophoresis were directly transferred to nitrocellulose membranes and probed with antisera against cold-induced winter rye GLP, CLP, and TLP. The results of these experiments confirmed the immunoblotting results obtained with SDS-PAGE (data not shown).

To examine further whether the observed cold-induced GLP, CLP, and TLP were indeed physically associated together, two sets of immuno-experiments were conducted. Because these experiments required the use of higher antiserum concentrations, the specificity of each of the three antisera was determined using a dilution of 1:3 in immunoblots of denatured apoplastic polypeptides

from CA plants. Under these conditions, the anti-GLP antiserum detected two polypeptides with apparent molecular masses of 32 and 35 kD, the anti-CLP antiserum detected two polypeptides at 28 and 35 kD, and the anti-TLP antiserum detected two polypeptides at 16 and 25 kD (Fig. 4). In the first immuno-experiment, glucanase activity in CA apoplastic extracts was assayed after incubating CA apoplastic extracts with specific rye anti-GLP, anti-CLP, or anti-TLP antisera. As shown in Figure 5, glucanase activity was dramatically inhibited in the presence of anti-GLP antiserum. Antisera produced against cold-induced CLP and TLP also inhibited glucanase activity by 93% and 95%, respectively. In other experiments, chitinase activity in the apoplastic extracts was inhibited by adding antisera against either GLP, CLP or TLP (data not shown). Secondly, immunoprecipitation of AFPs by anti-GLP, anti-CLP, and anti-TLP antisera was examined (Fig. 6). The anti-GLP antiserum precipitated not only 32 and 35 kD GLPs, but also a 35 kD CLP and a 25 kD TLP, as evident in the results of SDS-PAGE (Fig. 6A, lane 1) and immunoblotting (Figs. 6B, C and D, lane 1). Different groups of polypeptides were immunoprecipitated by anti-CLP antiserum (Fig. 6, lane 2) and by anti-TLP antiserum (Fig. 6, lane 3), but each of these antisera also precipitated a GLP, a CLP and a TLP (Fig. 6B, C and D).

**Purification and Characterization of NP3**

The separation of native and denatured proteins presented in Figures 1 and 3 could result from the co-migration of proteins similar to GLPs, CLPs and/or TLPs. To test this possibility, we isolated one complex from apoplastic extracts of CA rye leaves by chitin-affinity chromatography (Huynh et

al., 1992; Hon et al., 1994). In this procedure, only chitinases or lectins that have a chitin-binding domain specifically bind to the colloidal chitin used to pack the affinity column. By changing the pH of the washing buffer from pH 8 to 4.5, the non-specifically bound proteins were washed off the column. The specifically bound chitinases were then washed off the column using 20 mM acetic acid, pH 3.0. In our experiments, only one protein was bound specifically to the chitin affinity column. When separated by native PAGE, this protein had an Rf value similar to that of NP3 in the crude CA apoplastic extract (Fig. 7A). When denatured and separated by SDS-PAGE (Fig. 7B), this purified protein was composed of four polypeptides with molecular masses of 10, 12, 35 and 35 kD. The two 35-kD polypeptides were positively detected by antisera produced against cold-induced winter rye GLP and CLP (Fig. 7C).

**Antifreeze Activities of Native Proteins**

The antifreeze activities of individual native proteins eluted from native gels were compared with NP3 purified by chitin-affinity chromatography, crude apoplastic extract from NA leaves, and crude apoplastic extract from CA leaves. The protein concentration of each sample was adjusted to about 0.5 mg protein $mL^{-1}$. As summarized in Table 1, the highest antifreeze activity was found in the CA crude apoplastic extract, which was composed of mixture of all AFPs (rated 5), followed by NP4 which contained GLP, CLP and TLP (rated 4), NP 2 and NP3 which contained CLP and GLP (rate 3), NP5 which contained GLP and TLP (rated 3) and NP6 and NP7 which contained TLP (rated 2). NP1, NP8, and NP9 and crude apoplastic extract from NA leaves did not have any antifreeze

activity.

At this time, we cannot explain why NA apoplastic extracts lack antifreeze activity after they have been concentrated because they contain NP2, NP4, NP5 and NP6 (Fig. 1), all of which exhibit antifreeze activity when isolated from CA extracts (Table 1). It may be that different isozymes of GLPs, CLPs and/or TLPs accumulate at 20°C or that the proteins produced at 5°C are post-translationally modified in some way to acquire antifreeze activity. Either of these possibilities could explain the differences in polypeptide composition between CA and NA extracts observed by SDS-PAGE (Fig. 2).

# DISCUSSION

## Association of Winter Rye AFPs *in vivo*

Our biochemical and immunological evidence shows that apoplastic extracts from CA winter rye leaves contain nine proteins (Fig. 1). Seven of these native proteins are composed of multiple polypeptides when they are denatured and separated by SDS-PAGE (Fig. 3A). Surprisingly, GLPs, CLPs and TLPs are associated with six of the native proteins (Fig. 3B, 3C, and 3D). The association of glucanase with other proteins has been observed previously (Ballance and Manners, 1978; Ji et al., 1995). For example, native ß-1,3-glucanase purified from germinated rye by ion exchange chromatography on DEAE- and CM-cellulose followed by gel filtration on BioGel P-60 was associated with three unidentified proteins (Ballance and Manners, 1978). Moreover, an acidic ß-1,3-glucanase extracted from cucumber leaves infected by tobacco necrosis virus was associated with a class III chitinase because both enzymes migrated together in native PAGE (Ji and Kuc, 1995).

It is possible that the different proteins have the same mobility on a native gel by chance so that they form a single band on a native gel but migrate as several polypeptides by SDS-PAGE. To distinguish between these possibilities, NP3 (Fig. 7) was isolated by chitin-affinity chromatography and electrophoresed using the native gel system in the absence of other apoplastic proteins. Only one native protein was visible (Fig. 7A) and this protein contained both GLP and CLP as determined by immunoanalysis (Fig. 7C). The binding between GLP and CLP appeared to be strong because GLP and CLP remained in association when other proteins were washed off the chitin affinity column

using solutions with pHs ranging from 8.0 to 4.8. The physical association between GLP, CLP and TLP was also evident in the results of immunoinhibition experiments in which ß-1,3-glucanase activity in cold-acclimated crude apoplastic extracts was dramatically inhibited by the presence of either anti-GLP or anti-CLP or anti-TLP antisera, but not by preimmune serum (Fig. 5) and in immunoprecipitation experiments where anti-GLP, anti-CLP or anti-TLP antisera precipitated all three AFPs, GLPs, CLPs and TLPs, from CA apoplastic extracts (Fig. 6). We interpret these results to indicate that all of the polypeptides (GLP, CLP and TLP) are exposed on the surface of the native protein complex. Alternatively, if the complexes are small, then the binding of any one of the antisera may hinder access of the polymeric substrate to the glucanase's active site.

Why are there so many different native proteins in the apoplastic extract? We hypothesize that the individual native proteins are produced by different cell types. For example, immunolocalization studies of AFPs in winter rye revealed that all three classes of AFPs, GLPs, CLPs, and TLPs, are localized in the epidermis of CA leaves (Antikainen et al., 1996). Thus NP4, NP5, NP6 and/or NP7 may be produced by epidermal cells. In contrast, just two classes of AFPs, GLPs and CLPs, accumulate in cell walls surrounding the intercellular spaces in the mesophyll (Antikainen et al., 1996; Pihakaski-Maunsbach et al., 1996). We would expect NP2 and NP3 to be secreted by mesophyll cells. Therefore, each native protein identified in Figure 1 may have a different, tissue-specific location within the apoplast of a rye leaf. Other workers have also reported tissue-specific locations of glucanase and chitinase. In tomato plants, pathogen-induced ß-1,3-glucanase and chitinase are both present in the abaxial epidermal layer near stomata (Wubben et al., 1993). Ethylene-induced chitinase and ß-1,3-glucanase have also been localized together in abaxial

epidermal cells and in parenchymmal cells adjacent to vascular strands in bean leaves (Mauch et al., 1992). Additional evidence for the physical association between ß-1,3-glucanase and chitinase was obtained by immunolocalization of both ß-1,3-glucanase and chitinase in the large electron-dense aggregates located in the vacuoles of lower epidermal cells of ethylene-treated bean leaves (Mauch et al., 1992). The common compartmental location of glucanase and chitinase is consistent with our hypothesis that the two enzymes are physically associated with each other.

The arrangement of specific proteins and enzymes as part of functional complexes has been found in many systems. For example, the cellulose (mainly ß-1,4-glucanase) activity of many cellulolytic bacteria occurs in discrete multifunctional, multienzyme complexes called cellulosomes. These organized complexes account for the efficient solubilization of insoluble cellulose (Bayer et al., 1994). A second example of a multienzyme complex is acetyl-CoA carboxylase (ACCase), which consists of biotin carboxylase and biotin-carboxyl carrier protein and is a regulatory enzyme of fatty acid synthesis (Roesler et al., 1996).

Although ß-1,3-glucanase and chitinase are encoded by two different small gene families (Linthorst 1991), the expression of the two genes is often coordinately regulated upon pathogen infection and exposure to other environmental stresses such as wounding, drying and flooding (Ohashi et al., 1992; Stintzi et al., 1993). Glucanase and chitinase enzymatic activities increase concomitantly in many plants not only in response to pathogen attack but also in response to pathogen-derived elicitors and the plant hormone ethylene (Mauch et al., 1992). Moreover, it has been reported that ß-1,3-glucanase or chitinase purified from pea can not inhibit the growth in culture of most of the fungi tested when used individually. However, a combination of these enzymes

effectively inhibits the growth of most fungi tested (Mauch et al., 1988), which suggests that the two enzymes act synergistically in plant defense.

It is possible that GLPs and CLPs, as well as TLPs, play a synergistic role in improving the winter survival of winter rye plants. First of all, ß-1,3-glucanase and chitinase activities are both high in apoplastic extracts from CA winter rye leaves, so they may act together to provide resistance to pathogens (Hon et al., 1995). Secondly, GLPs, CLPs and TLPs may work in concert to modify the growth of intercellular ice as part of the mechanism of freezing tolerance. These proteins are all present in the apoplast of rye leaves after cold acclimation (Hon et al., 1995) and are all located in the epidermis and in cell walls lining the intercellular spaces of cold-acclimated leaves where they may interact with ice (Antikainen et al., 1996; Pihakaski-Manunsbach et al., 1996). The GLPs and CLPs appear to be physically associated with each other (Fig. 7), which is significant because the antifreeze activity of complexes that contain both GLPs and CLPs is higher than that of complexes that lack these proteins (Table 1).

**Possible Roles of AFP Complexes**

The general mechanism of action of AFPs can be explained by the adsorption-inhibition theory described in detail by Raymond and DeVries (1977). An ice crystal normally grows as a broad front with a low radius of curvature. However, when ice crystals are grown in a solution containing AFPs, the AFPs interact with ice in two unique ways. First, AFPs adsorb onto the nonbasal planes of ice at the ice-water interface (Raymond et al., 1989) and exert a concentration-dependent effect

on ice crystal growth morphology (DeVries, 1986). Second, AFPs adsorbing onto the ice surface block the binding of additional water molecules, which creates an ice crystal surface with many highly curved fronts and a high surface free energy. Consequently, the growth of these fronts is halted because it is less energetically favorable for water molecules to bind to this surface. The temperature must be lowered further to decrease free energy before crystal growth proceeds. As a result, the freezing point of the solution is depressed. Theoretically, one way to increase the effectiveness of an AFP is to increase the size of the protein so that it blocks a greater area on the ice crystal surface. Wu et al (1991a) demonstrated that this size effect does, in fact, occur. They showed that an insect AFP conjugated with rabbit anti-AFP IgG, which has no antifreeze activity by itself, plus goat anti-rabbit IgG antibody, which has no antifreeze activity by itself, exhibits greater antifreeze activity than the insect AFP alone. Furthermore, they showed that a second protein isolated from insect hemolymph can bind to the insect AFP and enhance its activity (Wu et al., 1991b). Thus, the high level of antifreeze activity observed in crude hemolymph extracts obtained from overwintering larvae probably requires the interaction of the insect AFP (12 to 22 kD) and its activator protein (70 kD).

The AFPs in winter rye may function in a similar fashion to the insect AFP plus its activator protein. By forming complexes composed of GLPs, CLPs and/or TLPs, the AFPs may block a larger area of the ice surface, thus making it more difficult for the ice to overgrow the complex(es). As a result, the growth of ice crystals is inhibited to a greater extent and antifreeze activity increases. The fact that the apoplastic extract, which is mixture of antifreeze proteins, has the highest antifreeze activity, followed by the complex containing GLP, CLP, and TLP and then complexes consisting of

GLP and TLP or GLP and CLP (Table 1), provides indirect evidence to support our hypothesis.

Physical damage caused by ice can occur in frozen tissues when small ice crystals condense into larger ones, which is a process known as recrystallization (Knight and Duman, 1986). Although recrystallization occurs slowly during prolonged freezing at very low temperatures, it can happen very quickly at temperatures near the melting point of ice. In nature, the inhibition of ice recrystallization may be the primary role of AFPs in freezing-tolerant organisms (Knight and Duman, 1986). Winter rye leaves form extracellular ice during winter (Pearce, 1988) and are exposed to fluctuating subzero temperatures that promote the recrystallization of ice. Winter rye AFPs inhibit ice recrystallization effectively at very low concentrations (25 μg protein $L^{-1}$) (Griffith and Antikainen, 1996). The ability of rye AFPs to inhibit ice recrystallization at low protein concentrations may be related to both the size of proteins and the presence of multiple ice-binding sites on each protein. For example, it is possible that each AFP complex can interact with more than one ice crystal because each of the components of the complex is able to interact with ice. In summary, we conclude that winter rye AFPs form oligomeric complexes in the apoplast that enhance the ability of these proteins to inhibit the growth and recrystallization of ice.

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## Figure Legends

**Figure 1.** Separation of apoplastic proteins by native polyacrylamide gel electrophoresis. Apoplastic proteins were extracted from NA and CA rye leaves by vacuum infiltration followed by centrifugation. The apoplastic proteins were separated from equal volumes of unconcentrated apoplastic extracts (lanes CA and NA1) and from equal amounts (10 μg) of apoplastic proteins (lanes CA and NA2) in an 8% continuous native polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250. Numbers on the left refer to individual native apoplastic proteins.

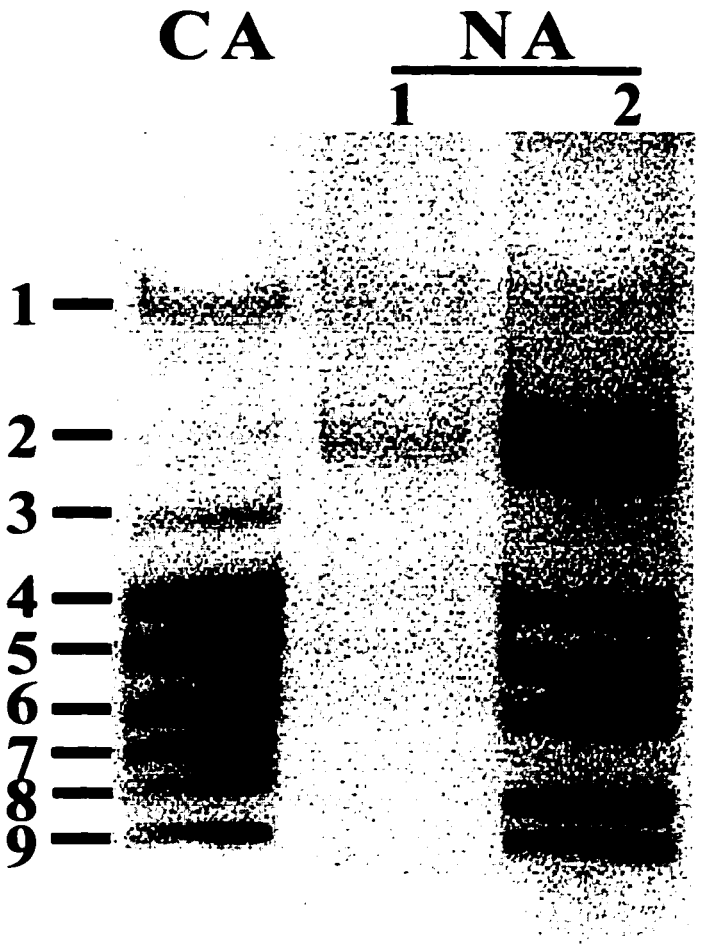

CA
NA
1
2
1
2
3
4
5
6
7
8
9

**Figure 2.** Examination of polypeptides in CA and NA apoplastic extracts by SDS-PAGE and immunoblotting. **(A)** SDS-PAGE (15% acrylamide) of equal volumes of crude CA and NA apoplastic extracts (lanes 1 and 2, respectively) and equal amounts (5 μg) of CA and NA apoplastic proteins (lanes 1 and 3, respectively). Polypeptides were stained with Coomassie brilliant blue R-250. Low range prestained SDS-PAGE molecular mass standards from BioRad are shown on the left (M). SDS-PAGE gels with the same sample loading scheme as shown in panel A were blotted and probed with **(B)** anti-GLP antiserum, **(C)** anti-CLP antiserum, and **(D)** anti-TLP antiserum. Positive immunodetection of polypeptides is indicated by arrows on the right.

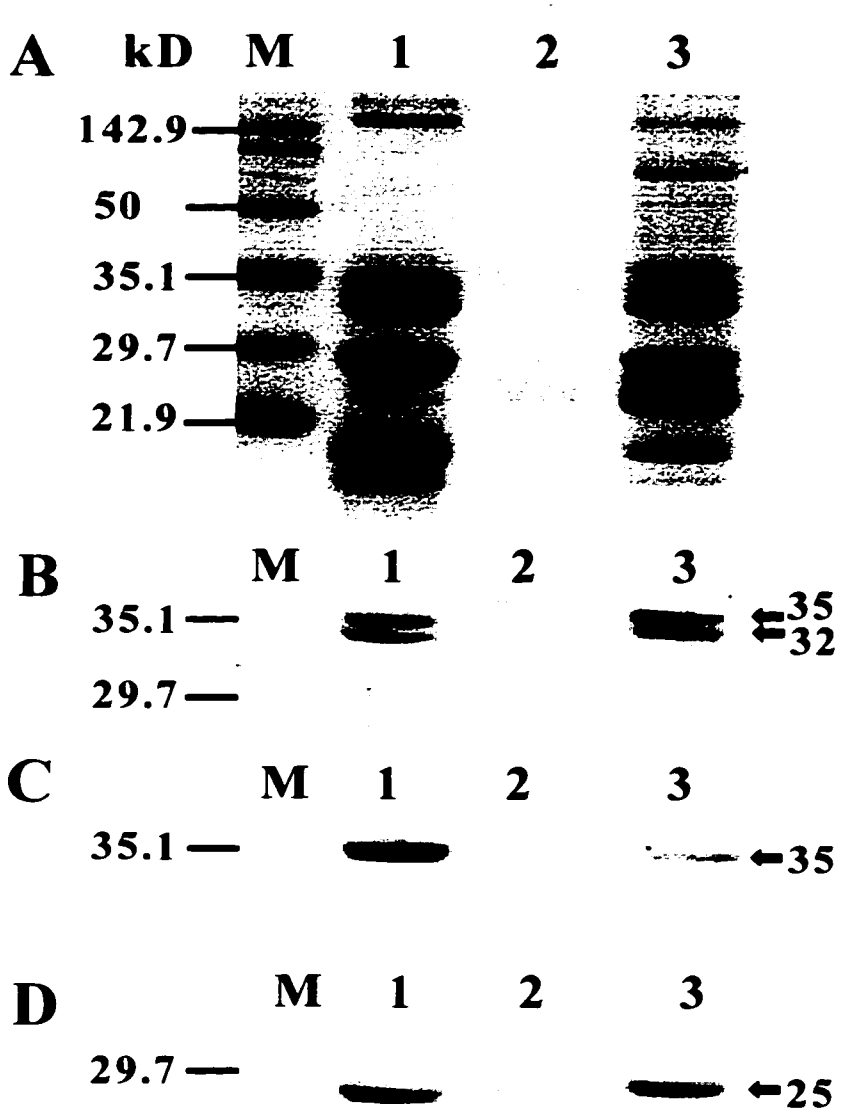

A
kD
M
1
2
3
142.9
50
35.1
29.7
21.9
B
M
1
2
3
35.1
29.7
35
32
C
M
1
2
3
35.1
35
D
M
1
2
3
29.7
25

**Figure 3.** Examination of native apoplastic proteins by SDS-PAGE and immunoblotting. **(A)** Individual CA apoplastic proteins were eluted from the native gel shown in Figure 1, denatured, separated by 15% SDS-PAGE and stained with Coomassie brilliant blue R-250. Equal amounts of proteins (5 mg) were loaded on each lane, and lanes 1 through 9 correspond to NP1 through NP9. The separation of low range SDS-PAGE molecular mass standards from BioRad is shown on the left (M). SDS-PAGE gels loaded with equal amounts (1 μg per lane) of individual native apoplastic proteins and with crude CA apoplastic extract in lane 10 were blotted and probed with **(B)** anti-GLP antiserum, **(C)** anti-CLP antiserum, and **(D)** anti-TLP antiserum. Positive immunodetection of polypeptides in apoplastic extracts is indicated on the right by arrows.

