Characterization and expression patterns of five Winter Rye β-1,3-endoglucanases and their role in cold acclimation

By

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Biology

Waterloo, Ontario, Canada, 2007

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Winter rye produces ice-modifying antifreeze proteins upon cold treatment. Two of these antifreeze proteins are members of the large, highly conserved, β-1,3-endoglucanase family. This project was designed to identify glucanase genes that are expressed during cold acclimation, wounding, pathogen infection, drought or treatment with the phytohormones ethylene and MeJa. Additionally, a more detailed proteomic analysis was to be carried out to evaluate the glucanase content of the apoplast of cold-acclimated (CA) winter rye.

Results of 2D SDS-PAGE analysis revealed that non-acclimated whole leaf protein extracts contain at least two β-1,3-endoglucanses while CA whole leaf protein extracts contain at least three β-1,3-endoglucanses. Subsequent 2D SDS-PAGE analysis was conducted on the apoplast extracts of NA and CA winter rye plants revealed the limitations of standard 1D SDS-PAGE. The 2-dimensional gel analysis revealed that there is a minimum of 25 proteins within the apoplast of CA winter rye, including at least 5 β-1,3-endoglucanases.

Genome walking was used to isolate cold-responsive glucanase genes. The five genes isolated were designated scGlu6, scGlu9, scGlu10, scGlu11 and scGlu12. The cis-element pattern within the promoter of each gene was evaluated using online databases of documented plant cis elements. As expected, all of the promoters contained elements associated with cold, biotic and abiotic stresses, light regulation, and development. The expression patterns predicted by the cis elements in each promoter were compared to the mRNA abundance produced by each gene as detected by semi-quantitative reverse transcriptase PCR. In most cases, the abundance of transcripts arising from each gene loosely corresponded to the expression pattern predicted by the cis elements the corresponding promoter. Transcripts of scGlu9, 10 and 11

were present in cold-treated tissues and are candidates for β-1,3-endoglucanases with antifreeze activity.

The results presented in this thesis provide additional insight into the apoplast proteome of CA winter rye plants as well as the complexity of the signals controlling the proteins that reside there. Although there are still a number of unresolved questions, this research opens new directions for future studies in the cold acclimation process in winter rye and specifically for the contribution of β -1,3-endoglucanses.

Acknowledgements

First and foremost I would like to thank my supervisor Dr. Marilyn Griffith. She believed in me, and taught me to believe in myself. This thesis would never have been written were it not for her support and encouragement. I will never forget her.

Another huge thank-you goes out to Dr. Barb Moffatt who took on the huge task of my supervision after Marilyn's passing. My appreciation for her guidance and support through the difficult times, and through the trials of my research cannot be put in words.

A special thanks also goes out to Dr. Mahmoud Yaish who taught me the basic molecular biology techniques I needed to succeed.

This thesis could not have been finished if not for the generosity of Dr. Tom Hsiang, Professor, Dept. Environmental Biology, University of Guelph who donated pink snow mould used for pathogen infection experiments. And to Dr. Thomas Elthon at The Center for Biotechnology University of Nebraska-Lincoln for his help and troubleshooting during my 2D SDS-PAGE adventures.

I would also like to thank all the members of the Griffith and Moffatt labs I have worked with throughout the years. Your help and friendship made this experience worth while.

And finally a big thank-you to my family and my boyfriend who put up with my stress and crazy schedule.

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Abbreviations

ABA	Abscisic acid
ABRE	ABA response element
AFP	Antifreeze protein
CA	Cold-acclimated
CBF	CRT binding factors
CRT	Cold response element
COR	Cold-regulated
DRT	Dehydration response element
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
Ethephon	2-chloroethylphosphoric acid
FW	Fresh weight
GA	Gibberellic acid
ICE	Inducer of cold-expression
LTI	Low temperature induced
LTP	Lipid Transfer Protein
MeJa	Methyl Jasmonate
NA	Non-acclimated
PCR	Polymerase Chain Reaction
PPFD	Photosynthetic photon flux density
PR	Pathogenesis-related
RT	Reverse transcriptase
RWC	Relative water content
SDS	Sodium Dodecyl sulfate
TW	Turgid weight
UTR	Untranslated region

1 Introduction

Plants are poikilotherms, that is, they are incapable of controlling their own temperature and so they assume the temperature of their environment. They cannot avoid environmental extremes and have developed species dependant mechanisms to survive temperature shifts. Tropical plants, such as sugar cane or banana, suffer chilling damage only at temperatures below 15°C (Levitt, 1980). Plants native to the arctic, temperate, and high-altitude zones withstand seasonal sub-zero temperatures.