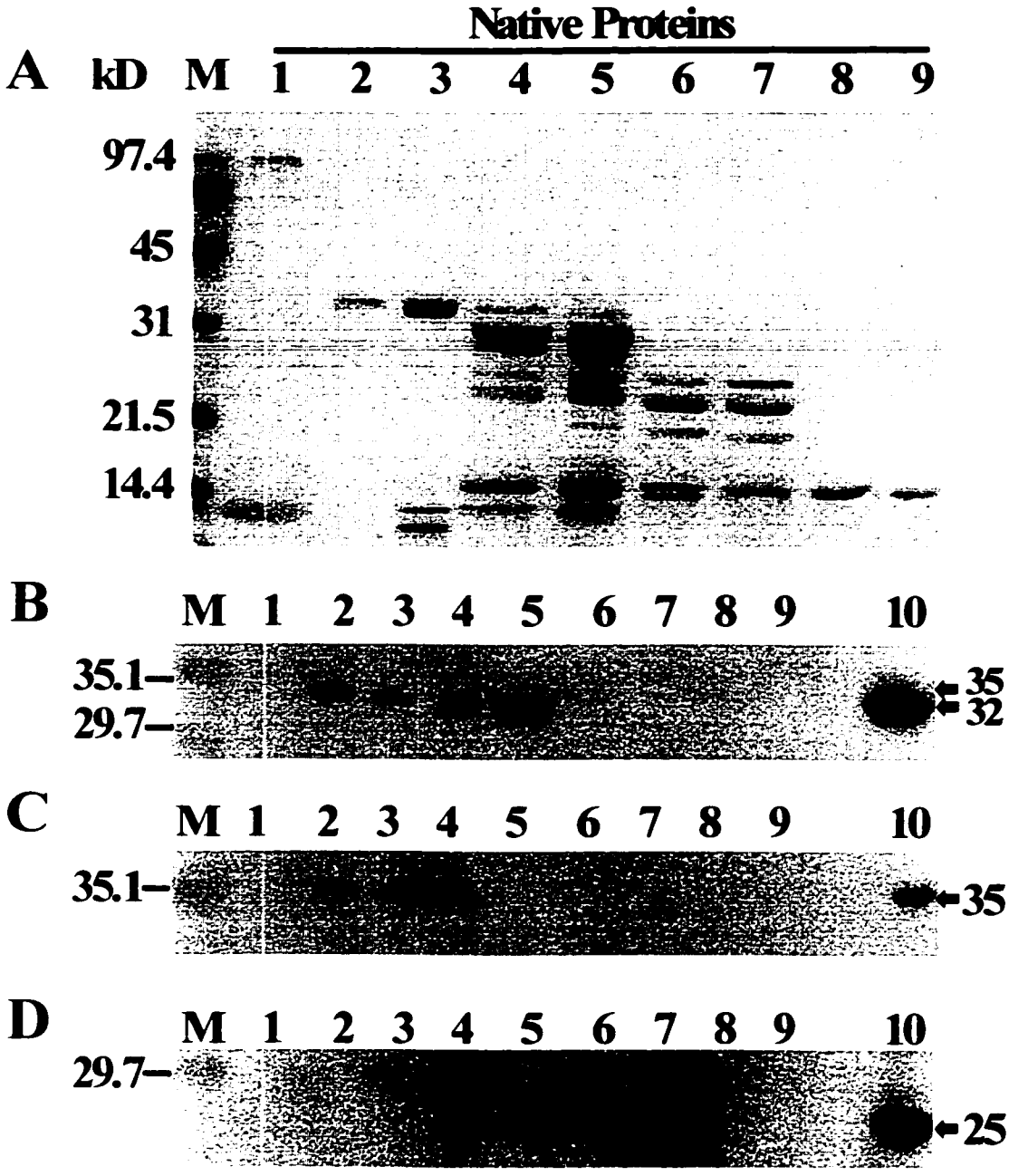

Native Proteins
A
kD
M
1
2
3
4
5
6
7
8
9
97.4
45
31
21.5
14.4
B
M
1
2
3
4
5
6
7
8
9
10
35.1
29.7
35
32
C
M
1
2
3
4
5
6
7
8
9
10
35.1
35
D
M
1
2
3
4
5
6
7
8
9
10
29.7
25

**Figure 4.** Specificities of anti-GLP, anti-CLP, and anti-TLP antisera. CA apoplastic proteins (5 μg) were denatured, separated in a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250 (lane CA). Similar gels were blotted and probed with antisera at a dilution of 1:3. Lane GLP was probed anti-GLP antiserum, lane CLP with anti-CLP antiserum, and lane TLP with anti-TLP antiserum. Low range prestained SDS-PAGE standards (M) were used to determine the molecular masses (kD). The molecular mass of each polypeptide immunodetected by an antiserum is indicated on the right.

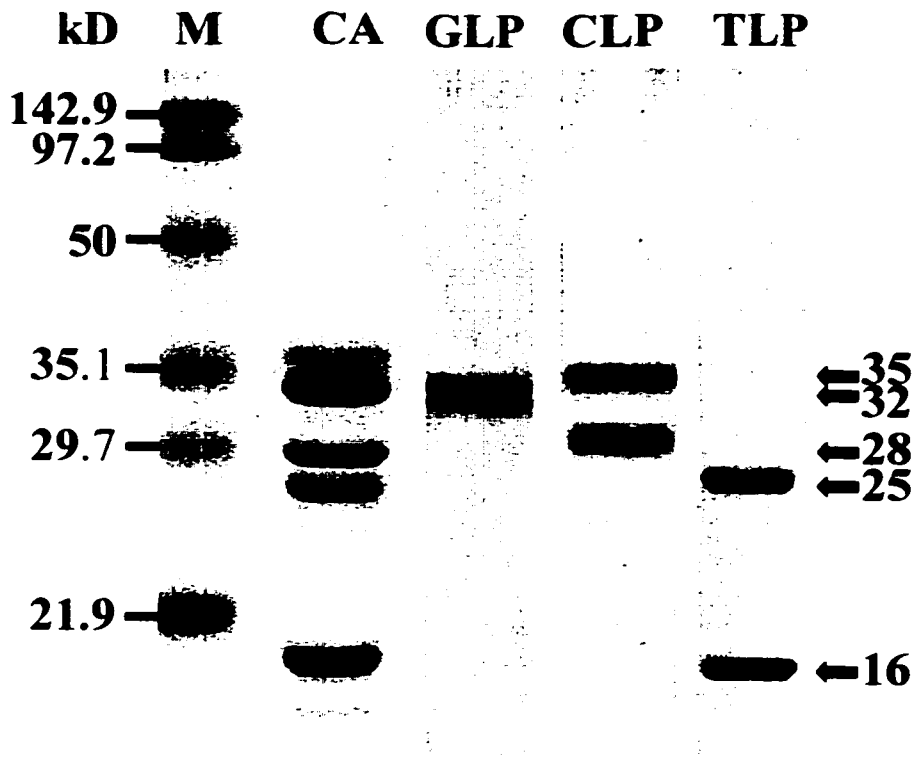

kD
M
CA
GLP
CLP
TLP
142.9
97.2
50
35.1
29.7
21.9
35
32
28
25
16

**Figure 5.** Inhibition of glucanase activity by antisera produced against AFPs. Glucanase activity was assayed in NA and CA apoplastic extracts, and in CA extracts incubated with antisera produced against cold-induced GLP (G), CLP (C), TLP (T), or preimmune serum (P). The glucanase specific activity was normalized as a percentage of the specific activity present in the CA apoplastic extract and is shown as the mean ± SE, n=3.

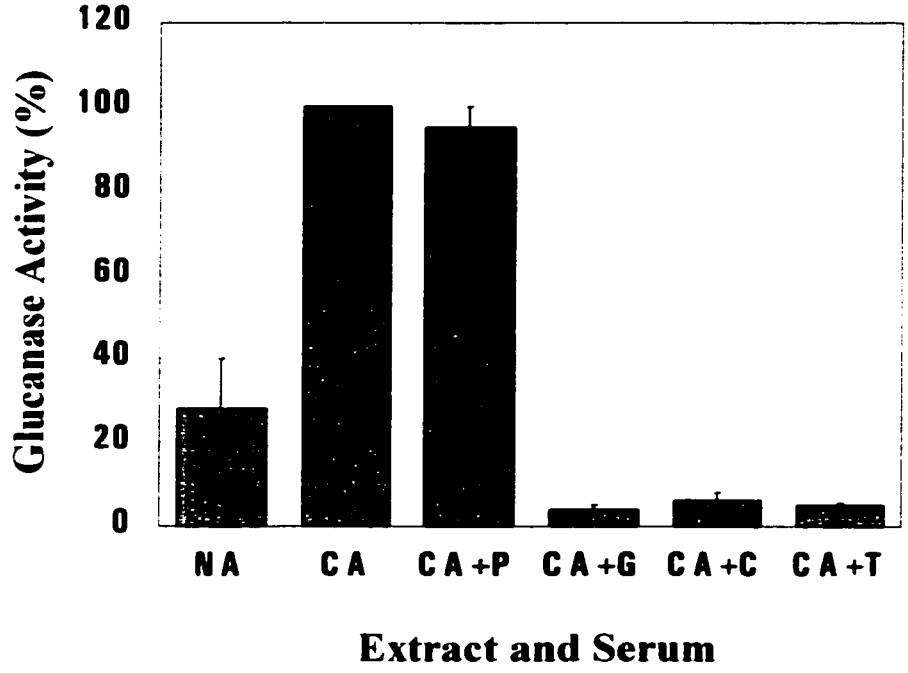

120
100
80
60
40
20
0
Glucanase Activity (%)
NA
CA
CA+P
CA+G
CA+C
CA+T
Extract and Serum

**Figure 6.** Immunoprecipitation of native AFPs by antisera produced against specific AFPs. Equal amounts (5 μg per lane) of proteins immunoprecipitated by anti-GLP (lane 1), anti-CLP (lane 2), anti-TLP (lane 3) antisera, or by preimmune serum (lane 4) were denatured and separated in a 15% SDS-polyacrylamide gel. CA apoplastic extract is shown as a positive control in lane 5. **(A)** A gel stained with Coomassie brilliant blue R-250. Gels were blotted and probed with **(B)** anti-GLP antiserum, **(C)** anti-CLP antiserum, and **(D)** anti-TLP antiserum. BioRad low range SDS-PAGE standards (M) were used in SDS-PAGE and prestained SDS-PAGE standards (M) were used in immunoblotting analysis to determine the molecular masses (kD).

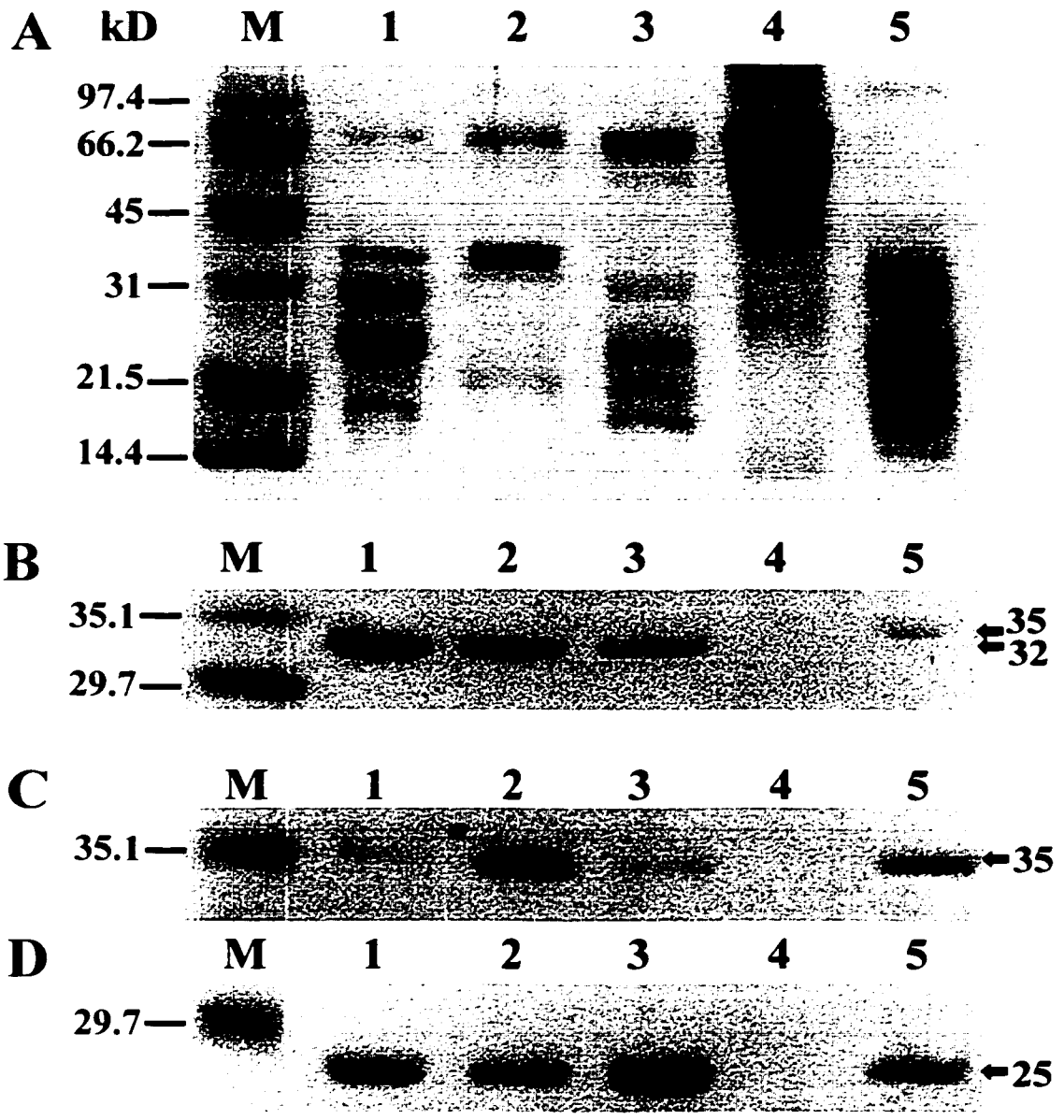

A
kD
M
1
2
3
4
5
97.4
66.2
45
31
21.5
14.4
B
M
1
2
3
4
5
35.1
29.7
35
32
C
M
1
2
3
4
5
35.1
35
D
M
1
2
3
4
5
29.7
25

**Figure 7.** Purification and identification of NP3. CA rye apoplastic extract and purified NP3 were examined by Native-PAGE (A), SDS-PAGE (B) and immunoblotting (C). NP3 was purified from rye apoplastic extract by chitin-affinity chromatography. Low range prestained SDS-PAGE standards (M) were used to determine molecular masses (kD). The gels (A and B) were stained with Coomassie brilliant blue R-250. Anti-GLP antiserum and anti-CLP antiserum were used to detect GLP and CLP, respectively.

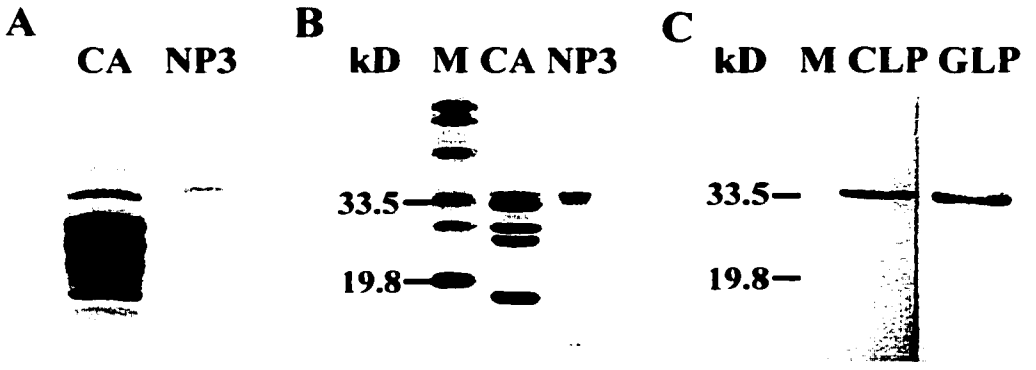

A
CA
NP3
B
kD
M
CA
NP3
33.5
19.8
C
kD
M
CLP
GLP
33.5
19.8

**Table 1**. Comparison of antifreeze activity of individual native proteins with the activity of crude CA and NA apoplastic extracts. The individual proteins were separated by native-PAGE (Fig. 1), eluted and assayed for antifreeze activity. Antifreeze activity of each sample was rated by 0 to 5 based upon the shape of ice crystals grown in solution (see Material and Methods) with 5 representing highest activity and 0 representing no activity. The protein concentration of each sample was determined using the Bradford (1976) method, as modified by BioRad, and is presented as the mean ± SE, n=3.

| Sample | Protein Concentration ($mg\ mL^{-1}$) | Antifreeze Activity |
|---|---|---|
| CA apoplastic extract | 0.45 ± 0.12 | 5 |
| NA apoplastic extract | 0.56 ± 0.07 | 0 |
| NP1 | 0.40 ± 0.09 | 0 |
| NP2 | 0.50 ± 0.14 | 3 |
| NP3 | 0.45 ± 0.10 | 3 |
| NP4 | 0.52 ± 0.08 | 4 |
| NP5 | 0.42 ± 0.08 | 3 |
| NP6 | 0.48 ± 0.09 | 2 |
| NP7 | 0.50 ± 0.10 | 2 |
| NP8 | 0.45 ± 0.08 | 0 |
| NP9 | 0.52 ± 0.15 | 0 |

CHAPTER THREE

# Antifreeze Proteins Accumulate in Response to Cold and Drought, but Not Abscisic Acid

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AFP, antifreeze protein; BCIP, 5-bromo-4-chloro-3-indolylphosphate-toluidine salt; CA, cold-acclimated; CLP, chitinase-like protein; Ethephon, 2-chloroethylphosphonic acid; GLP, β-1,3-glucanase-like protein; NA, non-acclimated; NBT, nitro blue tetrazolium; NP, native protein; PR proteins: pathogenesis-related proteins; TLP, thaumatin-like protein;

## ABSTRACT

Winter rye (*Secale cereale* L.) plants accumulate antifreeze proteins (AFPs) in the leaf apoplast during cold acclimation. In this study, experiments were conducted to assess the role of drought and ABA in the regulation of AFPs. The concentration of apoplastic proteins quickly increased in leaves of plants in response to drought at a temperature noninducive for cold acclimation. The level of apoplastic proteins induced by drought within 192 h was equivalent to the level of apoplastic proteins accumulated when plants were acclimated to cold temperature for 7 weeks. These drought-induced apoplastic proteins with molecular masses ranging from 11 to 35 kD contained two glucanases, two chitinases, and two thaumatin-like proteins (TLPs), as identified with antisera raised against cold-induced rye glucanase, chitinase, and TLP, respectively. Antifreeze activity was detected as early as 24 h of drought treatment and increased as the level of apoplastic proteins increased. ABA induced the accumulation of apoplastic proteins within 96 h to a level similar to that obtained when plants were drought-stressed for 192 h and cold-acclimated for 7 weeks. Although ABA-induced apoplastic proteins were immunologically similar to two GLPs and two TLPs they did not have the ability to modify the growth of ice crystals. Moreover, the ABA biosynthesis inhibitor fluridone could not prevent the accumulation of AFPs in the leaf apoplast of cold-acclimated plants. Our results show that cold acclimation and drought share a common mechanism in regulating the accumulation of AFPs in winter rye plants and that the accumulation of AFPs in winter rye leaves during cold acclimation is ABA-independent. Endogenous ethylene production increased transiently in drought-treated plants at 72 hr, followed by the accumulation of apoplastic proteins and increased antifreeze

activity at 96 hr. This raises the possibility of ethylene involved in the drought regulating AFPs accumulation in winter rye.

# INTRODUCTION

Overwintering plants require a period of acclimation to cold temperatures to develop freezing tolerance. This process requires the accumulation of proteins whose synthesis increases at low temperature. Some of these proteins include dehydrins (Close, 1997, Wisniewski et al., 1999), proteins involved in carbohydrate metabolism, 14-3-3 proteins, kinase regulators (Jarillo et al., 1994), and CAP160 and CAP85 (Guy et al., 1992) as well as a number of proteins with unknown functions. These proteins are all located within the cells. In addition, three types of proteins accumulate outside the cells during cold acclimation. These include antifreeze proteins (AFPs) that interact with ice (Griffith et al., 1992), pathogenesis- related (PR) proteins (Hiilovaara-Teijo et al., 1999) that protect the plants against disease-causing organisms, and cell wall-modifying proteins (Showalter, 1993).

There is evidence that many cold-regulated proteins also respond to drought stress and/or ABA. For example, a drought stress imposed on spinach seedlings grown at 25°C induces maximal freezing tolerance and the accumulation of 160- and 85-kD (CAP160 and CAP85, respectively) proteins that are also responsive to low temperature (Guy et al., 1992). Some of the cold-regulated proteins exhibit both common biochemical properties and sequence homology with the drought-responsive proteins known as late embryogenesis abundant (LEA) proteins. For example, spinach CAP85 (Neven et al., 1993), wheat WCS120 (Houde et al., 1992) and Arabidopsis COR47 (Gilmour et al., 1992; Lin et al., 1990) are all similar proteins that are highly hydrophilic, heat-stable, are rich in glycine, histidine or lysine and contain a lysine-rich repeated sequence that is present in all LEAII proteins (Close, 1997). Exogenously added ABA can substitute for low temperature in inducing

freezing tolerance and the synthesis of cold-induced proteins in several plant species (Bray, 1988; Chen et al., 1983; Chen and Gusta, 1983; Lång et al., 1989; Tseng and Li, 1990). In addition, a number of cold-responsive genes, such as *KIN*1 (Hajela et al., 1990) and *KIN*2 (Kurkela and Franck, 1990), *COR*15A (Lin and Thomashow, 1992), and *RAB*18 (Lång and Palva, 1992) genes of Arabidopsis plants, *A*13 (Zhu et al., 1993) of *Solanum commersonii*, and *BLT*4 gene of barley (White et al, 1994), are regulated by drought and/or ABA (Hughes and Dunn, 1996).

Cold acclimation of winter cereals at 5 $^{0}$C induces accumulation of antifreeze proteins (AFPs) in the leaf apoplast where ice forms during freezing (Marentes et al., 1993; Hon et al., 1995; Antikainen and Griffith 1997, Chun et al., 1998). The accumulation of these AFPs is correlated with increased freezing tolerance in winter rye (Marentes et el., 1993) and with higher rates of winter survival of winter wheat plants (Chun et al., 1998). Moreover, leaves from cold-acclimated winter rye plants become less freezing-tolerant after the concentration of apoplastic proteins is reduced by extraction (Marentes et al., 1993). Because AFPs bind to the surface of ice crystals and modify their growth (Griffith et al., 1992), they are thought to play a role in the mechanism of freezing survival of winter rye, possibly by inhibiting the recrystallization of extracellular ice formed when plants are exposed to fluctuating and/or prolonged subzero temperatures (Griffith et al., 1997).

The regulation of the synthesis of AFPs has not been studied in any plant. Because rye AFPs accumulate in response to cold temperature, it seemed reasonable to determine whether these proteins are also induced by factors known to effect the accumulation of other cold-induced proteins. In this study, we examined the accumulation of apoplastic proteins in leaves of winter rye plants treated with ABA or drought at warm temperature and compared those apoplastic proteins with the AFPs that

accumulate at low temperature by SDS-PAGE, immunoblotting, and assays of antifreeze activity. We also studied the effect of the ABA synthesis inhibitor fluridone on the accumulation of AFPs in leaves of rye plants grown at low temperature. Our results show that drought induces the accumulation of AFPs at a temperature noninducive for cold acclimation, while ABA induces the accumulation of apoplastic proteins that are immunologically similar to the cold-responsive proteins but do not exhibit antifreeze activity. We conclude that the accumulation of AFPs in response to low temperature is ABA-independent. Because ABA was not involved in regulating the synthesis of AFPs, we examined the possibility that ethylene may be involved in the regulation of drought-induced genes.

# MATERIALS AND METHODS

## Plant Materials and Growth Conditions

Seeds of winter rye (*Secale cereale* L. cv Musketeer) were obtained from Dr. G. McLeod (Agriculture Canada, Swift Current, SK, Canada). Seeds surface-sterilized with a 0.28% sodium hypochlorite solution for 5 min were planted in 15-cm pots of coarse vermiculite and germinated at 20/16°C (day/night) with a 16-h daylength and a PPFD of 300 μmol $m^{-2}s^{-1}$ for a week. Nonacclimated plants (NA) were grown under these conditions for an additional two weeks. Cold-acclimated plants (CA) were transferred to 5/2°C (day/night) with an 8-h daylength and a PPFD of 300 μmol $m^{-2}s^{-1}$ for an additional seven weeks. NA plants grown at 20/16°C for 3 weeks have similar physiological age to the CA plants grown at 5/2°C for 7 weeks (Krol et al., 1984; Griffith and McIntyre, 1993). Plants were watered as needed with Hoagland solution (Hoagland and Arnon, 1950).

## Drought and Chemical Treatments

For drought treatments, 3-week-old NA plants were not watered until wilt was visible. Wilting symptoms first appeared within two days from the time the watering regime was stopped (time zero). Starting at 72 hr, the drought-stressed plants were rewatered daily with 100 mL of Hoagland nutrient solution per pot to keep the plants alive, whereas control plants were watered daily to excess with 500 mL of Hoagland nutrient solution per pot. For ABA treatment, leaves of 3-week-

old NA plants were sprayed to run-off at dark daily with 100 μM ABA (mixed isomers, Sigma, St. Louis, MO, USA) (Guo et al., 1992) and 0.005% (v/v) Tween-20 for 8 days. Control plants were sprayed daily with 0.005 % (v/v) Tween-20. For the treatment with the ABA inhibitor fluridone, leaves of NA plants were sprayed first with 100 μM ABA at 24 hr and then with 100 μM fluridone (a gift from Dr. Malcolm Drew, Department of Horticulture Sciences, Texas A&M University, TX, USA) (Siddiqui et al., 1998) and 0.005% (v/v) Tween-20 at 48 hr. These alternating treatments of fluridone and ABA were repeated for another 96 hr. Control plants were sprayed with 0.005% (v/v) Tween-20. To study the effect of fluridone on the accumulation of AFP in apoplast at low temperature, leaves of NA plants were sprayed with 100 μM fluridone and 0.005% (v/v) Tween-20 immediately after the plants were transferred to 5°C. Control plants were sprayed with 0.005% (v/v) Tween-20. The treatment was continued every other day for 8 weeks. Three independent experiments were carried out for each of the above treatments. The samples harvested at each time point in each experiment were also independent. Leaves of ABA-, drought-treated, fluridone-treated NA plants and control plants were harvested for apoplastic extraction at 24-h intervals. Leaves of fluridone-treated CA plants and controls were harvested every week for apoplastic extraction.

**Relative Water Content Measurement**

The water content of NA leaves and NA leaves treated by drought and ABA was determined by measuring the difference between leaf fresh weight (FWT) and dry weight (DWT). Dry weight of leaf samples was measured after 24 hr of drying at 80°C when no further weight loss occurred.

Relative water content of leaf samples was calculated as ((FWT-DWT)/FWT)) x 100.

**Apoplastic and Total Soluble Protein Extraction**

Apoplastic proteins were extracted as described in Hon et al. (1994). Briefly, the leaves were cut into 3-cm sections, rinsed several times with deionized water, and then vacuum-infiltrated with extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$, followed by centrifugation at 900 g to recover the apoplastic contents. Total soluble proteins were extracted from leaf tissues using a borate buffer containing 50 mM sodium borate, 50 mM ascorbic acid, and 1 mM phenylmethylsulfonyl fluoride (pH 8.5) (Wisniewski et al., 1999). Total apoplastic protein content was measured using the Bradford (1976) method, as modified by BioRad (Mississauga, Canada), with BSA as the standard protein.

**Protein Electrophoresis and Immunoblotting**

Apoplastic proteins extracted from rye leaves with drought and different chemical treatments were denatured and the polypeptides were separated in a 15% (w/v) SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250 (Bio-Rad) according to Laemmli (1970). An equal volume (30 μL) of each apoplastic extract per gram leaf fresh weight was loaded onto each lane. In immunoblotting experiments, an equal volume (10 μl) of each apoplastic extract per gram leaf fresh weight was loaded onto each lane. Low-range unstained SDS-PAGE molecular weight standards

(Bio-Rad) were used to determine the apparent molecular masses of apoplastic polypeptides.

Polypeptides separated by SDS-PAGE were transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad) using the Mini Trans-Blot cell (Bio-Rad) according to the manufacturer's instructions. A buffer composed of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3, was used to transfer polypeptides. The blots were probed with rabbit 35 kD GLP antiserum (dilution 1:2000), or 35 kD CLP antiserum (dilution 1:2000), or 25 kD TLP antiserum (dilution 1:10000) produced against isolated winter rye AFPs (Hon et al., 1994; Antikainen et al., 1996), or dehydrin antiserum produced against a dehydrin consensus peptide TGEKKGIMDKIKEKLPGQH (dilution 1:1000) (a gift from Dr. Timothy J Close, Department of Botany and Plant Sciences, University of California, Riverside, CA, USA). The immunoreactions were detected by alkaline phosphatase conjugated to goat anti-rabbit IgG (Sigma) with 5-bromo-4-chloro-3-indolylphosphate-toluidine salt (BCIP) (BioShop, Burlington, Ontario, Canada) and nitro blue tetrazolium (NBT) (Sigma) as substrates. Low-range prestained SDS-PAGE molecular standards (Bio-Rad) were used to determined the apparent molecular masses of polypeptides. Broad-range prestained SDS-PAGE molecular standards (Bio-Rad) were used to determined the apparent molecular masses of total soluble polypeptides.

**Antifreeze Activity**

Antifreeze activity of drought and chemically treated apoplastic extracts and their controls at 24 hr interval was assayed qualitatively by examining the morphology of ice crystals (DeVries, 1986)

grown in apoplastic extracts obtained from rye leaves subjected to different treatment. The growth of ice crystals in each sample was controlled by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) and the morphology of ice crystals was examined using a phase-contrast photomicroscope (model BHT, Olympus).

**Ethylene Measurement**

To determine the endogenous ethylene production by rye, a single 3-week-old NA seedling was transferred without injury into a test tube (2.5 cm in diameter, 25 cm in height) and grown hydroponically in Hoagland solution for 48 hr. The drought treatment began by quickly surface-drying the seedling using paper tower. The seedling was retransferred into the same test tube without nutrient solution. Before each of the ethylene measurement, the test tube was sealed with a rubber cap. After 30 min, a 1-mL of gas sample was withdrawn through the cap with a gas-tight syringe and analyzed by a Shimadzu gas chromatograph (model GC-17A, Shimadzu Scientific Instruments Inc, Columbia, MD, USA) equipped with an aluminum column (1.5 m long, 0.55 mm ID) and a flame ionization detector. The column temperature was isothermal at $100^0$C and the injector and detector oven temperature was $150^0$C. After each measurement, the rubber cap was removed till next measurement. An analyzed ethylene sample (Alltech, Deerfield, IL, USA) was used as a standard. Three independent experiments were conducted.

**Statistical Analysis**

The two-sample t test (2-tailed) (SAS Institute. 1998) was used at a 5% level of significance to detect differences in apoplastic protein accumulation between chemically treated and control plants at each time point.

# RESULTS

## Drought-Induced Apoplastic Protein Accumulation in Rye Leaves

A mild and constant level of drought stress was imposed on the plants over a period of 192 h when the relative water content of drought-treated plants remained about 10% lower than the controls (Table 1). The level of total apoplastic protein extracted from leaves of drought-treated plants was examined over a period of 192 h. As shown in Figure 1, the drought treatment resulted in a slow but steady accumulation of proteins in the apoplast. The concentration of apoplastic proteins in drought- treated plants began to increase at 24 h and the rate of accumulation of apoplastic proteins increased linearly until the end of the experiment. In contrast, the concentration of apoplastic proteins in control plants remained low throughout the experiment. At 192 h, the apoplastic proteins in drought-stressed plants reached a level of 0.30 ± 0.02 (mean ± SE, n=3) mg protein per gram leaf fresh weight, which was a statistically significant increase ($p = 0.05$) over control plants (0.06 ± 0.01 (mean ± SE, n=3) mg protein per gram leaf fresh weight) and was equivalent to the level of apoplastic proteins accumulated when plants are cold-acclimated for 7 weeks (Marentes et al., 1993).

## Analysis of Drought-Induced Apoplastic Proteins by SDS-PAGE and Immunoblotting

The apoplastic proteins extracted from drought-treated leaves and control leaves were denatured and examined by SDS-PAGE and immunoblotting. Compared with the controls, six

abundant polypeptides with molecular masses of 35-, 32-, 28-, 16-, 14-, and 11-kD were rapidly induced by drought (Fig 2A). The accumulation of these polypeptides closely followed the time course of total apoplastic proteins accumulated in NA leaves treated with drought as shown in Figure 1. At the end of the induction experiment, the pattern of drought-induced polypeptides (Fig 2A, lane 192h +) separated by SDS-PAGE was very similar to the pattern of cold-induced polypeptides (Fig 2A, lane CA) in terms of total numbers of polypeptides and the molecular mass of each polypeptide.

Immunoblots of the polypeptides separated by SDS-PAGE (Fig 2A) revealed that the six abundant polypeptides induced by drought were positively detected by the GLP, CLP, and TLP antisera (Hon et al., 1994; Antikainen et at., 1996). As shown in Figure 2B, a 35- and a 32- kD polypeptide were recognized by GLP antiserum. Although the 32-kD GLP was found in both drought-treated and control apoplastic extracts, it accumulated to a greater level in apoplast of drought-treated plants as the intensity of the band increased relative to the control. The 35-kD GLP was only found in apoplastic extracts of drought-treated plants (Fig. 2B). After 24 h, two polypeptides of 35-kD and 28-kD were positively detected by CLP antiserum in apoplastic extracts of drought-treated plants (Fig. 2C). TLP antiserum detected 25-kD and 16-kD TLPs in drought-treated apoplast and only a 25-kD TLP in control apoplast. As shown in Figure 2D, the 25-kD TLP gradually accumulated after 24 h of drought treatment, whereas the 16-kD TLP was not present until 96 h. The presence of the 32-kD GLP and 25-kD TLP in control apoplastic extracts, as shown in Figures 2A, B and D, indicates that their accumulation may be constitutive or developmentally regulated (Dong and Dunstan 1997), while the presence of the 35-kD GLP, 35- and 28-kD CLP and 16-kD TLP only in apoplastic extracts from drought-treated leaves suggests a specific role for drought in the induction

and accumulation of these proteins.

**Antifreeze Activity of Drought-Treated Samples**

Apoplastic extracts from leaves of drought-treated and control plants were assayed for antifreeze activity. Interestingly, a low level of antifreeze activity was detected in the leaf apoplastic extracts after 24-h of drought treatment (data not shown). After the plants were exposed to drought for 192 h, ice crystals grown in the apoplastic extracts were shaped like hexagonal columns, which indicated a high level of antifreeze activity in the leaf apoplast (Fig. 3 Drought+). A similar high level of antifreeze activity was also detected when winter rye plants were cold-acclimated for 7 weeks (Fig. 3 CA). In contrast, no antifreeze activity was detected in the NA control as indicated by the disc-shaped ice crystals that formed in the apoplastic extracts (Fig. 3 Drought-).

**Accumulation of Apoplastic Proteins in ABA-Treated Leaves**

In a preliminary experiment, we used dehydrin known as an ABA-responsive protein (Close, 1997) as a positive control to determine the proper concentration of ABA. As shown in Figure 4, the application of 100 μM ABA induced accumulation of a 98 kD polypeptide in NA leaves in 96 hr (Fig. 4A). This polypeptide was identified as a dehydrin using antiserum produced against a dehydrin consensus peptide TGEKKGIMDKIKEKLPGQH (Fig. 4B). These results indicated that a dosage of 100 μM ABA can effectively induce an ABA-related response in winter rye plants.

The total apoplastic protein contents of leaves harvested from plants treated with 100 μM ABA were examined for a period of 192 h. Compared with drought treatment (Fig. 1), treatment of plants with ABA resulted in more rapid accumulation of proteins in the apoplast (Fig. 5). Within 24 h, the concentration of apoplastic proteins in ABA-treated plants increased one-fold over control plants. In less than 96 h the concentration of apoplastic proteins in ABA-treated plants reached a level similar to plants treated with drought for 192 h (Fig. 1) or cold-acclimated for 7 weeks (Marentes et al., 1993). At 96 h a peak value of 0.42 ± 0.01 (mean ± SE, n=3) mg protein per gram leaf fresh weight had accumulated in ABA-treated plants, while the level of apoplastic proteins in control plants remained low (0.1 ± 0.03, mean ± SE, n=3, mg protein $g^{-1}$ leaf fresh weight). The apoplastic protein content in ABA-treated leaves gradually decreased after 96-h of ABA treatment. The total apoplastic protein contents in ABA-treated leaves were statistically different ($p = 0.05$) from the controls after 24 h.

**Analysis of ABA-Induced Apoplastic Proteins by SDS-PAGE and Immunoblotting**

The apoplastic proteins extracted from ABA-treated leaves and control leaves were denatured and examined by SDS-PAGE and immunoblotting. Six polypeptides with molecular masses of 35-, 32-, 28-, 16-, 14-, and 11-kD were rapidly induced by ABA (Fig. 5A). The accumulation of these polypeptides closely followed the time course of total apoplastic proteins accumulated in NA leaves treated with ABA (Fig. 4). At 96 h of the ABA-induction experiment, the numbers of polypeptides and the molecular mass of each polypeptide induced by ABA (Fig. 5A, lane 96+) were similar to

those induced by drought (Fig. 2A, lane 192+) and low temperature (Fig. 5A, lane CA).

Immunoblots of the polypeptides separated by SDS-PAGE (Fig. 6A) revealed that four of the six abundant polypeptides induced by ABA were positively detected by GLP and TLP antisera. No CLP was detected in ABA-treated apoplastic extracts (Fig. 6C). As shown in Figure 6B, a 35- and a 32- kD polypeptide were recognized by GLP antiserum. Although the 32-kD GLP was found in both ABA-treated and control apoplastic extracts, it accumulated to a greater level in apoplastic extracts of ABA-treated plants as the intensity of the band increased relative to the control. The 35-kD GLP was only found in apoplastic extracts of ABA-treated plants (Fig. 6C). TLP antiserum detected 25-kD and 16-kD TLPs in ABA-treated apoplast and only a 25-kD TLP in control apoplast (Fig. 2D). The 25-kD TLP gradually accumulated after 24 h of ABA treatment, whereas the 16-kD TLP was not present until 96 h. The presence of the 32-kD GLP and 25-kD TLP in control apoplastic extracts, as shown in Figures 6A and C, indicates that their accumulation may be constitutive or developmentally regulated (Dong and Dunstan 1997), while the fact that the 35-kD GLP and 16-kD TLP were present only in apoplastic extracts from ABA-treated leaves suggests a specific role for ABA in the induction and accumulation of these proteins.

**Antifreeze Activity of ABA-Treated Samples**

Apoplastic extracts from leaves of ABA-treated and control plants were assayed for antifreeze activity. No antifreeze activities were found in the apoplastic extracts of ABA-treated samples (Fig. 3 ABA+ and ABA-).

**Effect of Fluridone on the Accumulation of AFPs**

If ABA were an important regulator of AFP accumulation, then we would expect applications of fluridone to inhibit the accumulation of AFPs at low temperature. Therefore, we transferred plants to cold temperature and treated them with fluridone every other day. A concentration of 100 μM fluridone was used because it effectively inhibited the accumulation of a 79 kD dehydrin in NA leaves treated with ABA (Fig. 4). As shown in Fig. 7, the concentration of apoplastic proteins in CA leaves sprayed with fluridone was no different than controls over a period of 8 weeks. In addition, the levels of antifreeze activity in fluridone-treated samples were similar to those found in controls (Fig. 3 Fluridone+). These results indicated that the accumulation of AFPs during cold acclimation was independent of ABA.

**Endogenous Ethylene Production In Drought Plants**

We also measured endogenous ethylene production in NA plants in response to drought. As shown in Figure 8, a transient peak of ethylene production was detected at 72 hr of drought treatment and quickly declined afterwards. No endogenous ethylene was produced in controls.