Plants survive sub-zero temperatures through physiological, biochemical and molecular adaptations. Annual plants will over winter as seeds, eliminating the need to protect their tissues from freezing-damage. Plant species that remain vegetative throughout the winter must avoid or tolerate ice formation within their tissues.

A number of species have been studied to evaluate the effect of cold on plants. Although *Arabidopsis thaliana* cannot survive sub-zero temperatures, it has been used extensively as a molecular model in cold-related studies, primarily because its complete genome has been sequenced and mutants are readily obtained. Plants that survive sub-zero temperatures such as poplar and winter cereals have also been used for cold studies, but to a lesser extent because these species are less suitable for molecular genetic analysis. This research focuses on winter rye (*Secale cereale* L.), an over-wintering cereal. It tolerates freezing temperatures by excreting AntiFreeze Proteins (AFPs) into the apoplast after a period of cool, non-freezing temperatures in a process known as cold acclimation (Levitt, 1980).

1.1 Freezing in Plants -- A general Overview

Although plants differ in their ability to withstand cold and sub-zero temperatures the process by which their tissues freeze is universal. Vegetative plants freeze when they cannot prevent ice nucleation and growth within their tissues (Levitt, 1980). Ice nucleation occurs when water molecules cluster and form ice spontaneously, or in response to an intrinsic or extrinsic ice nucleating substance. Certain biological molecules, such as some proteins, cell structures or inorganic debris serve as ice-nucleators. In addition, various species of bacteria are capable of acting as, or producing ice nucleators (reviewed in Griffith & Yaish, 2004). This process is exaggerated in moist climates where heterogeneous nucleation (nucleation in response to a chemical or organic substance) is a problem (Saki & Larcher, 1987).

1.1.1 Freezing Avoidance vs. Freezing Tolerance

Plant species that avoid ice crystal formation within their tissues do so by two known mechanisms. The first method involves using the ground or snow cover as an insulating blanket that prevents heat loss (Andrews, 1996). The second method is supercooling (Levitt, 1980), which involves cooling of the liquids within a plant below their freezing points without the formation of ice crystals. Supercooling is only possible in the absence of a nucleator. In fact, the introduction a nucleator or disturbance of any kind, a light touch for example, will cause instant freezing. Supercooling is a limited cold survival mechanism as water will flash freeze at -38°C and the solute concentrations in plants only allow them to supercool to -47°C. Therefore, plants that live in environments where temperatures may drop to as low as -60°C, such as trees in boreal forests, must have survival mechanisms in addition to supercooling (Levitt, 1980).

When compared to supercooling, freezing tolerance is a better long term cold-temperature survival strategy. Freezing tolerance develops after a period of cold acclimation and allows plants to endure ice formation within their non-cytoplasmic tissues (Levitt, 1980). Comparisons between non-acclimated and cold-acclimated plants show that cold acclimation causes changes at the anatomical, physical and molecular levels. Without these changes, extracellular ice formation can severely harm or even kill a plant through membrane damage and dehydration (Levitt, 1980).

Several factors affect the degree of freezing tolerance induced by cold acclimation. The temperature at which a plant cold acclimates is species dependant but is generally a few degrees above the point at which freezing damage would first occur in non-acclimated plants (Guy, 2003). In addition to temperature, cold acclimation and freezing tolerance is affected by light levels. In temperate zones, the onset of winter is characterized by cooler temperatures and an overall decrease in light levels. Many plants, such as barley (*Hordeum vulgare*) are more freezing tolerant if cold-acclimated with cool temperatures and a short-day length compared to plants acclimated solely by cooler temperatures (Guy, 2003).

1.1.2 The Freezing Process and Consequences of Freezing in Plants

Ice first forms externally at the surface of the plant (Burke *et al.,* 1976) followed by internal development within extracellular spaces, with cell walls and xylem acting as ice nucleators (Jeffree *et al.,* 1987; Olien, 1967). Ice continues to spread through the extracellular spaces, but is prevented from entering the intracellular spaces by the plasma membrane (Levitt, 1980). Although ice in the extracellular spaces can cause mechanical shearing of plasma membranes, create adhesions between the cell wall and the cell membrane, denature proteins

and produce reactive oxygen species, the main consequence of ice formation within plant tissue is cellular dehydration. Ice formed in extracellular spaces has a chemical potential less than that of the liquid water inside the cell, and as the internal water potential drops, water tends to move out of the cell. The extent of cellular water loss is correlated with the amount of ice within the extracellular tissues. Therefore, if ice continues to nucleate beyond a critical point, the plant will die from dehydration. Plants that can cold-acclimate have developed many mechanisms to tolerate extracellular ice formation, minimize membrane shearing and dehydration. Various plant models are used to delineate the molecular basis for these adaptations.