# DISCUSSION

## Freezing Tolerance and Drought-Induced AFPs in Winter Rye

Overwintering plants are often subjected to fluctuating and/or prolonged subzero temperatures. As the temperature drops below 0°C, ice initially forms in the intercellular spaces of plants. Because the water potential of ice is less than that of liquid water at a given temperature, the formation of extracellular ice leads to the movement of unfrozen water from the inside of the cell to the ice located in intercellular spaces, causing cellular dehydration. Therefore, it has been suggested that the factor that determines the lethal freezing temperature in plants could be related to the ability of the cells to tolerate freezing-induced dehydration (Levitt, 1980). If this is true, then the mechanism by which a plant achieves intracellular drought tolerance should be similar to the mechanism by which plants become freezing-tolerant. Indeed, there is evidence that this is the case. Chen and Li (1978) demonstrated that water stress induces freezing tolerance in red osier dogwood stems. A drought stress imposed on young seedlings grown at 25 $^{0}$C also led to the induction of maximal freezing tolerance in spinach plants (Guy et al., 1992). Moreover, winter rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) plants exposed to a mild drought stress (40% relative humidity) at ambient temperature for 24 h also became as freezing-tolerant as plants subjected to cold acclimation for 4 weeks at 4$^{0}$C (Cloutier and Andrews, 1984; Cloutier and Siminovitch, 1982; Siminovitch and Cloutier, 1982). Although freezing tolerance can be increased in overwintering plants by exposure to either cold temperature or drought, the pathway(s) by which environmental changes are sensed

is(are) not well characterized.

In this study, we have shown that, in response to drought at warm temperature, winter rye quickly accumulates apoplastic proteins to a level equivalent to the level of apoplastic proteins induced in plants that have been cold-acclimated for 7 weeks (Fig.1). In characterizing the drought-induced apoplastic proteins, we found that (1) they exhibit the same number of polypeptides with similar molecular masses as apoplastic proteins from CA plants (Fig. 2A), (2) they include GLPs, CLPs, and TLPs similar to those found in CA plants (Fig. 2B, C, and D), and, most interestingly, (3) they exhibit high levels of antifreeze activity similar to CA plants (Fig. 3 Drought +). Our results show that drought and cold both induce the accumulation of AFPs in the rye leaf apoplast.

During cold acclimation, the relative water content of winter rye leave gradually decreased from 87 % to 76 % in about 3 weeks (Krol et al., 1984). The lower relative water content was associated with an increase in leaf dry weight (Krol et al., 1984) and apoplastic AFP accumulation (Hon et al., 1995). When the relative water content of leaves in drought plants decreased to 76.1 % at 96 hr (Table 1), both levels of apoplastic proteins (Fig. 1) and antifreeze activity (Fig. 3) began to increase. The association of decreased water content with increased AFP accumulation in both cold-acclimated and drought-treated winter rye leaves supports the hypothesis that responses to cold and drought in plants involve common mechanisms, possibly through sensing the water status (Guy et al., 1992).

Ethylene has been found to induce the expression of many PR genes and their products, including β-1,3-glucanase and chitinase (Felix and Meins, 1987; Mauch et al., 1992; Xu et al., 1994:). Promoter deletion analysis revealed that an ethylene-responsive element (ERE) or GCC box is

present in the promoter regions of the β-1,3-glucanase gene (Ohme-Takagi and Shishi, 1995), basic chitinase gene (Zhou et al., 1997), and *PR-5d* gene encoding an isoform of the thaumatin-like protein (Sato et al., 1996) of tobacco plant. In addition, the accumulation of EREBPs is required for the expression of PR genes in pathogen-infected tobacco leaves and germinated seeds (Ohme-Takagi and Shishi,1995; Leubner-Metzger et al., 1998). It is thought, therefore, that ethylene-mediated expression of PR genes and their products is through the activation of ethylene signaling pathway, acting via EREBPs with the ERE as the target. Because winter rye AFPs are PR proteins composed of β-1,3-glucanase, chitinase and thaumatin-like proteins (Hon et al., 1995), they may be regulated by ethylene. In this study, a transient increase in endogenous ethylene production was found in drought-treated plants at 72 hr (Fig. 8), followed by the accumulation of apoplastic proteins (Figs. 1 and 2) and increased antifreeze activity (Fig. 3) at 96 hr. Petruzzelli et al. (1999) reported that ß-1,3-glucanase and chitinase activities increased during pea seed germination after 20 hr of ethylene treatment. If ethylene is an inducer of AFPs composed of ß-1,3-glucanase and chitinase, increased endogenous ethylene should be able to activate the expression of AFP genes resulting in accumulation of AFPs within 24 hr. More evidence is needed to prove the possible role of ethylene in the regulation of accumulation of AFPs in winter rye in response to drought and cold.

**ABA-Induced Accumulation of Apoplastic Proteins in Winter Rye**

A number of studies have shown that some low-temperature responsive proteins are also induced by ABA. For example, a group of high molecular weight proteins (150 to 176 kD)

accumulates in winter wheat in response to both low temperature and ABA and increases more in a wheat cultivar with higher freezing tolerance than in one with lower freezing tolerance (Abromeit et al., 1992). The accumulation of spinach COR85 protein (Kazuoka and Oeda, 1992), wheat WCS120 protein (Dallaire et al., 1994), a 140-kD protein in Arabidopsis (Lång et al., 1989), and a plasma membrane polypeptide AWPM19 in wheat suspension-cultured cells (Koike et al., 1997), which are all induced during cold acclimation and associated with the development of freezing tolerance, are also responsive to ABA. These results, along with the observations that ABA can substitute for cold acclimation and rapidly induce freezing tolerance in several plant species at a nonacclimating temperature (Chen et al, 1983; Ishikawa et al, 1990; Orr et al., 1986; Veisz et al 1996; Bravo et al., 1998), imply that ABA may play a role in the signaling pathway for environmental acclimation in plants.

Evidence also indicates that ABA may function in the regulation of AFPs. Kurkela and Franck (1990) and Kurkela and Borg-Franck (1992) found that two cold- and ABA-induced genes from *Arabidopsis thaliana*, *KIN1* and *KIN2* encode proteins similar to an AFP of winter flounder. In response to ABA treatment, embryogenic cell lines of *Picea abies* secrete a 70-kD protein whose N-terminal amino acid sequence has 40% identity to AFPs of several species of polar water fishes and may be associated with the high freezing tolerance (-15 $^{0}$C) in these cell lines (Sabala et al., 1996). Although none of these proteins have shown to have antifreeze activity, it is possible that ABA may be involved in the regulation of AFPs in winter rye plants. However, the present studies showed that, in response to ABA treatment, NA rye plants quickly accumulated apoplastic proteins in the leaves within 192 h to a level that is equivalent to that of plants cold-acclimated for 7 weeks (Marentes et

al., 1993) (Fig. 5). Although these ABA-induced apoplastic proteins contained proteins immunologically similar to cold-induced GLPs and TLPs (Fig. 6), they did not exhibit antifreeze activity (Fig. 3 ABA+). Moreover, the ABA biosynthesis inhibitor fluridone could not prevent the accumulation of AFPs in plants grown at low temperature (Fig. 7). These results show that the accumulation of cold-regulated winter rye AFPs is independent of ABA.

**Conclusion**

There are no previous studies of the regulation of AFP synthesis in plants. Our results show that AFPs accumulate in response to cold and/or drought, but not in response to ABA. Therefore, an ABA-independent signaling pathway must be involved in the plant's perception of cold and drought and in the accumulation of AFPs. This signaling pathway for AFPs may involve ethylene.

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## Figure Legends

**Figure 1.** Time course of accumulation of apoplastic proteins in leaves from drought-treated rye plants. Apoplastic proteins were extracted at 24-h intervals from leaves of NA rye plants during drought treatment (■) and from NA rye leaves (□) as a control by vacuum infiltration followed by centrifugation. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

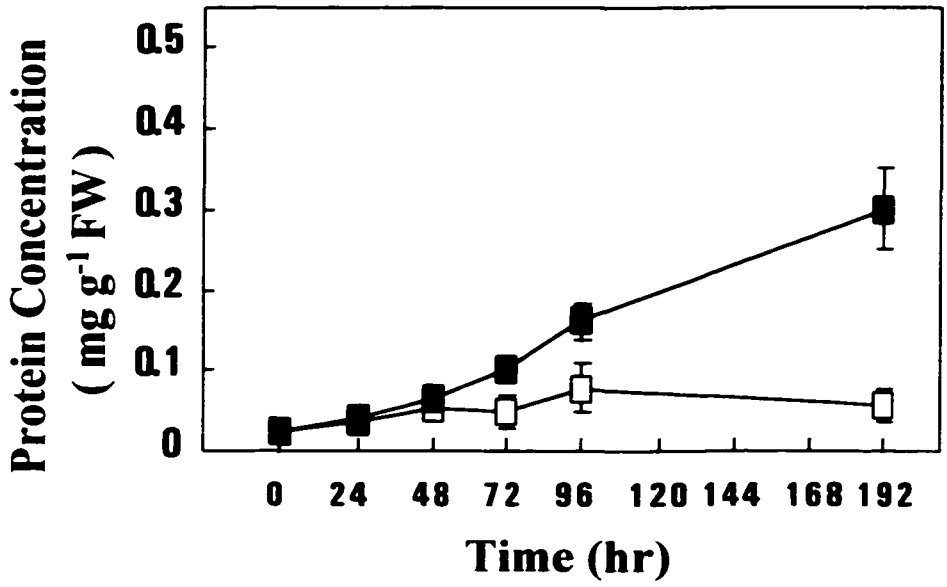

Protein Concentration
( mg g-1 FW )
0.5
0.4
0.3
0.2
0.1
0
0 24 48 72 96 120 144 168 192
Time (hr)

**Figure 2.** Examination of proteins in apoplastic extracts from leaves of drought-treated plants by SDS-PAGE and immunoblotting. (A) Apoplastic protein extracted at 24-h intervals from rye leaves of drought-treated NA plants (+) and NA plants (-) as control and from CA leaves (CA) as positive control were denatured, separated by an 15% SDS-PAGE, and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-polyacrylamide gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the cold-induced winter rye 32-kD GLP, (C) antiserum produced against the cold-induced winter rye 35-kD CLP, and (D) antiserum produced against the cold-induced winter rye 25-kD TLP. Low-range SDS-PAGE standards (M) were used in SDS-PAGE and low-range prestained SDS-PAGE standards were used in immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.

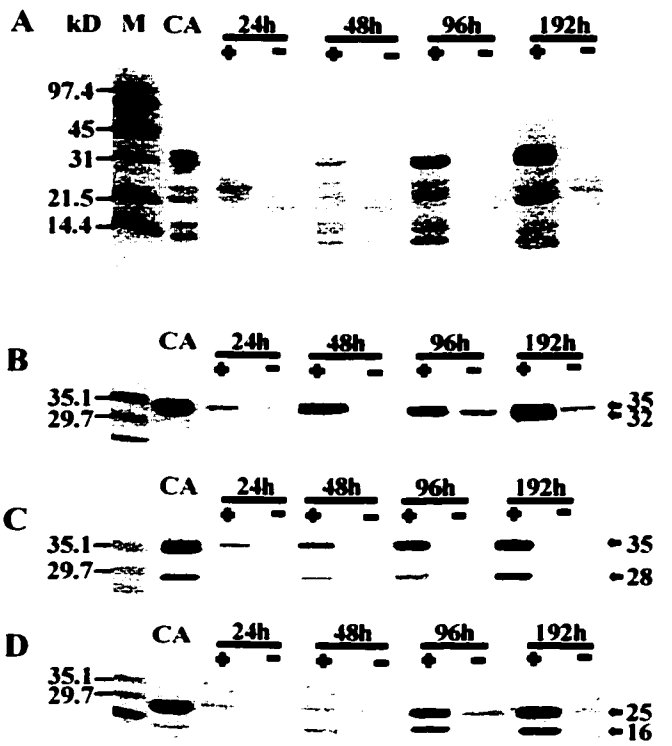
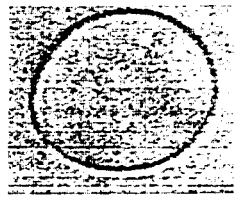

A
kD
M
CA
24h
48h
96h
192h
97.4
45
31
21.5
14.4
B
CA
24h
48h
96h
192h
35.1
29.7
35
32
C
CA
24h
48h
96h
192h
35.1
29.7
35
28
D
CA
24h
48h
96h
192h
35.1
29.7
25
16

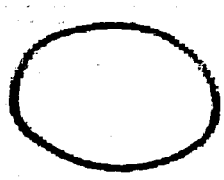
**Figure 3.** Antifreeze activity of apoplastic proteins extracted from drought- and ABA-treated leaves. Antifreeze activity was determined for each leaf apoplastic extract by observing the morphology of ice crystals grown in crude leaf apoplastic extracts of NA plants treated with drought (Drought +) and its control (Drought-) at 96 hr; 100 μM ABA (ABA+) and its control (ABA-) at 96 hr; 100 μM Fluridone (Fluridone +) and its control (Fluridone -) at 96 hr; and cold-acclimated (CA) plants at 7-week. A representative crystal image of three independent measurements was shown. Magnification bar represents 10 μm.



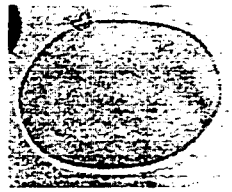
**Drought+**



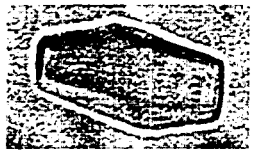
**NA (Drought -)**



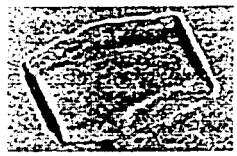
**ABA+**



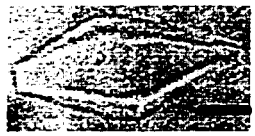
**ABA-**



**Fluridone +**



**Fluridone -**



**CA**

**Figure 4.** Examination of proteins in fluridone-treated total soluble extracts by SDS-PAGE and immunoblotting. (A) Total soluble protein extracted at 96 h from NA rye leaves (lane 1) as a negative control, from NA leaves sprayed with 100 μM ABA (lane 2), and from NA leaves sprayed alternately with 100 μM ABA, followed by 100 μM fluridone (lane 3) were denatured, separated by a 10% SDS-PAGE and stained with Coomassie brilliant blue R-250. An equal volume of each total soluble extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-PAGE gels loaded with 10 μL per lane of each total soluble extract were blotted and probed with antiserum produced against a dehydrin consensus peptide TGEKKGIMDKIKEKLPGQH (dilution 1:1000). Broad-range prestained SDS-PAGE standards (M) were used in both SDS-PAGE and immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.

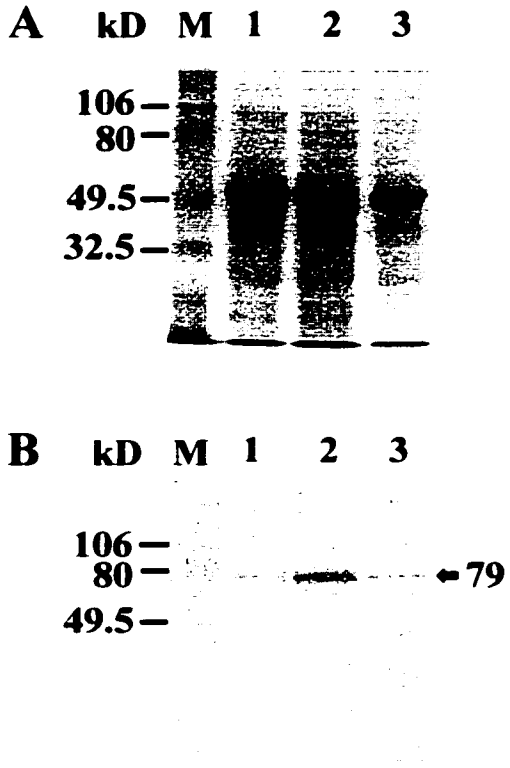

A
kD
M
1
2
3
106
80
49.5
32.5
B
kD
M
1
2
3
106
80
49.5
79

**Figure 5.** Time course of accumulation of apoplastic protein in ABA-treated rye leaves. Apoplastic protein was extracted at 24-h intervals from NA rye leaves sprayed with 100 μM ABA (■) and from NA apoplastic extracts as control (□) by vacuum infiltration, followed by centrifugation. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

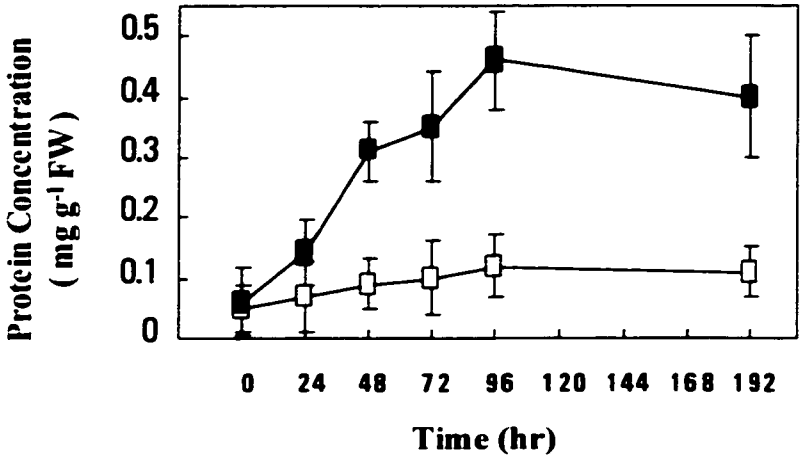

Protein Concentration
( mg g-1 FW )
0.5
0.4
0.3
0.2
0.1
0
0 24 48 72 96 120 144 168 192
Time (hr)

**Figure 6.** Examination of proteins in ABA-treated apoplastic extracts by SDS-PAGE and immunoblotting. (A) Apoplastic protein extracted at 24-h intervals from NA rye leaves sprayed with 100 μM ABA (+), from NA leaves (-) as a negative control, and from CA leaves (CA) as a positive control were denatured, separated by a 15% SDS-PAGE and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-PAGE gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the winter rye 32-kD GLP, (C) antiserum produced against the winter rye 35-kD CLP, and (D) antiserum produced against the winter rye 25-kD TLP. Positive immunodetection of each of the corresponding polypeptides in CA apoplastic extracts is indicated as CA. Low range SDS-PAGE standards (M) were used in SDS-PAGE and low range prestained SDS-PAGE standards were used in immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.

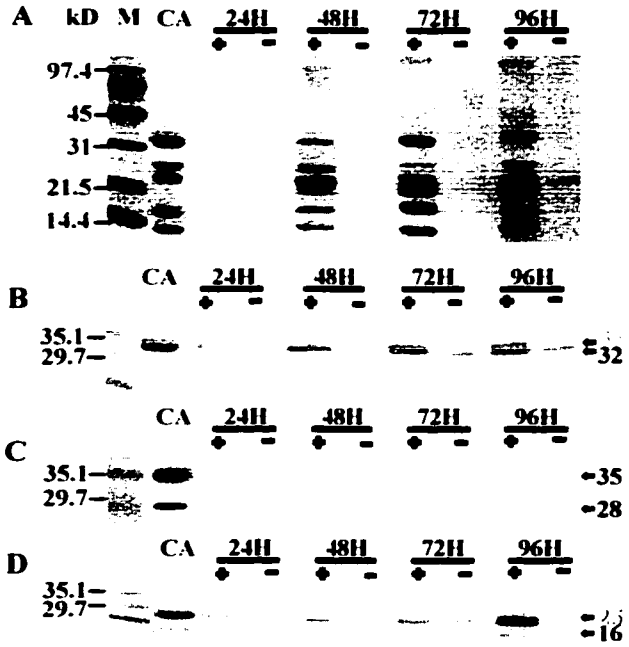

A
kD
M
CA
24H
48H
72H
96H
97.4
45
31
21.5
14.4
B
CA
24H
48H
72H
96H
35.1
29.7
32
C
CA
24H
48H
72H
96H
35.1
29.7
35
28
D
CA
24H
48H
72H
96H
35.1
29.7
16

**Figure 7.** Time course of accumulation of apoplastic proteins in fluridone-treated rye leaves. Apoplastic proteins were extracted from leaves of CA rye plants sprayed with 100 μM fluridone (■) and from CA plants sprayed with water (□) as control. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

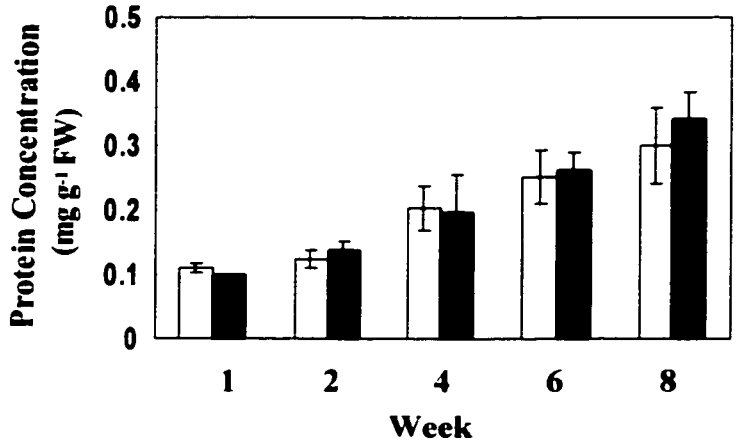

Protein Concentration
(mg g-1 FW)
0.5
0.4
0.3
0.2
0.1
0
1
2
4
6
8
Week

**Figure 8.** Time course of endogenous ethylene production in drought plants ( ■ ). Ethylene content was measured using a Shimadzu gas chromatograph (model GC-17A) with an analyzed ethylene standard and is presented as the means ± SE (n =3).

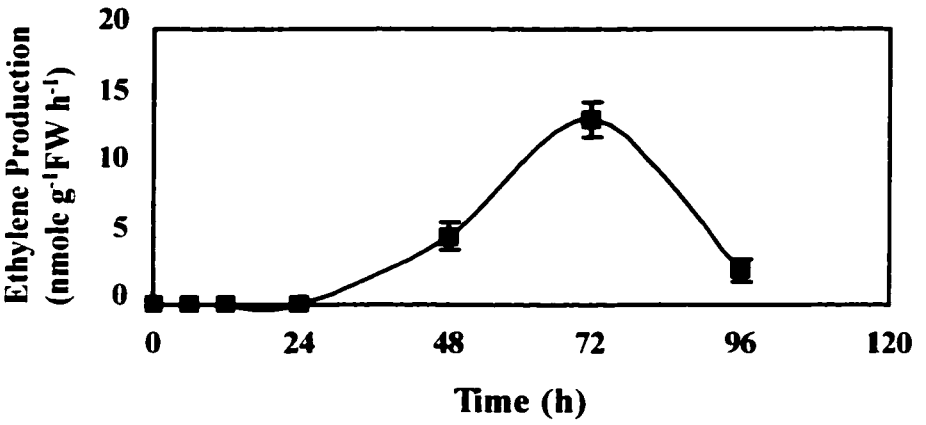

Ethylene Production
(nmole g-1FW h-1)
20
15
10
5
0
0
24
48
72
96
120
Time (h)

**Table 1.** Relative Water Content of NA Rye Leaves Treated with Drought and ABA.

Relative water content of leaf samples was calculated as ((FWT-DWT)/FWT)) x 100 and is presented as the mean ± SE in %, n = 3. FWT: leaf fresh weight; DWT: leaf dry weight.

| Time | Relative Water Content (%) | | |
|---|---|---|---|
| (hr) | NA Control | Drought-treated | ABA-treated |
| 0 | 85.4 ± 0.1 | 85.4 ± 0.1 | 85.4 ± 0.2 |
| 24 | 85.1 ± 0.1 | 84.8 ± 0.2 | 87.6 ± 0.2 |
| 48 | 85.1 ± 0.1 | 82.9 ± 0.1 | 86.3 ± 0.1 |
| 72 | 85.1 ± 0.1 | 80.1 ± 0.2* | 86.9 ± 0.2 |
| 96 | 85.1 ± 0.2 | 76.1 ± 0.2* | 86.1 ± 0.1 |
| 120 | 85.7 ± 0.1 | 75.8 ± 0.2* | 86.7 ± 0.3 |
| 144 | 85.0 ± 0.1 | 76.2 ± 0.1* | 86.3 ± 0.2 |
| 168 | 85.9 ± 0.2 | 75.9 ± 0.1* | 85.9 ± 0.1 |

* Different from the control at p = 0.05

CHAPTER FOUR

# Induction of Antifreeze Proteins in Winter Rye Leaves by Ethylene[1]

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**Footnotes:**

1. This work was supported by a research grant from the National Science and Engineering Research Council of Canada to M.G.

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AFP, antifreeze protein; BCIP, 5-bromo-4-chloro-3-indolylphosphate-toluidine salt; CA, cold-acclimated; CLP, chitinase-like protein; Ethephon, 2-chloroethylphosphonic acid; GLP, β-1,3-glucanase-like protein; NA, non-acclimated; NBT, nitro blue tetrazolium; NP, native protein; PR proteins: pathogenesis-related proteins; TLP, thaumatin-like protein;

## ABSTRACT

Antifreeze proteins (AFPs) accumulate at low temperature in the apoplast of winter rye leaves. The individual polypeptides with antifreeze activity are similar to three pathogenesis-related (PR) proteins: glucanase-like (GLPs), chitinase-like (CLPs) and thaumatin-like (TLPs) proteins. The objective of this study was to study the regulation of AFPs in response to ethylene and salicylic acid, which are both known regulators of PR proteins. Apoplastic protein concentration and antifreeze activity increased in winter rye leaves treated with ethylene within 120 hr. The accumulated proteins consisted of two GLPs, two CLPs, and two TLPs, as identified with antisera raised against cold-induced rye GLP, CLP, and TLP, respectively. The induction of accumulation of AFPs by ethylene was further confirmed by exposing NA plants to either the ethylene-releasing agent ethephon or the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). Both ethephon and ACC induced similar high levels of apoplastic proteins and antifreeze activity. Moreover, the effect of ethephon was blocked by the ethylene inhibitor $AgNO_3$. Endogenous ethylene production rose in intact rye plants within 12 hr of exposure to $5^0$C and were sustained at this high rate of production for the week studied. Production of individual AFPs was detected 36 hr later. Salicylic acid induced the accumulation of three classes of apoplastic PR proteins immunologically similar to the cold-induced proteins within 168 hr; however, these proteins did not exhibit antifreeze activity. Our results suggest that ethylene may regulate the accumulation of AFPs involved in freezing resistance in winter rye.

# INTRODUCTION

When plants become freezing-tolerant, they accumulate novel proteins that presumably function to increase plant survival at subzero temperatures (Guy, 1990). These proteins accumulate at low temperatures as a result of cold-regulated gene expression. Other factors that have been shown to induce the expression of cold-regulated genes include ABA and drought (Hughes and Dunn, 1996)

During cold acclimation, winter cereals accumulate antifreeze proteins AFPs (Antikainen and Griffith, 1997). These proteins have the ability to bind to the ice and modify its growth and are located in the apoplast where ice forms during freezing (Griffith et al., 1992). In characterizing the AFPs from winter rye, we discovered that they are pathogenesis-related (PR) proteins (Hon et al., 1995). Because the AFPs retain their enzymatic activities, they also have antifungal properties and function in disease resistance, particularly to low temperature pathogens such as snow molds (Hiilovaara-Teijo et al., 1999).

PR proteins are known to be induced by ethylene and salicylic acid (SA). For example, exogenously applied ethylene induces accumulation of β-1,3-endoglucanase and endochitinase and a large increase in their enzymatic activities in bean leaves (Abeles et al., 1971; Mauch et al., 1992). Chitinase and β-1,3-endoglucanase activities increase in chickpea plant in response to treatment with the ethylene-releasing compound, 2-chloroethylphosphonic acid (ethephon) (Cabello et al., 1994). In addition, ethylene-responsive sequences have been identified in the promoter region of a bean chitinase gene (Roby et al., 1991) and a β-1,3-endoglucanase gene (Ohme-Takagi and Shishi, 1995). Salicylic acid can efficiently induce PR protein accumulations in leaves of tobacco plants upon

pathogen attack (White 1979) and is considered to be endogenous signal responsible for triggering systemic acquired disease resistance in plants (Malamy et al., 1990; Kessmann et al., 1994).

Although there are no reports of ethylene or SA playing a role in the expression of cold-regulated genes involved in freezing tolerance, we hypothesized that ethylene and/or SA could regulate of the accumulation of AFPs similar to PR proteins. Our approach was (1) to treat plants grown at warm temperature with regulators known to induce the accumulation of PR proteins, (2) to examine the apoplastic proteins by SDS-PAGE and immunoblotting, and (3) to assay antifreeze activities of apoplastic proteins extracted from chemically treated leaves. The results presented here indicate, for the first time to my knowledge, that ethylene induces the accumulation of AFPs that are normally induced by low temperature in leaves of winter rye plants. Although SA induces the accumulation of apoplastic PR proteins immunologically similar to those induced by low temperature, these proteins exhibit no antifreeze activity and may be products of a different set of genes.

# MATERIALS AND METHODS

## Plant Materials and Growth Conditions

Seeds of winter rye (*Secale cereale* L. cv Musketeer) were obtained from Dr. G.McLeod (Agriculture Canada, SwiftCurrent, SK, Canada). Seeds surface-sterilized with a 0.28% sodium hypochlorite solution for 5 min were planted in 15-cm pots of coarse vermiculite and germinated at 20/16°C (day/night) with a 16-h daylength and a PPFD of 300 μmol $m^{-2}s^{-1}$ for a week. Nonacclimated plants (NA) were grown under these conditions for an additional two weeks. Cold-acclimated plants (CA) were transferred to 5/2°C (day/night) with an 8-h daylength and a PPFD of 300 μmol $m^{-2}s^{-1}$ for an additional seven weeks. NA plants grown at 20/16°C for 3 weeks have similar physiological age to the CA plants grown at 5/2°C for 7 weeks (Krol et al., 1984; Griffith and McIntyre, 1993). Plants were watered as needed with Hoagland solution (Hoagland and Arnon, 1950).

## Chemical Treatments

For ethylene treatment, one pot of NA plants was transferred into a 143 L, sealed plastic incubator. Ethylene treatment was administered by injecting 143 mL of 1000 μL $L^{-1}$ ethylene (Alltech, Deerfield, IL, USA) to make a concentration of 1 μL $L^{-1}$ in the incubator. Control plants were kept in a similar sized incubator with no ethylene. For ethephon treatment, leaves of NA plants were sprayed daily with 10 mM ethephon (Sigma, St. Louis, MO, USA) ( Brederode et al., 1991;

Cabello et al., 1994) and 0.005% Tween-20 at the beginning of dark period. Control treatments were performed to assess the effects that were not related to ethylene. Control plants were sprayed daily with 2 mM hydrochloric acid, 2 mM phosphoric acid and 0.005 % Tween-20. For ACC treatments, NA plants were sprayed daily with 10 mM ACC (CalBiochem, San Diego, CA, USA) and 0.005% Tween-20. Control plants were sprayed with only 0.005% Tween-20. For ethylene inhibitor treatments, NA plants were watered daily with 200 μM $AgNO_3$ (Smalle et al., 1997) in Hoagland nutrient solution or sprayed with 10 mM ethephon and 0.005% Tween-20. Control plants were sprayed daily with 2 mM hydrochloric acid and 2 mM phosphoric acid as well as 0.005% Tween-20 and watered daily with Hoagland solution. For salicylic acid treatment, NA plants were sprayed daily with 200 μM salicylic acid (Sigma) (Janda et al., 1999) and 0.005% Tween-20. Control plants were sprayed with 0.005% Tween-20.

Three independent experiments were carried out for each of the above treatments and each treatment lasted for 168 hr. Leaves of chemically treated and control plants were harvested for apoplastic extraction at 24-hr intervals.

**Apoplastic Protein Extraction**

Apoplastic proteins were extracted as described in Hon et al. (1994). Briefly, the leaves were cut into 3-cm sections, rinsed several times with deionized water, and then vacuum infiltrated with extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$, followed by centrifugation at 900 g to recover the apoplastic contents. Total apoplastic protein content was measured using the

Bradford (1976) method, as modified by BioRad (Mississauga, Canada), with BSA as the standard protein.

**Protein Electrophoresis and Immunoblotting**

Apoplastic proteins extracted from rye leaves with different chemical treatments were denatured and the polypeptides were separated in a 15% (w/v) SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250 (Bio-Rad) according to Laemmli (1970). An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. In immunoblotting experiments, an equal volume of each apoplastic extract per gram leaf fresh weight (10 μL) was loaded on each lane. Low-range prestained SDS-PAGE molecular standards (Bio-Rad) were used to determined the apparent molecular masses of polypeptides.

Polypeptides separated by SDS-PAGE were transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad) using the Mini Trans-Blot cell (Bio-Rad) according to the manufacturer's instructions. A buffer composed of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3, was used to transfer polypeptides. The blots were probed with rabbit anti-GLP antiserum (dilution 1:2000), or anti-CLP antiserum (dilution 1:2000), or anti-TLP antiserum (dilution 1:10000) produced against isolated winter rye AFPs similar to 35-kD GLP, 35-kD CLP, and 25-kD TLP, respectively (Hon et al., 1994; Antikainen et al., 1996). The immunoreactions were detected by alkaline phosphatase conjugated to goat anti-rabbit IgG (Sigma) with BCIP (BioShop, Burlington, Ontario, Canada) and NBT (Sigma) as substrates.

**Antifreeze Activity**

Antifreeze activity of chemically treated apoplastic extracts was assayed at 24 hr interval qualitatively by examining the morphology of ice crystals (DeVries, 1986) grown in apoplastic extracts from different chemically treated rye leaves. The growth of ice crystals in each sample was controlled by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) and the morphology of ice crystals was examined using a phase-contrast photomicroscope (Olympus BHT).

**Ethylene Measurement**

To determine the endogenous ethylene production by rye, a single 3-week-old seedling treated with cold was transferred without injury into a test tube (2.5 cm in diameter, 25 cm in height) sealed with a rubber cap. The roots were immersed in Hoagland nutrient solution. After 30 min, a 1-mL of gas sample was withdrawn through the cap with a gas-tight syringe and analyzed by a Shimadzu gas chromatograph (model GC-17A, Shimadzu Scientific Instruments Inc, Columbia, MD, USA) equipped with an aluminum column (1.5 m long, 0.55 mm ID) and a flame ionization detector. The column temperature was isothermal at $100^0$C and the injector and detector oven temperature was $150^0$C. Analyzed ethylene (Alltech, Deerfield, IL, USA) was used as a standard. Three independent experiments were conducted.

**Statistical Analysis**

The two-sample t test (2-tailed) (SAS Institute, 1998) was used at 5% level of significance to detect differences in apoplastic protein accumulation between chemically treated and control plants at each time point.

# RESULTS

## Accumulation of Apoplastic Proteins in Ethylene-Treated Leaves

Total apoplastic proteins extracted from leaves of NA plants treated with 1 $\mu L\ L^{-1}$ ethylene were examined for a period of 120 hr. As shown in Figure 1, apoplastic proteins accumulated slowly in leaves of NA plants after 24 hr of ethylene treatment. At 120 hr of induction, total apoplastic proteins in ethylene-treated leaves accumulated to a level of 0.19 ± 0.02 (mean ± SE, n=3) mg apoplastic protein per gram leaf fresh weight while only 0.08 ± 0.04 (mean ± SE, n=3) mg apoplastic protein per gram leaf fresh weight was found in control leaves.

## Analysis of Ethylene-Induced Apoplastic Proteins by SDS-PAGE and Immunoblotting

The apoplastic proteins extracted from ethylene-treated leaves at 120 hr were denatured and examined by SDS-PAGE and immunoblotting. Compared with the controls, six abundant polypeptides with molecular masses of 35, 32, 28, 25,16, and 14 kD were induced by ethylene (Fig 2A). Immunoblots of the polypeptides separated by SDS-PAGE (Fig 2A) revealed that the six abundant polypeptides induced by ethylene were positively detected by AFP antisera (Hon et al., 1994; Antikainen et al., 1996). As shown in Figure 2B, a 35- and a 32- kD polypeptide were recognized by GLP antiserum in extracts of cold-acclimated and ethylene-treated leaves. Although the 32-kD GLP was found in both ethylene-treated and control apoplastic extracts, the intensity of

the band increased in apoplastic extracts of ethylene-treated leaves relative to the control. CLP antiserum detected a 35-kD CLP in both ethylene-treated and control apoplastic extracts, but the band for the 35-kD CLP was more intense in ethylene-treated apoplastic extracts. In contrast, the 28-kD CLP was only detected in ethephon-treated apoplastic extracts by CLP antiserum at 120-hr (Fig. 2C). The 25-kD and 16-kD polypeptides were identified as TLPs in ethylene-treated apoplast by TLP antiserum. The fact that the 35-kD GLP, 28-kD CLP and 25- and 16-kD TLP were present only in apoplastic extracts from ethylene-treated leaves suggests a specific role for ethylene in the induction and accumulation of these proteins.

**Antifreeze Activity of Ethylene-Treated Samples**

Apoplastic extracts from leaves of ethylene-treated and control plants were assayed for antifreeze activity. A low level of antifreeze activity was detected in NA leaves after 73-hr exposure to ethylene (data not shown). After the plants were treated by ethylene for 120 hr, ice crystals grown in the apoplastic extracts were shaped like hexagonal columns, which indicated a high level of antifreeze activity in the leaf apoplast (Fig. 3 Ethylene+). A similar high level of antifreeze activity was detected when winter rye plants were cold-acclimated for 7 weeks (Fig. 3 CA). In contrast, no antifreeze activity was detected in the NA control as indicated by the disc-shaped ice crystals that formed in the apoplastic extracts (Fig. 3 Ethylene-).

**Accumulation of Apoplastic Proteins in Ethephon-Treated Leaves**

During ethylene induction experiment, the NA plants that were kept in a closed chamber showed symptoms of wilting and yellowish after 72 hr of ethylene treatment, possibly caused by ethylene itself or/and other factor such as low $O_2$ and high $CO_2$ concentration in the chamber. To eliminate the possible effect(s) of non-ethylene factors, the ethylene releasing agent ethephon was used to spray the NA plants. The total apoplastic proteins extracted from leaves of NA plants treated with 10 mM ethephon were examined for a period of 168 hr. As shown in Figure 4, treatment of NA plants with ethephon resulted in rapid accumulation of proteins in the apoplast. Between 0- and 72-hr of ethephon treatment, the total apoplastic protein content gradually increased in both ethephon-treated and control leaves with slightly higher accumulation in ethephon-treated leaves. However, after 72-hr of ethephon treatment, the total apoplastic protein content rapidly increased in ethephon-treated leaves compared with constant low levels of apoplastic proteins in control leaves. At 144-hr of induction, a total level of 0.30 ± 0.02 (mean ± SE, n=3) mg apoplastic protein per gram leaf fresh weight was accumulated in ethephon-treated leaves, which was equivalent to the level of apoplastic proteins accumulated in leaves cold-acclimated at low temperature for 7 weeks (Marentes et al., 1993), while only 0.08 ± 0.03 (mean ± SE, n=3) mg apoplastic protein per gram leaf fresh weight was found in control leaves. There was no difference in apoplastic protein concentrations of NA plants and NA plants sprayed with the acids. The rate of accumulation of apoplastic proteins in ethephon-treated leaves gradually slowed down after 144-hr of ethephon treatment and reached a peak of 0.33 ± 0.03 (mean ± SE, n=3) mg apoplastic protein per gram leaf fresh weight at 168 hr. The total

apoplastic protein contents in ethephon-treated leaves were statistically different ($p = 0.05$) from the controls after 72 hr.

When taken up by plants, ethephon decomposes into ethylene, hydrochloric acid and phosphoric acid. Therefore, the induction of apoplastic proteins in NA leaves by ethephon could be the effect of either ethylene or hydrochloric acid or phosphoric acid or a combination of the three compounds. To assess these possibilities, the control plants were sprayed with 2 mM hydrochloric acid and 2 mM phosphoric acid. The concentration of 2 mM of acids was chosen because treatment with 10 mM of either acid caused severe necrosis of the leaves, which was not seen in leaves treated with 10 mM ethephon. Secondly, the decomposition of ethephon into ethylene and acids is a gradual process (Abeles,1973), which does not normally result in a high concentration of acids released at one time on the leaf surface. Thirdly, the spray of 2 mM acids on leaves caused some pinpoint necroses that were similar to those that appeared on leaves treated with 10 mM ethephon, which suggested that 2 mM of acids may mimic the level of acids released from 10 mM ethephon over time. The fact that apoplastic proteins accumulated specifically in ethephon-treated leaves, but not in the control leaves treated with acids (Fig. 4), indicates that it was the ethylene, not the acid, that resulted in the ethephon-induction of apoplastic proteins in NA rye leaves.

**Analysis of Ethephon-Induced Apoplastic Proteins by SDS-PAGE and Immunoblotting**

The apoplastic proteins extracted from ethephon-treated leaves and acid-treated control leaves were denatured and examined by SDS-PAGE and immunoblotting. Compared with the controls,

seven abundant polypeptides with molecular masses of 35, 32, 28, 25,16, 14 and 11 kD were rapidly induced by ethephon (Fig 5A). The accumulation of these polypeptides closely followed the time course of total apoplastic proteins accumulated in NA leaves treated with ethephon shown in Figure 4. At the end of the induction experiment, the pattern of ethephon-induced polypeptides (Fig 2A, lane 168 h +) separated by SDS-PAGE was very similar to the pattern of cold-induced polypeptides (Fig 2A, lane CA) in terms of total numbers of polypeptides and the molecular mass of each polypeptide.