1.2 Models in Cold Research

Early research on cold tolerance involved plants of agronomic importance, such as cereal and vegetable crops. Winter rye and other winter cereals became popular due to their outstanding ability to withstand cold temperatures. In the late 1980s, increased interest in *A. thaliana*, a small, easily transformable dicot with a short generation time, drew cold-researchers away from un-transformable monocots such as rye. Although not highly cold-tolerant, *A. thaliana* gave researchers the ability to study cold tolerance through mutant analysis. This has led researchers further from cereal studies and enabled them to conduct genome-wide cold analysis of changes induced by low temperatures (for reviews see Chinnuswammy *et al.,* 2006; Van Buskirk & Thomashow, 2006)

1.2.1 Winter Rye as a Model Organism for Plant Cold Response

Winter rye is a member of the Poaceae family and is closely related to barley and wheat. Rye is used to make the dark, hard bread commonly consumed in Northern and Eastern

Europe, and is also consumed whole, rolled like oats, or refined into alcoholic beverages such as whisky and vodka. This over-wintering plant is recognized as being more drought resistant and cold tolerant than winter wheat; it can survive to -22°C (Krol *et al.,* 1984).

Initial studies of winter rye's cold tolerance were focused on the physiological and biochemical changes associated with low temperatures. In 1992, Marilyn Griffith and colleagues determined that apoplastic extracts of Cold-acclimated (CA) winter rye had both ice nucleation and antifreeze activity (Griffith *et al.,* 1992). This research lead to the discovery of six major and seven minor CA apoplastic polypeptides. Of these polypeptides, seven modified the growth of ice crystals *in vitro* and displayed thermal hysteresis. Thermal hysteresis refers to an observed difference between melting and freezing temperatures of a liquid. These properties identified the polypeptides as antifreeze proteins, which until that time were known to be produced only by fish and insects (reviewed in Griffith & Yaish, 2004). The winter rye AFPs have the molecular masses of 13, 26, 29, 32, 35 and 36 kDa, are enriched with Asp/Asn, Glu/Gln, Ser, Thr, Gly, and Ala and contain 0-5% Cys (Hon *et al.,* 1994). Interestingly, N-terminal sequencing revealed these polypeptides have striking identities to plant Pathogeneisis-Related (PR) proteins (Hon *et al.,* 1995). Further studies using immunoblotting and enzyme activity assays confirmed the PR nature of the polypeptides. Two proteins of 32 and 35 kDa were identified as β-1,3-endoglucanases, the 29 and 36 kDa polypeptides were endochitinases, and the 13 and 26 kDa polypeptides were thaumatin-like proteins and the 13 kDa polypeptide proved to be a lipid transfer-like protein (Hon *et al.,* 1995).

In addition to cold treatment, winter rye can also gain antifreeze activity and produce these 6 apoplastic PR proteins after treatment with the plant hormone ethylene, the ethylene-releasing chemical 2-chloroethylphosphoric acid (ethephon) or the ethylene precursor 1-

aminocyclopropane-1-carboxylate (ACC) (Yu *et al.,* 2001). AFPs are not produced in response to the growth regulator salicylic acid or abscisic acid (ABA) (Yu *et al.,* 2001a). However, there is evidence that methyl Jasmonate (MeJa) and hydrogen peroxide may be involved in cold stress signaling.

MeJa is a well known cellular regulator of stress responses against pathogens and is also involved in a wide array of developmental processes (Cheong & Choi, 2003). The regulation pathway of MeJa is closely related to that of ethylene as MeJa can trigger the production of ethylene (Hudgins *et al.,* 2004). The fact that MeJa is closely linked to both the pathogen response pathway and the ethylene response pathway strongly suggests that MeJa may play a role in AFP production in winter rye. Preliminary work by Jennifer Merz, a German dipolme student in the Griffith lab, has shown that MeJa not only produces apoplastic proteins with antifreeze activity, but that it produces a greater concentration of apoplastic proteins compared to ethephon treatment (59.48 µg ± 13.77 g-1 FW versus 38.71µg ± 6.46 µg g-1 FW, respectively) (Merz, 2005). Although the concentration of accumulated apoplastic proteins appears to be different in the two cases, this may not correspond to the amount of antifreeze activity each treatment produces.