Immunoblots of the polypeptides separated by SDS-PAGE (Fig 5A) revealed that six of the seven abundant polypeptides induced by ethephon were positively detected by AFP antisera (Hon et al., 1994; Antikainen et al., 1996). As shown in Figure 5B, a 35- and a 32- kD polypeptide were recognized by GLP antiserum. Although the 32-kD GLP was found in both ethephon-treated and control apoplastic extracts, it accumulated to a greater level in ethephon-treated apoplast relative to the control. The 35-kD GLP was found only in apoplastic extracts of ethephon-treated plants (Fig. 5B). Although CLP antiserum weakly detected a 35-kD CLP in both ethephon-treated and control apoplastic extracts before 48-hr of ethephon treatment, the 35-kD CLP increased dramatically in apoplastic extracts after 72-hr of ethephon induction as indicated by the increased color intensity of the band. In contrast, the 28-kD CLP was detected only in ethephon-treated apoplastic extracts by CLP antiserum after 48-hr (Fig. 5C). TLP antiserum detected 25-kD and 16-kD TLPs in ethephon-treated apoplast and only a 25-kD TLP in control apoplast. As shown in Figure 5D, the 25-kD TLP gradually accumulated after 24 hr of ethephon treatment, whereas the 16-kD TLP was not present until 96 hr. The presence of 35-kD GLP, 28-kD CLP, and 16-kD TLP were presented only in apoplastic extracts from ethephon-treated leaves suggests a specific role for ethephon in the induction

and accumulation of these proteins.

**Antifreeze Activity of Ethephon-Treated Samples**

Apoplastic extracts from leaves of ethephon-treated and control plants were assayed for antifreeze activity. A low level of antifreeze activity was detected in NA leaves after 73-hr exposure to ethephon (data not shown). After the plants were sprayed by ethephon for 168 hr, ice crystals grown in the apoplastic extracts were shaped like hexagonal bipyramids, which indicated a high level of antifreeze activity in the leaf apoplast (Fig. 3 Ethephon+). A similar high level of antifreeze activity was also detected when winter rye plants were cold-acclimated for 7 weeks (Fig. 3 CA). In contrast, no antifreeze activity was detected in the NA control as indicated by the disc-shaped ice crystals that formed in the apoplastic extracts (Fig. 3 Ethephon-).

**ACC-Induced Apoplastic Protein Accumulation in NA Rye Leaves**

To further test the hypothesis that ethylene plays a role in the induction of AFPs in NA winter rye, ACC, an immediate precursor to ethylene in the ethylene biosynthetic pathway (Yang and Hoffman, 1984), was used to elevate endogenous levels of ethylene. As shown in Figure 6, the total apoplastic protein gradually increased in leaves of NA plants after a 72-hr application of 10 mM ACC and reached a maximum level of 0.27 ± 0.03 (mean ± SE, n=3) mg protein per gram leaf fresh weight at 144 hr, which was statistically higher (4.5-fold) than that of control (0.06 ± 0.02 (mean ± SE, n=3)

mg apoplastic protein per gram leaf fresh weight). Compared with the time course of induction of apoplastic proteins by ethephon (Fig. 4), there was an 24-hr delay in the accumulation of apoplastic protein in leaves treated with ACC (Fig. 6), suggesting that additional time was required to convert ACC to ethylene.

**Analysis of ACC-Induced Apoplastic Proteins by SDS-PAGE and Immunoblotting**

When the apoplastic proteins extracted from ACC-treated and control leaves were denatured and examined by SDS-PAGE, no changes in polypeptides were found in ACC-treated and control leaves during the first 72 hr. However, seven polypeptides with molecular masses of 35, 32, 28, 25,16, 14 and 11 kD were rapidly induced in ACC-treated leaves after 96-hr of ACC application (Fig 7A). The accumulation of these polypeptides closely followed the time course of total apoplastic proteins accumulated in NA leaves treated with ACC, as shown in Figure 6. Our results show that these seven polypeptides were the major apoplastic polypeptides induced by ACC. At the end of the induction experiment, the pattern of ACC-induced polypeptides (Fig 7A, lane 168 h +) was very similar to the patterns of ethephon-induced polypeptides (Fig.5, panel A) and cold-induced polypeptides (Fig 5, lane CA) in terms of total numbers of polypeptides and the molecular mass of each polypeptide.

Immunoblots of the polypeptides separated by SDS-PAGE (Fig 7A) revealed that six of the seven abundant polypeptides induced by ACC were positively detected by anti-GLP antiserum, anti-CLP antiserum or by anti-TLP antiserum (Hon et al., 1994). As shown in Figure 5B, the 35- and 32-

kD polypeptides were recognized by anti-GLP antiserum. The 32-kD GLP was found in both ACC-treated and control apoplast extracts. However, the 35-kD GLP was found only in ACC-treated apoplastic extracts. Anti-CLP antiserum detected a 35-kD and a 28-kD polypeptide in ACC-treated apoplastic extracts and weakly recognized a 35-kD polypeptide in the control apoplastic extracts (Fig. 7C). The 35-kD polypeptide was detected by anti-CLP antiserum in both ACC-treated and controls apoplast after 24-hr of ACC treatment. In contrast, the 28-kD CLP was only detected in ACC-treated samples by anti-CLP antiserum (Fig. 7C). Anti-TLP antiserum detected 25-kD and 16-kD TLPs in apoplastic extracts of ACC-treated plants and only a 25-kD TLP in the apoplast of control plants. As shown in Figure 7D, the 25-kD TLP gradually accumulated in ACC-treated apoplasts after 24-hr, whereas the 16-kD TLP was not present until 96-hr of ACC treatment.

Like ethephon, ACC specifically induced the accumulation of the 35-kD GLP, 28-kD CLP and 16-kD TLP because these proteins were detected only in the ACC-treated plants (Fig. 7B, C, and D, lane 168h). The timing of the accumulation of the four proteins was different between ACC- and ethephon-treated plants. The 35-kD GLP (Fig. 7B) and 28-kD CLP (Fig. 7C) started accumulating in the apoplast at 72 h of ACC treatment, while they were detected in ethephon-treated plants as early as 24 h and 48 h (Fig. 5B and C), respectively. The 16-kD TLP was induced at 96 h by both ACC (Fig. 7D) and ethephon (Fig. 5D).

**Antifreeze Activity of ACC-Treated Samples**

Antifreeze activity was detected in crude apoplastic extracts from ACC-treated leaves. The

highest activity was found in the crude apoplastic extracts from leaves treated with ACC for 144 hr (Fig. 3 ACC+) when all six AFPs reached peak levels (Fig. 6 and Fig. 7). The level of the ACC-induced antifreeze activity was equivalent to the levels found in both ethephon-induced (Fig. 3 Ethephon+) and cold-induced (Fig. 3 CA) samples because ice crystals with the hexagonal bipyramid shape were grown in these samples that had similar protein concentration. In addition, the ACC-induced antifreeze activity disappeared at the same dilution (20-fold) as used in diluting ethephon-induced and cold-induced antifreeze activities (ice crystal not shown). In contrast, no antifreeze activity was detected in the control extract as indicated by the growth of a disc-shaped ice crystal (Fig. 3 ACC-).

**Ag Inhibition**

The effect of ethephon on the induction of AFPs in NA rye plants (Figs. 5 and 6) was blocked when ethephon-treated NA rye plants were watered with 200 μM $AgNO_3$. As shown in Figure 8, there was no additional apoplastic protein accumulation in NA rye plants sprayed with ethephon and watered with $AgNO_3$. When apoplastic extracts from NA leaves treated with ethephon and $AgNO_3$ were denatured and examined by SDS-PAGE, four polypeptides with molecular masses of 35-, 32-, 28- and 25-kD were present at low levels in both ethephon- and $AgNO_3$-treated and control apoplastic extracts (Fig. 9A). Immunoblots of polypeptides separated by SDS-PAGE showed that the 35-kD polypeptide was weakly recognized as a CLP in both control and ethephon-and $AgNO_3$-treated apoplasts by CLP antiserum (Fig. 9C), the 32-kD polypeptide was identified as a GLP by GLP

antiserum in both control and ethephon-and $AgNO_3$-treated apoplastic extracts (Fig. 9B), and the 25-kD polypeptide was detected as a TLP by TLP antiserum in both control and ethephon-and $AgNO_3$-treated apoplasts (Fig. 9D). No antifreeze activities were found in either control (Fig. 3Ag-) or ethephon-and $AgNO_3$-treated apoplastic extracts (Fig. 3Ag+). Silver ions are known to inhibit ethylene action (Davies et al., 1990; Sisler et al., 1990). The inhibitory effects of $AgNO_3$ on the induction of AFPs by ethephon provided additional evidence that ethylene plays a role in ethephon-induced AFP accumulation in winter rye.

**Endogenous Ethylene Production**

As shown in Figure 10, a peak rate of endogenous ethylene production of 22.62 ± 3.21 (SE), n=3, nmole per gram leaf fresh weight per hour was detected in plants cold-acclimated for 12 hr and the production of ethylene was sustained for a week. No endogenous ethylene was detected in controls.

**Induction of Apoplastic Protein Accumulation in NA Plants by Salicylic Acid**

As shown in Figure 11, total apoplastic proteins gradually accumulated in NA plants treated with 200 μM salicylic acid and reached a maximal level of 0.19 ± 0.02 (SE), n=3, mg protein per gram fresh leaf weight at 96 hr, which was a statistically significant increase (two-fold) compared with the control. A concentration of 200 μM salicylic acid was used because there were no apoplastic

proteins accumulated in leaves sprayed with SA at concentrations less than 200 μM (50 and 100 μM) for 192 hr in a preliminary experiment (data not shown) and the concentration of 200 μM SA induced SA-related response in other plant (Janda et al., 1999). When apoplastic extracts taken from SA-treated and control plants at 96 hr and plants cold-acclimated for 7 weeks were denatured, separated by SDS-PAGE and examined with antisera produced against AFPs from CA rye, polypeptides with same molecular masses were produced in response to both cold temperature (CA) and SA treatment (Fig. 12A). In addition, immunologically similar PR proteins (a 32- and 35-kD β-1,3-glucanase, a 35- and 28-kD chitinase and a 25- and 16-kD TLP were also accumulated in both CA and SA-treated NA plants (Fig. 12B, C, and D). Interestingly, antifreeze activity was found only in CA apoplastic extracts and not in apoplastic extracts from SA-treated plants (Fig. 3 SA+). These results suggest that salicylic acid does not play a role in the induction of AFPs in winter rye.

Ideally, a PR-1 protein, a known SA-responsive protein, or the systemic acquired disease resistance response should be used as a positive control for SA (Kessmann et al., 1994). Because of unavailability of PR-1 protein antiserum or of resources to conduct a disease treatment in winter rye, no positive control for SA treatment was used in this study. However, the accumulation of PR proteins, the 32-kD β-1,3-glucanase, a 35- and 28-kD chitinase and a 16-kD TLP, in 200 μM SA-treated rye plants, but not in the controls (Figs. 12B,C, and D) or not in 50 and 100 μM SA-treated rye plants (data not shown) was an indicator of the SA response often found in other plant-disease systems (Kessmann et al., 1994)

# DISCUSSION

## Ethylene-Induced AFPs in Winter Rye

Antifreeze proteins have been detected in more than twenty plant species, including both dicotyledonous and monocotyledonous plants (Antikainen and Griffith, 1997; Chun et al., 1998). These proteins gradually accumulate in the leaf apoplast in response to low temperatures. For example, over a period of 7 weeks of cold acclimation, apoplastic AFPs accumulate to levels of 0.3 mg protein per gram leaf fresh weight in winter rye plants and they decrease dramatically in concentration within few days if the plants are returned to 20°C to deacclimate (Marentes et al., 1993). During 7 weeks of cold acclimation, 21 cold-acclimated winter wheat chromosome substitution lines accumulate apoplastic AFPs to a mean value of 70 μg protein per gram leaf fresh weight (Chun et al., 1998). We have now shown that, in response to ethylene treatment at warm temperature, winter rye quickly accumulates apoplastic proteins to a level of 0.19 mg protein per gram leaf fresh weight in leaves within 120 hr (Fig.1). These ethylene-induced apoplastic proteins are very similar to the cold-induced apoplastic proteins because they exhibit polypeptides with similar molecular masses as apoplastic proteins from CA plants (Fig. 2A), they consist of pairs of GLPs, CLPs, and TLPs similar to those found in CA plants (Fig. 2B, C, and D), and, most interestingly, they exhibit high levels of antifreeze activity (Fig. 3 Ethylene +) similar to CA plants (Fig. 3CA). The induction of the accumulation of AFPs in winter rye by ethylene at warm temperature was further confirmed by application of the ethylene-releasing agent ethephon or the precursor of ethylene biosynthesis ACC.

Both ethephon and ACC induced the accumulation of similar sets of apoplastic GLPs, CLPs, and TLPs ( Figs. 5 and 7) similar to those proteins induced by low temperature (Figs.5 and 7, lane CA) and ethylene (Fig. 2). Moreover, both ethephon- and ACC-induced apoplastic proteins had the ability to modify the growth of ice crystals (Fig. 3 Ethephon + and ACC +). The fact that apoplastic proteins do not accumulate in plants treated with the ethylene action inhibitor silver ion (Fig. 8) and no antifreeze activity was found in apoplastic extracts from Ag-treated plants (Fig. 3 Ag-) provides additional evidence to suggest a role of ethylene in induction of accumulation of AFPs in winter rye.

Is ethylene involved in the cold-regulated accumulation of AFPs in winter rye? Evidence suggests that it may be. First of all, winter rye plant produced endogenous ethylene within 12 hr of transfer to cold acclimation (Fig. 10), followed by an increase in antifreeze activity (Hon et al., 1995). The peak rate of ethylene production was sustained for at least a week, at which time AFPs were first detected (Hon et al., 1995). Secondly, exogenous application of ethylene induced both the accumulation of AFPs (Figs 1 and 2) and a high level of antifreeze activity (Fig. 3) in NA apoplastic extracts at warm temperature. Thirdly, the accumulation of AFPs and the appearance of antifreeze activity are both inhibited by $AgNO_3$, an known competitor for the binding site of ethylene (Davies et al., 19900. Finally, others have shown by promoter deletion analysis that an ethylene-responsive element (ERE) or GCC box is present in the promoter regions of the β-1,3-glucanase gene (Ohme-Takagi and Shishi, 1995), basic chitinase gene (Zhou et al., 1997), and *PR-5d* gene encoding an isoform of the thaumatin-like protein (Sato et al., 1996) of tobacco plant. Because winter rye AFPs are PR proteins composed of β-1,3-glucanase, chitinase and thaumatin-like proteins (Hon et al., 1995), it is reasonable to hypothesize that the transfer to low temperature activates endogenous

ethylene production in winter rye. Increased ethylene may trigger the expression of AFP genes possibly through ERE located in the promoter regions of GLP, CLP, and TLP genes, which, in turn, results in accumulation of AFPs. Alternatively, ethylene may serve as an sensitizer making the plants more sensitive to other hormones.

**Salicylic Acid-Induced PR Proteins in Winter Rye**

We also studied the responses of NA winter rye to exogenous salicylic acid at warm temperature. As observed with salicylic acid treatment, the NA plants accumulated three classes of apoplastic PR proteins immunologically similar to cold-induced GLPs, CLPs, and TLPs in response to treatment with salicylic acid (Fig. 11 and 12). However, these proteins did not have the ability to modify the growth of ice crystals as shown in Fig. 3SA+. These results suggest that SA may not be involved in regulating freezing resistance in winter rye, rather SA may regulate PR proteins involved in disease resistance. Many studies have demonstrated that SA is an endogenous activator of the accumulation of PR proteins and systemic acquired disease resistance (SAR) in plants. For example, the exogenous application of SA induces both resistance to tobacco mosaic virus (TMV) and the accumulation of PR proteins in tobacco plants (White 1979). TMV infected-tobacco plants (Malamy et al.,1990) and tobacco necrosis virus-infected cucumbers (Métrux et al.,1990) accumulate endogenously synthesized SA and the levels of endogenous SA are correlated with the induction of PR proteins (Yalpani et al., 1991). Moreover, transgenic tobacco that expresses the salicylate hydroxylase gene, which encodes the degratory enzyme salicylate hydroxylase, does not accumulate

SA or SAR-related PR proteins, and shows no resistance to pathogen infection because SA is hydrolyzed into catechol, which has no SAR-inducing activity (Gaffney et al., 1993).

Recently Hiilovaara-Teijo et al. (1999) found that winter rye plants accumulated three classes of PR proteins (GLPs, CLPs and TLPs) in the apoplast in response to the infection by snow mold (*Microdochium nivale*), a low-temperature parasitic fungus, at warm temperature. The snow mold-induced apoplastic proteins exhibited both glucanase and chitinase activities but lacked antifreeze activity. The accumulation of similar PR proteins in winter rye in response to either snow mold or SA indirectly suggests that SA could act as an endogenous signal triggering the expression of PR protein genes of winter rye upon pathogen attack.

Although ethylene (Fig. 2), SA (Fig. 12), and snow mold (Hiilovaara-Teijo et al., 1999) all induce the accumulation of PR proteins immunologically similar to the low temperature-induced PR proteins (Hon et al., 1994) in winter rye, the fact that only ethylene- and low temperature-induced PR proteins have antifreeze activity (Fig. 3) suggests that different genes encoding PR protein may be expressed in response to the different stimuli. One set of PR protein genes are expressed in response to pathogens and SA, whereas the second set is induced by low temperature and/or ethylene. This conclusion is consistent with studies by Yeh (1997) and Yeh et al. (2000) who have shown that the nucleotide sequences of genes encoding the 28-kD and 35-kD chitinases expressed at low temperature in winter rye both produce proteins with antifreeze activity.

In conclusion, ethylene is an important regulator of the accumulation of AFPs in response to cold because exogenous ethylene induces the accumulation of apoplastic AFPs in winter rye at warm temperature. Moreover, a peak of endogenous ethylene production arises at 12 hr of cold acclimation

and is sustained for at least a week and the accumulation of AFPs was followed 36 hr after the increase of endogenous ethylene.

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**Figure Legends**

**Figure 1.** Time course of accumulation of apoplastic proteins in ethylene-treated rye leaves. Apoplastic proteins were extracted at 24-hr intervals from NA rye leaves treated with 1 μL $L^{-1}$ ethylene (■) and NA rye leaves (□) as a control by vacuum infiltration followed by centrifugation. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

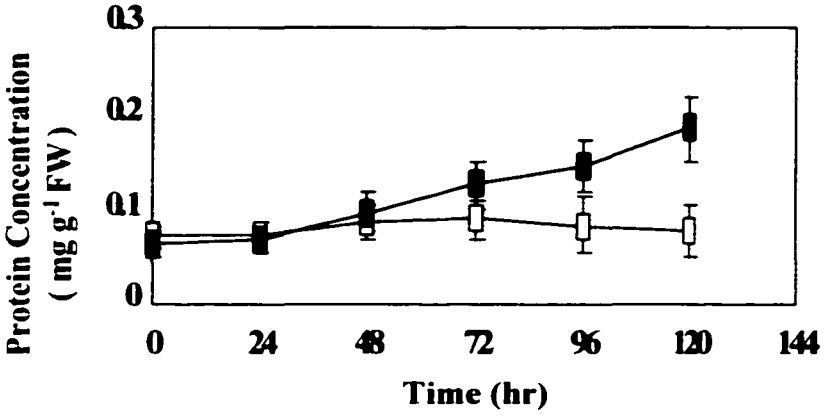

0.3
0.2
0.1
0
Protein Concentration
( mg g-1 FW )
0
24
48
72
96
120
144
Time (hr)

**Figure 2.** Examination of proteins in apoplastic extracts of ethylene-treated leaves by SDS-PAGE and immunoblotting. (A) Apoplastic protein extracted at 120 hr from NA rye leaves treated with 1 μL/L ethylene (+), from NA leaves (-) as control, and from CA leaves (CA) as positive control were denatured, separated by an 15% SDS-PAGE, and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-polyacrylamide gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the cold-induced winter rye 32-kD GLP, (C) antiserum produced against the cold-induced winter rye 35-kD CLP, and (D) antiserum produced against the cold-induced winter rye 25-kD TLP. Low-range prestained SDS-PAGE standards (M) were used in both SDS-PAGE and immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.