Hydrogen peroxide is a plant-signaling molecule that increases within leaf tissue in response to cold (Okuda *et al.,* 1991). There is some evidence to suggest that treatment with hydrogen peroxide improves cold tolerance in plants. Pre-spraying maize and mung bean with hydrogen peroxide increased the survival of seedlings after a period of chilling (Yu *et al.,* 2003). In addition, treatment of mung bean with 200mM hydrogen peroxide induced a chilling tolerance equivalent to that of cold acclimation, and had an effect on calcium levels within the leaves (Yang *et al.,* 2002). There is no evidence that hydrogen peroxide is involved in AFP

regulation, but the increased chilling tolerance and changes to calcium levels within leaves after spray treatment suggests that there may be a role for this signaling molecule in AFP accumulation (Stressman *et al.,* 2004).

Winter rye AFPs do not act as cyroprotectant, a property of many insect and fish AFPs have, nor do they dramatically lower the freezing point of plant tissues. Therefore, winter rye AFPs must have another mode of protecting plant leaves and crowns from ice propagation. There is strong evidence suggesting that winter rye AFPs interact directly with ice to modify its growth. Infrared video thermography shows that winter rye leaves producing AFPs have a lower freezing temperature but also display a diminished rate of ice propagation compared to leaves that do not produce AFPs (Griffith *et al.,* 2005).

The full cDNA sequences of the winter rye chitinases with antifreeze-activity have been established (Yeh *et al.,* 2000b). These chitinases, designated CHT9 and CHT46 encode signal peptides targeting for extracellular excretion followed by a sequence coding for the functional chitinase domains, which are greater than 90% similar to chitinases from related monocots. Transcripts of these genes increase by 5-10 fold in winter rye leaves and crowns during cold acclimation. Futhermore, these cold-induced chitinases retain their enzyme activity when expressed in either yeast and or *Escherichia coli* (Yeh *et al.,* 2000b). These proteins, therefore, do not require eukaryote post-translational modification for their enzyme or antifreeze activities.

Infection of winter rye plants by low-temperature pathogens such as snow mold does not induce AFP production in its crowns and leaves, but does induce homologous proteins without antifreeze activity. Surprisingly, CHT46a, the winter rye chitinase that lacks antifreeze

activity has the identical predicted amino acid sequence to that of the winter rye chitinase CHT46 that does have antifreeze activity. In addition, mass spectrometry analysis has shown that neither CHT46a nor CHT46 are post-translationally modified save for the removal of their signal peptide. Perhaps the differences between the proteins are due to interactions with other expressed proteins. For instance, interaction studies have shown that PR proteins assemble into complexes of glucanases, chitinases, thaumatin-like proteins and lipid-transfer proteins (Yu & Griffith, 1999). These protein complexes have an increased number of calcium-binding domains compared to complexes without antifreeze activity. This suggests that calcium maybe a regulator of antifreeze activity (Stressman *et al.,* 2004). Clearly, there are distinct signals and cellular conditions necessary to produce antifreeze activity.

A recent bioinformatic study involving 2D protein modeling of AFPs revealed that AFPs contain hydrophilic groups that are spaced at regular intervals on one of the outside faces of the protein, allowing it to bind loosely to the ice crystal lattice. Using this information, an algorithm was developed to search protein databases for candidate AFPs. This algorithm recognized and differentiated AFPs from non-AFPs and has lead to a theoretical model explaining how AFPs bind to ice and modify their growth (Doxey *et al.,* 2006). Interestingly, this study suggests that most winter rye β-1,3-endoglucanases possess antifreeze activity to some degree, indicating that the dramatic changes in the degree of antifreeze activity between CA and NA plants lies not with the β-1,3-endoglucanases clone but with the chitinases, thaumatin-like proteins or other apoplastic factors.

1.2.2 *A. thaliana* as a Model Organism for Plant Cold Response

A. thaliana has established itself as the dominant model of choice for plant molecular biologists (Somerville & Koornneef, 2002). This little plant's popularity is largely due to its small genome, short life cycle, the ease to which it is transformed, and its abundant seed production. Additional interest in this plant has been encouraged by the availability of its completely sequenced genome, easily accessible mutant collections, and microarrays of its complete transcriptome (Huala *et al.,* 2001). *A. thaliana* is not particularly cold tolerant and cannot withstand extreme freezing temperatures. Despite these drawbacks, *A. thaliana* has still proven itself useful for molecular cold-stress studies, particularly in gene regulation analyses.