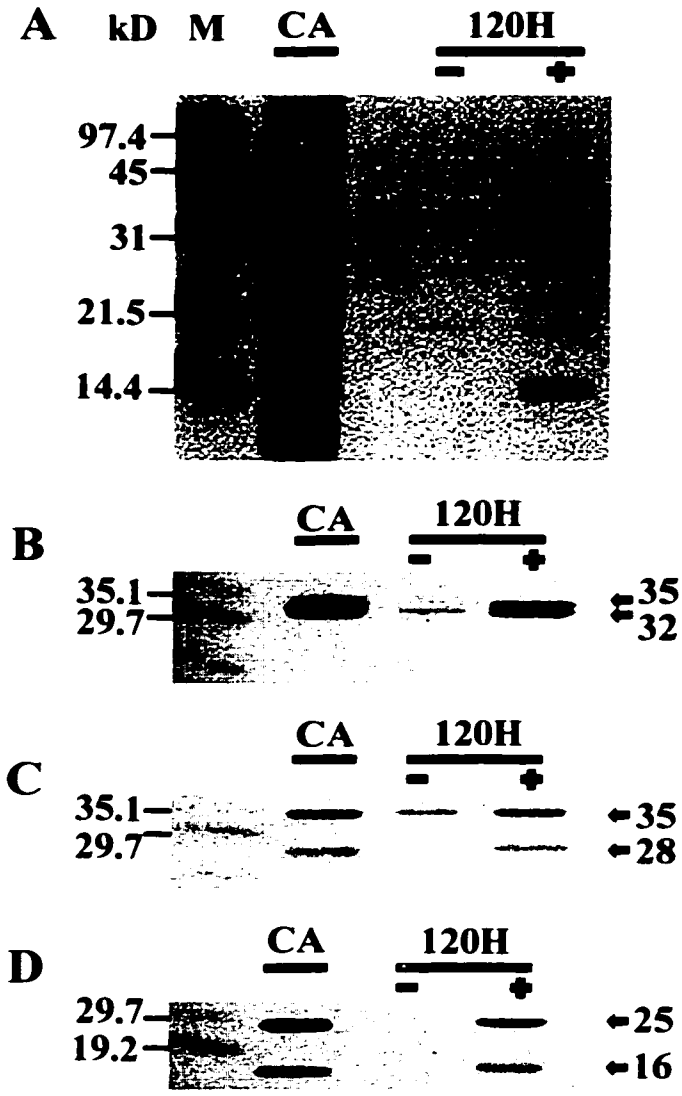
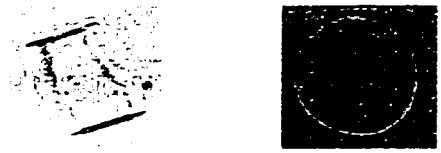

A
kD
M
CA
120H
97.4
45
31
21.5
14.4
B
CA
120H
35.1
29.7
35
32
C
CA
120H
35.1
29.7
35
28
D
CA
120H
29.7
19.2
25
16

**Figure 3.** Antifreeze activity of apoplastic proteins extracted from different chemically treated leaves. Antifreeze activity was determined for each leaf apoplastic extract by observing the morphology of ice crystals grown in crude leaf apoplastic extracts of plants treated with 10 mM ethephon at 168 hr (Ethephon +); 2 mM HCl- and 2 mM $H_2PO_3$ (Ethephon-) as control; 10 mM ACC at 168 hr (ACC+) and its control (ACC); 200 μM $AgNO_3$ (Ag+) and its control (Ag-) at 168 hr and 200 μM salicylic acid (SA+) and its control (SA-) at 192 hr; Ice crystals grown in cold-acclimated (CA) for 7-week and nonacclimated (NA) plants for 3-week are shown for comparison. A representative crystal image of three independent measurements was shown. Magnification bar represents 10 μm.



Ethylene+ Ethylene-



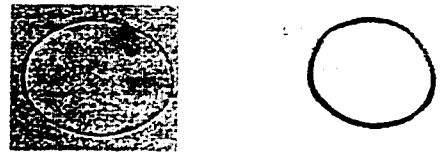
Ethephon+ Ethephon-



ACC+ ACC-



SA+ SA-



Ag+ NA (Ag-)



CA

**Figure 4.** Time course of accumulation of apoplastic proteins in ethephon-treated rye leaves. Apoplastic proteins were extracted at 24-hr intervals from NA rye leaves sprayed with 10 mM ethephon (■) and NA rye leaves sprayed with 2 mM HCl and 2 mM $H_3PO_3$ (□) as a control by vacuum infiltration followed by centrifugation. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

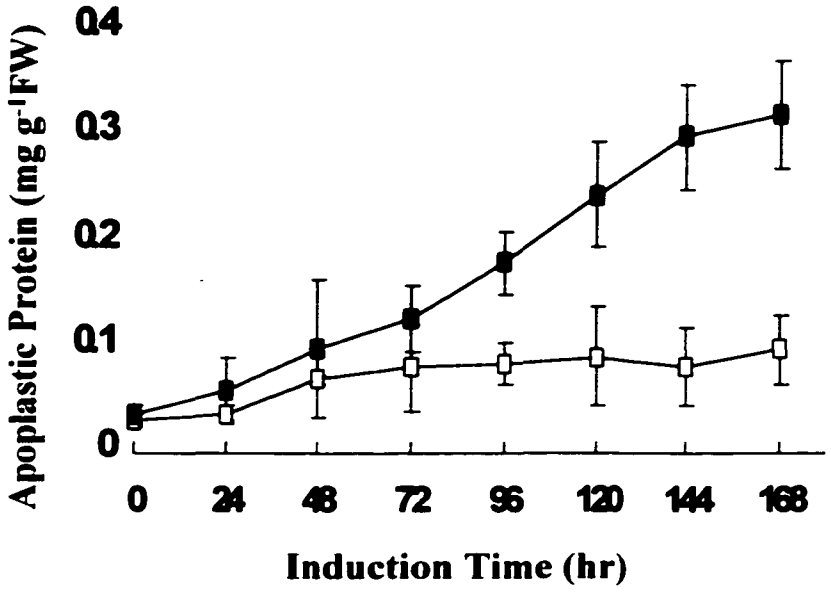

Apoplastic Protein (mg g-1FW)
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
Induction Time (hr)

**Figure 5.** Examination of proteins in apoplastic extracts of ethephon-treated leaves by SDS-PAGE and immunoblotting. (A) Apoplastic protein extracted at 24-hr intervals from NA rye leaves sprayed with 10 mM ethephon (+), from NA leaves sprayed with 2 mM HCl /$H_2PO_3$ (-) as control, and from CA leaves (CA) as positive control were denatured, separated by an 15% SDS-PAGE, and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-polyacrylamide gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the cold-induced winter rye 32-kD GLP, (C) antiserum produced against the cold-induced winter rye 35-kD CLP, and (D) antiserum produced against the cold-induced winter rye 25-kD TLP. Low-range prestained SDS-PAGE standards (M) were used in both SDS-PAGE and immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.

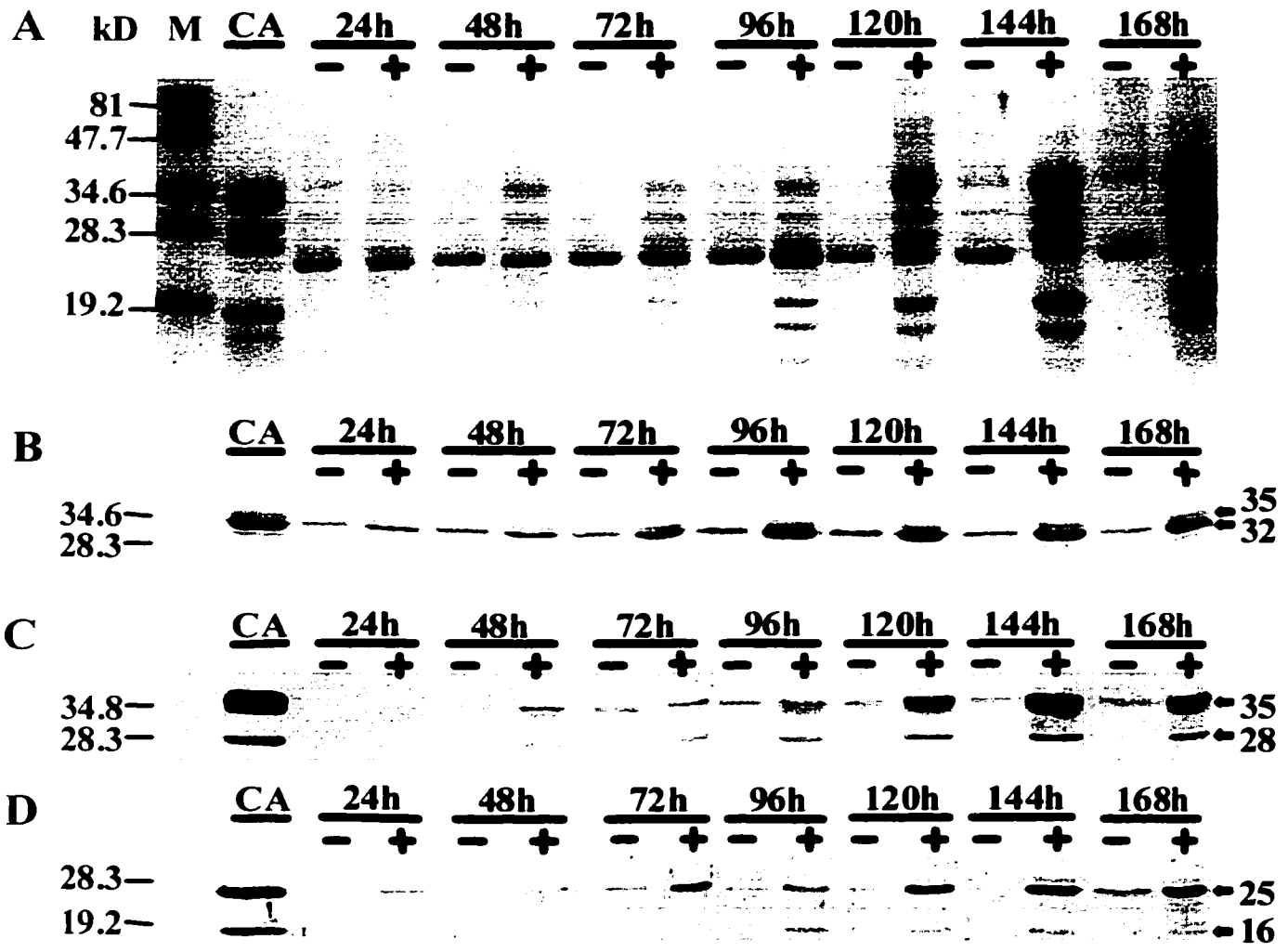

A
kD
M
CA
24h
48h
72h
96h
120h
144h
168h
81
47.7
34.6
28.3
19.2
B
34.6
28.3
35
32
C
34.8
28.3
35
28
D
28.3
19.2
25
16

**Figure 6.** Time course of accumulation of apoplastic protein in ACC-treated rye leaves. Apoplastic protein was extracted at 24-hr intervals from NA rye leaves sprayed with 10 mM ACC (■) and from NA apoplastic extracts as control (□) by vacuum infiltration, followed by centrifugation. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

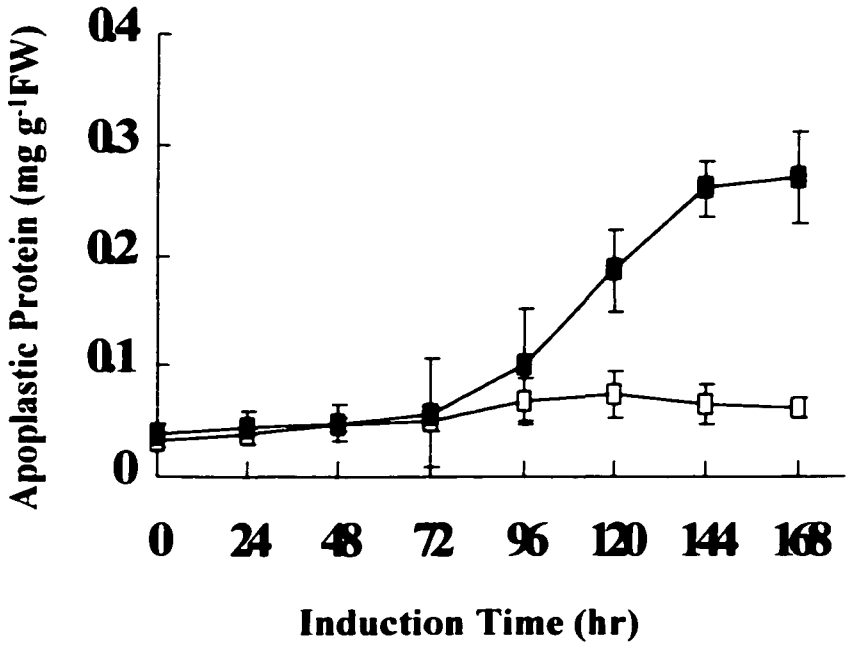

Apoplastic Protein (mg g-1FW)
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
Induction Time (hr)

**Figure 7.** Examination of proteins in ACC-treated apoplastic extracts by SDS-PAGE and immunoblotting. (A) apoplastic protein extracted at 24-hr interval from NA rye leaves sprayed with 10 mM ACC (+), from NA leaves (-) as a negative control, and from CA leaves (CA) as a positive control were denatured, separated by a 15% SDS-PAGE and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-PAGE gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the winter rye 32-kD GLP, (C) antiserum produced against the winter rye 35-kD CLP, and (D) antiserum produced against the winter rye 25-kD TLP. Positive immunodetection of each of the corresponding polypeptides in CA apoplastic extracts is indicated as CA. Low range prestained SDS-PAGE standards (M) were used in both SDS-PAGE and immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.

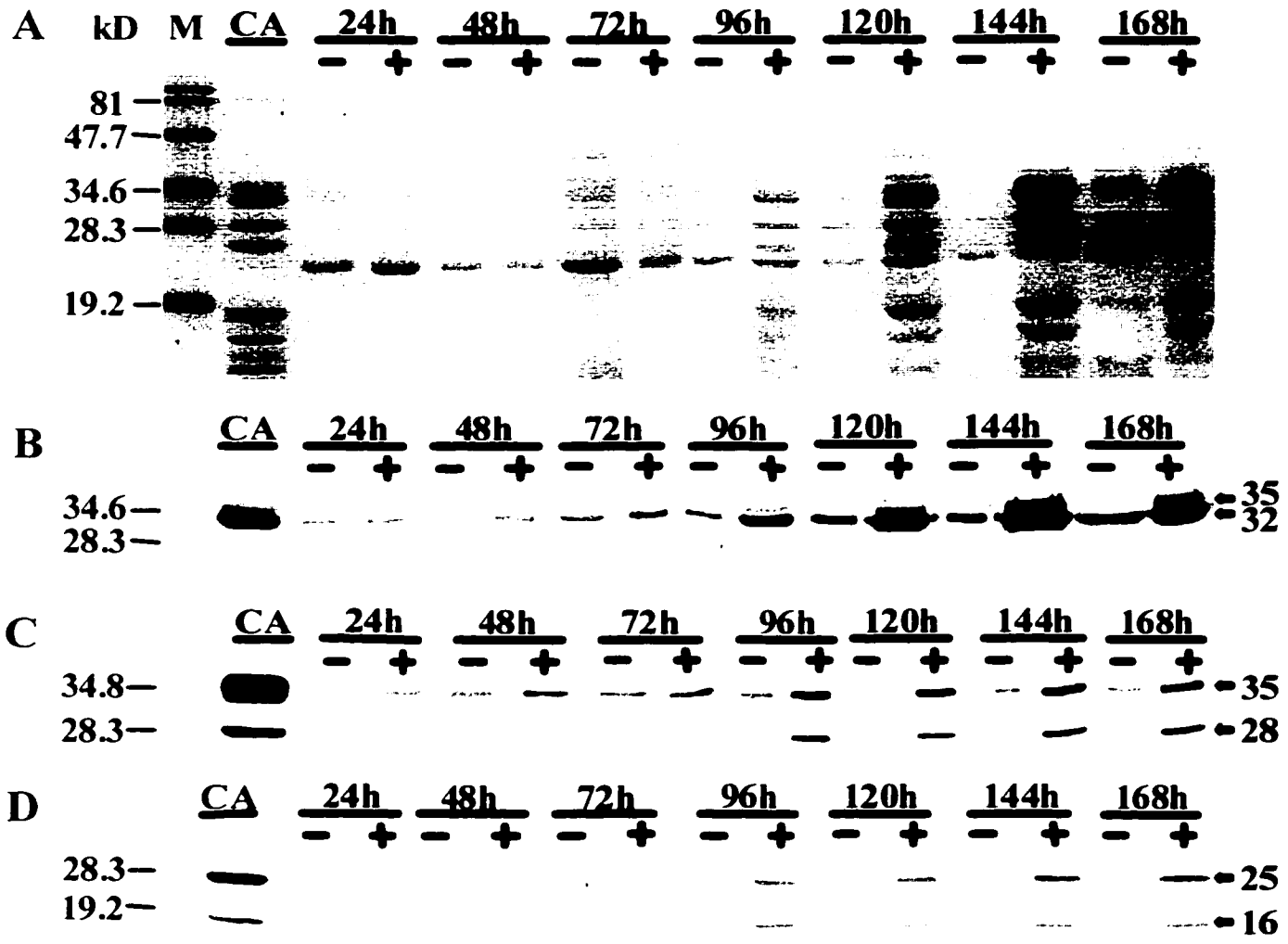

A
kD
M
CA
24h
48h
72h
96h
120h
144h
168h
81
47.7
34.6
28.3
19.2
B
34.6
28.3
35
32
C
34.8
28.3
35
28
D
28.3
19.2
25
16

**Figure 8.** Time course of accumulation of apoplastic proteins in $AgNO_3$-treated rye leaves. Apoplastic proteins were extracted at 24-hr intervals from leaves of NA rye plants watered with Hoagland solution containing 200 μM $AgNO_3$ and sprayed with 10 mM ethephon (■) and from NA plants sprayed with 2 mM $HCl/H_2PO_3$ (□) as control. Total apoplastic proteins were measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein and are presented as the means ± SE (n = 3).

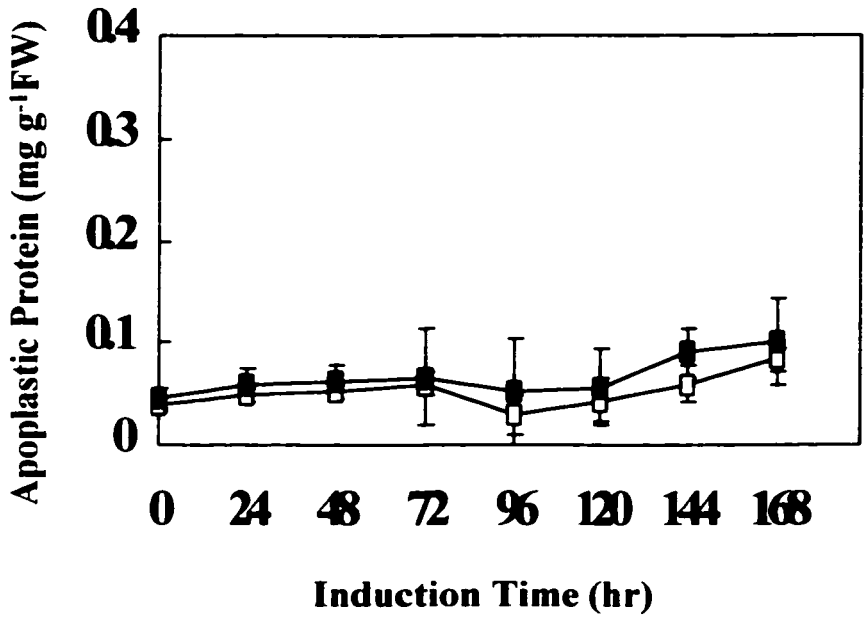

Apoplastic Protein (mg g-1FW)
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
Induction Time (hr)

**Figure 9.** Examination of protein in $AgNO_3$-treated apoplastic extracts by SDS-PAGE and immunoblotting. (A) Apoplastic proteins extracted at 24-hr intervals from NA rye plants watered with Hoagland solution containing 200 μM $AgNO_3$ and sprayed with 10 mM ethephon (+), from NA leaves sprayed with 2 mM $HCl/H_2PO_3$ (-) as negative control, and from CA leaves (CA) as positive control were denatured, separated by a 15% SDS-PAGE and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-PAGE gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the winter rye 32-kD GLP, (C) antiserum produced against the winter rye 35-kD CLP, and (D) antiserum produced against the winter rye 25-kD TLP. Positive immunodetection of each of the corresponding polypeptides in CA apoplastic extracts is indicated as CA. Low-range prestained SDS-PAGE standards (M) were used in both SDS-PAGE and immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each polypeptide immunodetected by an antiserum is indicated on the right.

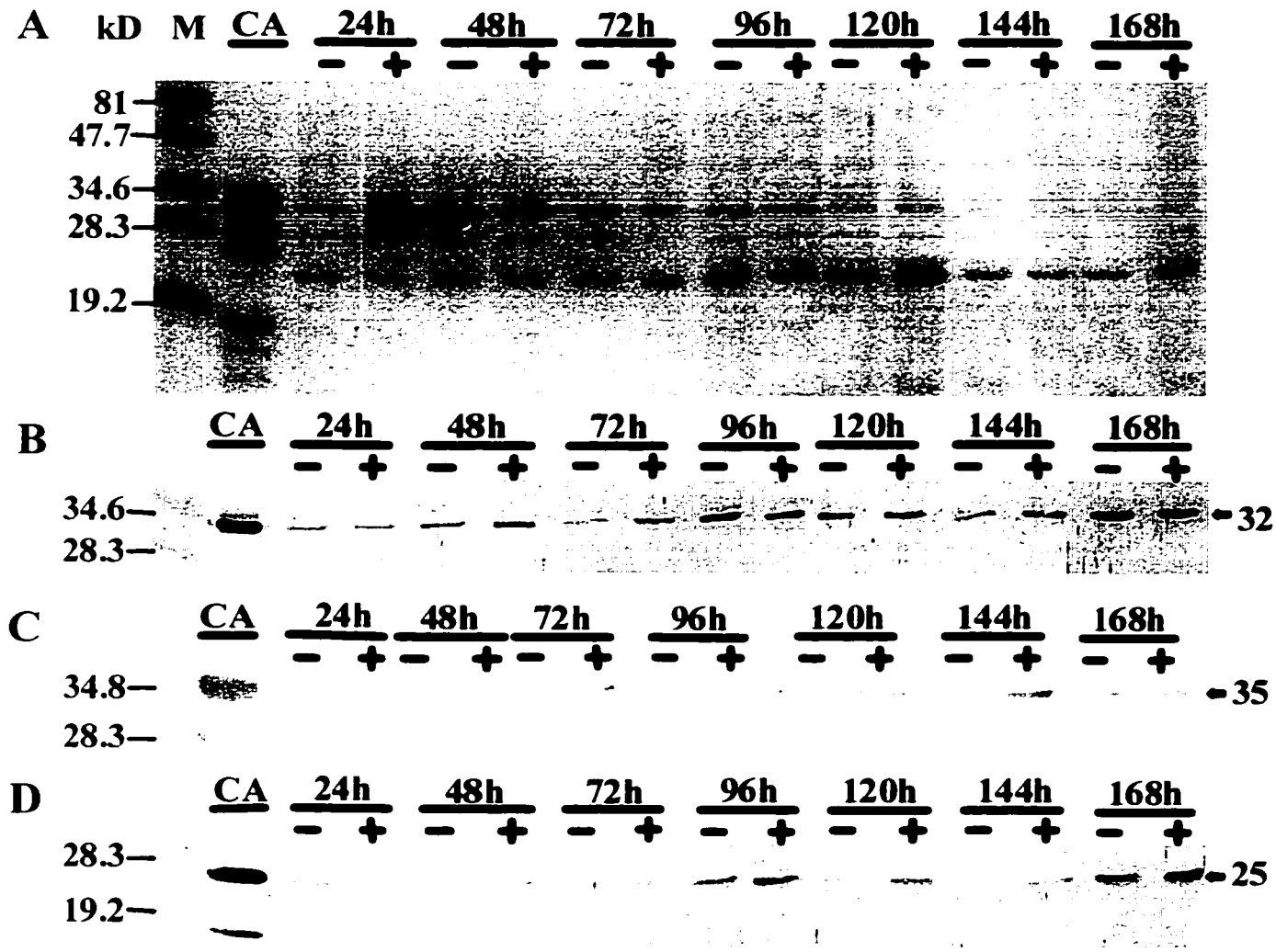

A
kD
M
CA
24h
48h
72h
96h
120h
144h
168h
81
47.7
34.6
28.3
19.2
B
34.6
28.3
32
C
34.8
28.3
35
D
28.3
19.2
25

**Figure 10.** Time course of endogenous ethylene production in plants transferred to low temperature( ■ ). Ethylene content was measured by gas chromatography with an analyzed ethylene as a standard and is presented as the means ± SE (n =3).

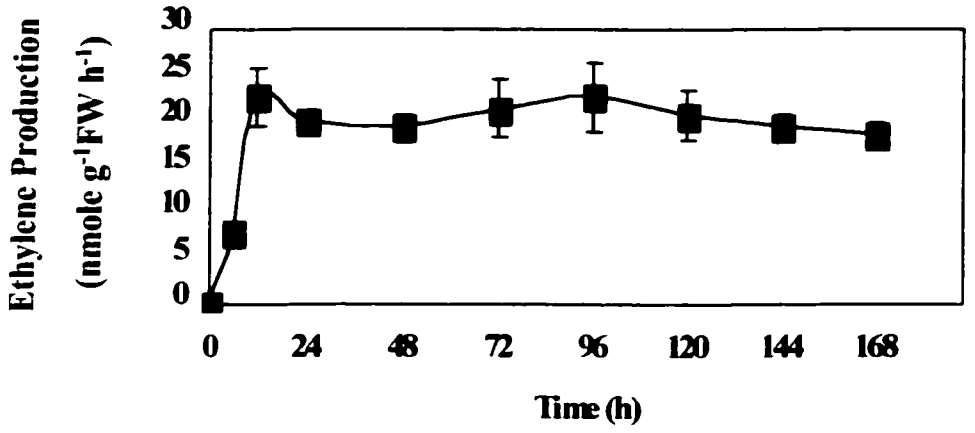

Ethylene Production
(nmole g-1FW h-1)
30
25
20
15
10
5
0
0
24
48
72
96
120
144
168
Time (h)

**Figure 11.** Time course of accumulation of apoplastic protein in salicylic acid-treated rye leaves. Apoplastic proteins were extracted at 24-hr intervals from NA rye leaves sprayed with 200 μM salicylic acid (■) and NA rye leaves sprayed with 0.005% Tween-20 (□) as a control. Total apoplastic protein was measured using the Bradford (1976) method, as modified by BioRad, with BSA as the standard protein. The data are presented as the means ± SE (n = 3).

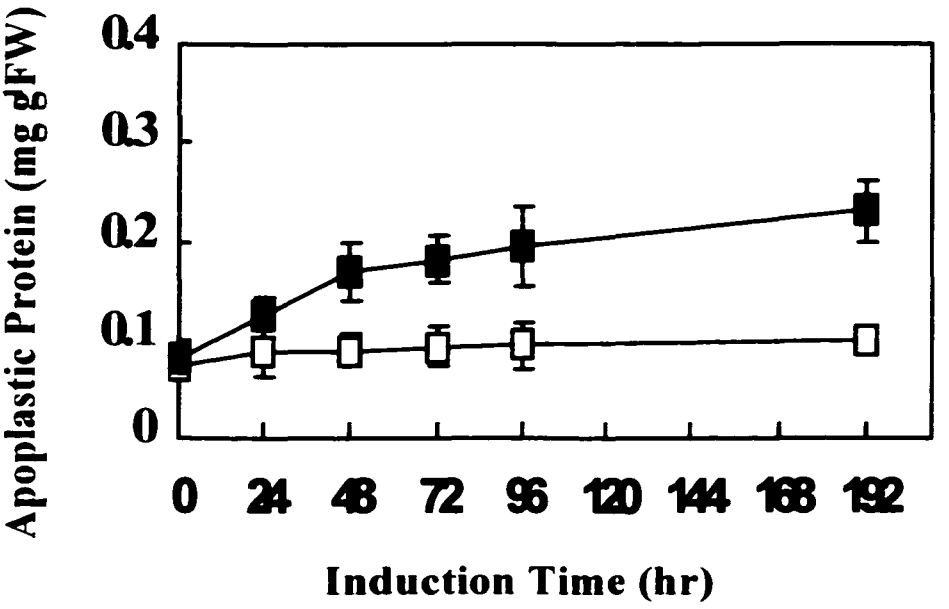
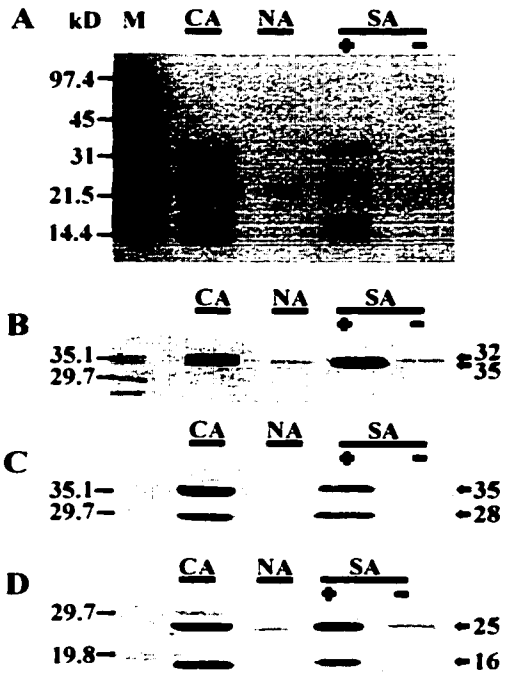

Apoplastic Protein (mg gFW)
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
192
Induction Time (hr)

**Figure 12.** Examination of proteins in salicylic acid-treated apoplastic extracts by SDS-PAGE and immunoblotting. (A) Apoplastic proteins extracted at 192 hr from NA rye leaves sprayed with 200 μM salicylic acid (+), from NA leaves (-) as negative control, and from CA leaves (CA) as a positive control were denatured, separated by a 15% SDS-PAGE and stained with Coomassie brilliant blue R-250. An equal volume of each apoplastic extract per gram leaf fresh weight (30 μL) was loaded on each lane. SDS-PAGE gels loaded with 10 μL per lane of each apoplastic extract were blotted and probed with (B) antiserum produced against the winter rye 32-kD GLP, (C) antiserum produced against the winter rye 35-kD CLP, and (D) antiserum produced against the winter rye 25-kD TLP. Positive immunodetection of each of the corresponding polypeptides in CA apoplastic extracts is indicated as CA. Low range prestained SDS-PAGE standards (M) were used in both SDS-PAGE and immunoblotting analysis to determine the molecular mass (kD). The molecular mass of each immunodetected polypeptide is indicated on the right.


A
kD
M
CA
NA
SA
97.4
45
31
21.5
14.4
B
CA
NA
SA
35.1
29.7
32
35
C
CA
NA
SA
35.1
29.7
35
28
D
CA
NA
SA
29.7
19.8
25
16

# CHAPTER FIVE

## SUMMARY OF EXPERIMENT RESULTS AND GENERAL DISCUSSION

### *Antifreeze Proteins in Winter Rye Leaves Form Oligomeric Complexes*

In Chapter Two, experiments were designed to characterize rye antifreeze proteins in their native forms. The results show that AFPs form oligomeric complexes *in vivo*. Nine proteins were separated by native-PAGE from apoplastic extracts of cold-acclimated winter rye leaves. Seven of these proteins exhibited multiple polypeptides when denatured and separated by SDS-PAGE. After isolation of the individual proteins, six were shown by immunoblotting to contain various combinations of glucanase- (GLP), chitinase- (CLP), and thaumatin-like (TLP) proteins in addition to other unidentified proteins. Antisera produced against individual cold-induced winter rye GLP, CLP, and TLP all dramatically inhibited glucanase activity in apoplastic extracts from cold-acclimated winter rye leaves, and each antiserum precipitated all three AFPs, suggesting that each of the polypeptides may be exposed on the surface of the complexes.

By forming complexes composed of GLPs, CLPs, and/or TLPs, the AFPs may block a larger area of the ice surface, thus making it more difficulty for the ice to overgrow the complexes. As a result, the growth of ice crystals is inhibited to a greater extent and antifreeze activity increases. The fact that the apoplastic extract, which is mixture of AFPs, has the highest antifreeze activity, followed by the complex containing

GLP, CLP, and TLP, and then complexes consisting of GLP and TLP or GLP and CLP (Chapter Two, Table 1), provides indirect evidence to support the hypothesis. The ability of rye AFPs to inhibit ice recrystallization at low protein concentration (Antikainen and Griffith, 1997) may be related to both the size of proteins and the presence of multiple ice-binding sites on each proteins. For example, it is possible that each AFP complex can interact with more than one ice crystal, because each component of the complex is able to interact with ice. In summary, winter rye AFPs form oligomeric complexes in the apoplast that enhance the ability of these proteins to inhibit the growth and recrystallization of ice, which contributes to the freezing tolerance of these plants.

*Antifreeze Proteins Accumulate in Response to Cold and Drought but Not Abscisic Acid*

The role of drought and ABA in the regulation of AFPs is presented in Chapter Three. Drought stress was imposed by stopping watering NA plants until wilt was visible. For ABA treatment, leaves of NA plants were sprayed to run-off daily with 100 μM ABA. The apoplastic extracts from drought- or ABA-treated plants were examined by SDS-PAGE and immunoblotting and assayed for antifreeze activity. The concentration of apoplastic proteins quickly increased in leaves of plants in response to drought at a temperature noninducive for cold acclimation. The level of apoplastic proteins induced by drought within 192 h was equivalent to the level of apoplastic proteins accumulated when plants were acclimated to cold temperature for 7 weeks. These drought-induced apoplastic proteins with molecular masses ranging from 11 to 35 kD contained two glucanases, two chitinases, and two thaumatin-like proteins (TLPs), as

identified with antisera raised against cold-induced rye glucanase, chitinase, and TLP, respectively. Antifreeze activity was detected as early as 24 h of drought treatment and increased as the level of apoplastic proteins increased. Endogenous ethylene level increased transiently in drought plant at 72 hr, followed by the accumulation of apoplastic proteins and increased antifreeze activity at 96 hr. ABA induced the accumulation of apoplastic proteins within 96 h to a level similar to that obtained when plants were drought-stressed for 192 h and cold-acclimated for 7 weeks. Although ABA-induced apoplastic proteins were immunologically similar to two GLPs and two TLPs they did not have the ability to modify the growth of ice crystals. Moreover, the ABA biosynthesis inhibitor fluridone could not prevent the accumulation of AFPs in the leaf apoplast of cold-acclimated plants. Our results show that cold acclimation and drought share a common mechanism in regulating the accumulation of AFPs in winter rye plants and that the accumulation of AFPs in winter rye leaves during cold acclimation is ABA-independent.

*Induction of Rye Antifreeze Proteins by Ethylene*

In Chapter Four, a pharmacological approach was used to assess the role of ethylene in the regulation of AFP accumulation in winter rye leaves in response to ethylene, the ethylene-releasing agent ethephon, the ethylene biosynthesis precursor ACC, and the ethylene inhibitor $AgNO_3$. NA plants were incubated with 1 $\mu L\ L^{-1}$ of ethylene or leaves of NA plants were sprayed daily with 10 mM ethephon or 10 mM ACC to elevate the concentration of endogenous ethylene. For ethylene inhibitor

treatment, NA plants were watered with 200 μM $AgNO_3$ and sprayed daily with 10 mM ethephon. Apoplastic proteins extracted from the chemical-treated plants were assayed for antifreeze activity and examined by SDS-PAGE and immunoblotting. Apoplastic protein concentration and antifreeze activity increased in winter rye leaves treated with ethylene within 120 hr. The accumulated proteins consisted of two GLPs, two CLPs, and two TLPs, as identified with antisera raised against cold-induced rye GLP, CLP, and TLP, respectively. The induction of accumulation of AFPs by ethylene was further confirmed by exposing NA plants to either the ethylene-releasing agent ethephon or the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). Both ethephon and ACC induced similar high levels of apoplastic proteins and antifreeze activity. Moreover, the effect of ethephon was blocked by the ethylene inhibitor $AgNO_3$. Endogenous ethylene levels rose in intact rye plants within 12 hr of exposure to $5^0$C and were sustained at this high level for the week studied. Production of individual AFPs was detected 36 hr later. Salicylic acid induced the accumulation of three classes of apoplastic PR proteins immunologically similar to the cold-induced proteins within 168 hr; however, these proteins did not exhibit antifreeze activity. Our results suggest that ethylene may regulate the accumulation of AFPs involved in freezing resistance in winter rye.

**Conclusions**

Winter rye AFPs have been identified to be composed of a 32- and a 35-kD GLP, a 28- and a 35-kD CLP, and a 16- and a 25-kD TLP (Hon et al., 1994). Each protein, isolated under denaturing condition, exhibits antifreeze activity only when it is renatured

*in vitro* (Hon et al., 1994). In the present study (Chapter Two), my evidence suggests that the cold-induced rye AFPs form oligomeric complexes containing various combinations of GLP, CLP, and TLP, in addition to other unidentified proteins *in vivo*. The highest antifreeze activity was found in the apoplastic extract that is a mixture of AFPs, followed by the complex containing GLP, CLP, and TLP, and then complexes consisting of GLP and TLP or GLP and CLP. The complexes of AFPs can restrict extracellular ice growth and penetration by modifying ice growth and inhibiting recrystallization of small ice into large ones more effectively than the individual AFP.

Winter rye AFPs accumulate in the leaf apoplast during cold acclimation and decrease dramatically in concentration within a few days if the plants are returned to $20^0$C to deacclimate (Marentes et al., 1993), suggesting that the accumulation of these proteins is cold-regulated. I now have provided evidence that the accumulation of rye AFPs is also regulated by drought (Chapter Three) and ethylene (Chapter Four) because both drought and ethylene induce the accumulation of apoplastic proteins similar to the cold-induced proteins at a temperature noninducive for cold acclimation. Moreover, the drought- and ethylene-induced apoplastic proteins have the ability to modify the growth of ice crystals. The presence of the 32-kD GLP, 35-kD CLP and 25-kD TLP in control apoplastic extracts in the drought and ethylene experiments indicates that their accumulation may be constitutive or developmentally regulated (Dong and Dunstan 1997), while the fact that the 35-kD GLP, 28-kD CLP, and 16-kD TLP were present only in apoplastic extracts from drought- and ethephon-treated leaves suggests a specific role for drought and ethylene in the induction and accumulation of these proteins. There is no evidence to suggest the involvement of drought and ethylene in the pathway for cold-

regulated accumulation of rye AFPs. However, the facts that both factors induce the accumulation of AFPs in winter rye plants (Chapters Two and Three), that drought can substitute for low temperature to induce similar high degree of freezing tolerance of winter rye as cold acclimation (Cloutier and Andrews, 1984; Cloutier and Siminovitch, 1982; Siminovitch and Cloutier, 1982), and that ethylene is involved in low temperature responses of many plants and induces chilling tolerance in tomato, as discussed previously, all suggest that drought and ethylene may play a role in regulating the development of freezing tolerance.

The cold-regulated accumulation of AFPs in winter rye is ABA-independent because, as shown in Chapter Three, ABA-induced apoplastic proteins at warm temperature have no antifreeze activity, and, secondly, the ABA biosynthesis inhibitor fluridone can not prevent the accumulation of AFPs in the leaf apoplast of cold-acclimated plants.

Although drought (Chapter Three), ethylene (Chapter Four), salicylic acid (SA) (Chapter Four), and the low temperature parasitic fungus known as snow mold (Hiilovaara-Teijo et al., 1999) all induce the accumulation of β-1,3-glucanase, chitinase, and thaumatin-like proteins immunologically similar to the low temperature-induced PR proteins (Hon et al., 1994) in winter rye, the fact that only drought-, ethylene- and low temperature-induced PR proteins have antifreeze activity suggests that different genes encoding PR proteins may be expressed in response to different stimuli. One set of PR protein genes are responsive to pathogens and SA, whereas the second set is induced by low temperature, drought, and/or ethylene. This conclusion is consistent with studies by Yeh (1997) and Yeh et al. (2000) who showed that the nucleotide sequences of genes

encoding the 28-kD and 35-kD chitinases expressed at low temperature in winter rye both produce proteins with antifreeze activity. Although genes encoding PR proteins without antifreeze activity have not yet been isolated, southern blotting indicates that each of the PR proteins are encoded by small gene family (Yeh et al., 2000).

*Future Studies*

Although the accumulation of rye AFPs during cold acclimation is positively correlated with increased freezing tolerance, direct evidence that these proteins have roles in freezing tolerance is not yet available. One approach to address this question is to isolate genes encoding individual AFPs, followed by making transgenic plants that overexpress individual AFPs or/and all AFP genes, and then comparing the level of freezing tolerance in the transgenic plants with that in controls. If AFPs are key contributors to freezing tolerance, I would expect to see increased freezing tolerance in transgenic plants that overexpress AFP genes.

Drought (Chapter Three) and ethylene (Chapter Four) are two inducers of rye AFP accumulation. A drought responsive-element or/and an ethylene responsive-element is(are) expected to be located in the promoter regions of AFP genes. More molecular studies on rye AFP genes and their promoters are needed. These studies include isolation of AFP genes and their promoters and Southern and northern analyses.

ABA (Chapter Three) and SA (Chapter Four) induce the accumulation of rye apoplastic proteins that are immunologically similar to the low temperature-, ethylene- (Chapter Four) and drought-induced (Chapter Three) PR proteins, but do not have

antifreeze activity. These results suggest that different genes encoding PR proteins may be expressed in response to the different stimuli. One set of PR protein genes would be expressed in response to ABA and SA, whereas the second set would be induced by low temperature or/ and ethylene or /and drought. Direct evidence will rely on the isolation of these two sets of genes from winter rye.

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