Studies, including those of *A. thaliana*, have shown that cold acclimation is associated with changes in gene expression. The genes involved in cold acclimation were given appropriate names such as Cold-Regulated (COR) and Low Temperature Induced (LTI) (reviewed by Shinozaki *et al.,* 2003) . It was hypothesized that changes in the expression of these genes help plants become cold tolerant. The majority of scientists who work to test this hypothesis chose *A. thaliana* as their model organism and have focused on the identification of transcription factors and mapped the transcriptional control pathways that are important during cold-acclimation.

A. thaliana research shows that there are two main transcriptional control pathways for cold-tolerance: one is ABscisic Acid (ABA)-dependant, the other is ABA-independent (reviewed by Shinozaki *et al.,* 2003). In the first pathway, many transcription factors act by binding to cis-elements known as ABA Response Elements (ABREs). Often there is the requirement for promoters to contain two copies of these ABREs or another twin cis-elements

known as a coupling element. The coupling elements are highly conserved in cold-promoter regions (reviewed by Van Buskirk, 2006) and are collectively known as Dehydration Response/Cold Response elements (DRT/CRT) as they are involved in transcription of cold and dehydration related genes. In fact, initial research on the ABA-independent cold transcription pathway found that it was identical to a large portion of the dehydration pathway (reviewed by Van Buskirk, 2006). This isn't surprising given that dehydration is a key component of cold stress.

Researchers using *A. thaliana* as a model organism discovered a family of transcription factors that are involved in both ABA-dependant and -independent pathways. These transcription factors are collectively known as CRT Binding Factors (CBFs) because they bind to genes containing the CRT cis-elements within promoters (reviewed by Van Buskirk *et al.,* 2006). The discovery of the CBF family of transcription factors was accomplished by isolating *A. thaliana* mutants with the ability to survive freezing temperatures, and identifying mutants that influence cold gene expression. Further mutant studies lead to the discovery of another cold-related transcription factor called Inducer of CBF Expression (ICE1) that binds directly to cis-elements within the promoter of CBF3 (Chinnusamy *et al.,* 2003). This transcription factor was called the "Master regulator of cold acclimation" in a recent review by Chinnusamy *et al.,* (2006) because transcriptome analysis revealed that a large proportion of CBF-dependant and independent cold regulated genes are either not induced or have severely decreased expression levels in ICE1 null mutants (Chinnusamy *et al.,* 2003). Other studies have shown that constitutive over expression of ICE1 enhances freezing tolerance (Chinnusamy *et al.,* 2003). In addition to ICE1, another ICE transcription factor (ICE2) has been suggested to be relavant to cold-acclimation, by more than one group (Van Buskirk & Thomashow, 2006). ICE2 binds to

cis-elements within CBF2 (and possibly CBF1) inducing CBF2 expression (Zarka *et al.,* 2003; Van Buskirk & Thomashow, 2006).

Other studies using *A. thaliana* have revealed a number of smaller pathways running parallel or downstream of CBF transcription factors (reviewed by Chinnusamy *et al.,* 2006). These additional pathways enable *A. thaliana* to fine tune its cold response. For instance, some zinc finger proteins and some double-stranded RNA binding proteins have been recognized as negative regulators of CBF expression (reviewed by Chinnusamy *et al.,* 2006). Other research has identified CBF-independent as well as post-transcriptional regulation of cold gene transcription (reviewed by Chinnusamy *et al.,* 2006). Identification of these additional cold-response pathways in *A. thaliana*, as well as identifying the role of all of the hundreds of genes (Saki *et al.,* 2001) shown to change their expression pattern after cold-stress are the next major goals of cold research in *A. thaliana*.

Although the use of *A. thaliana* has revealed the molecular response pathways involved in cold-regulation, it is not tolerant of extreme cold temperatures, nor does it produce antifreeze proteins. Winter rye and other plants able to survive extremes by producing antifreeze proteins must be used to study these aspects low-temperature survival.

1.3 β-1,3-endoglucanases

CA winter rye produces a number of AFPs, the research described in this thesis focuses on the two β-1,3-endoglucanases. These enzymes hydrolyze β-1,3-glucans, such as the linear form found in the carbohydrate laminarin. For this reason, β-1,3-endoglucanses are sometimes referred to as laminarinases. β-1,3-glucans are common structural components of the cells of bacteria, fungi, algae, higher plants and a few invertebrates (Bull & Chesters, 1963) and β-1,3-

endoglucanses are produced by all of these organisms where they facilitate the breakdown and turnover of β-1,3-glucan-containing structures. In plants, β-1,3-glucans are found in several plant structures including the wall of pollen tubes, endosperm cell walls, in the sieve elements of parenchyma cells, and in cell walls, especially those in fruiting bodies (Bull & Chesters, 1966). In addition to their role in protection against pathogens and their role in ice recrystallization, β-1,3-endoglucanases are involved in a number of key cellular processes including cell division, microsporogenesis, pollen germination and tube growth, fertilization, embryogenesis, fruit ripening, seed germination, mobilization of storage reserves in the endosperm of cereal grains and bud dormancy (Leubner-Metzger & Meinz, 1999). Stresses that are known to upregulate β-1,3-endglucanses include infection with a fungal pathogen, wounding, cold, ozone and UVB exposure (Jin *et al.,* 1999). These stresses are associated with increases in the phytohormones known to upregulate expression of β-1,3-endoglucanases such as ethylene, ABA and salicylic acid (Leubner-Metzger & Meinz, 1999). In addition, the jasmonates, such as MeJa, and signaling molecules such as hydrogen peroxide, are potentially involved in glucanase expression (Cheong & Choi, 2003).

Without a complete genome sequence it is impossible to determine the exact number genes coding for β-1,3-endoglucanases in a given species. A ball-park Figure can be obtained from analysis of the Arabidopsis genome. The genome of this plant contains at least 50 genes having the potential to code for an active β-1,3-endoglucanase (Yaish *et al.,* 2006).

1.4 Objectives

Winter rye has been shown to produce ice-modifying AFPs upon treatment with cold but not freezing temperatures which allow the plant to tolerate ice formation within its tissues

(Griffith, 1992). Two of these AFPs are represented by members of the β-1,3-endoglucanase family. Although the winter rye β-1,3-endoglucanase family is poorly characterized, studies of β-1,3-endoglucanases from prokaryotes and eukaryotes have revealed that these widely occurring enzymes have various functions. Plant glucanases are known to be involved with pathogen resistance, cell wall synthesis, and pollen development (for a review see Meins *et al.,* 1992). The members of the winter rye β-1,3-endoglucanase family that have been characterized show strong sequence homology to other plant β-1,3-endoglucanases. At least one β-1,3-endoglucanase with antifreeze activity is excreted into the apoplast of winter rye after treatment with ethylene and cold but not after treatment with ABA, salicylic acid, or pathogens (Yu & Griffith, 1999). In addition, some recombinant cold-induced glucanases have both antifreeze activity and retain partial hydrolytic activity at even subzero temperatures (Yaish *et al.,* 2006). The known winter rye cDNA sequences separate into two phylogenetic groups (Group I and Group II). The first group (Group I) has seven known members and up to 96% sequence identity with barley ß-1,3-endoglucanases while the second group has only one member and only an 80% sequence identity. All of the sequences contain the glycosyl hydrolase domain (Yaish *et. al.* 2006) that is indicative of glucanases. Because both groups contain members that display antifreeze activity, sequence alone cannot be used to identify glucanases with this property.

The goal of this project is to identify the genes encoding β-1,3-endoglucanases that are expressed during cold acclimation or after wounding, pathogen infection, drought or treatment with the phytohormones ethylene and MeJa. Treatments of cold, drought or ethylene are known to cause plants to excrete AFPs similar to PR proteins into their apoplast. There is preliminary evidence to suggest that MeJa treatment also results in apoplastic antifreeze

activity. Because there is a high sequence identity between β-1,3-endoglucanases, it is impossible to differentiate glucanases on their coding sequences alone. It is anticipated that promoters of cold-regulated glucanse genes will contain known cold-related cis-elements where the promoters of non-cold-related β-1,3-endoglucanases will contain cis-elements relating to other known roles of β-1,3-endoglucanases, such as flower development and cell wall production. These differences will allow researchers a means to easily distinguish the genes coding glucanases with and without cold-related functions. To approach this, full-length sequences, including the promoter sequence of a number of β-1,3-glucanases from winter rye will be obtained and the cis-element pattern within the promoter of each gene will be determined and compared to the expression pattern of each gene for verification.

Semi-quantitative Reverse Transcriptase (RT)-PCR analyses will be used to test whether putative cold-regulated β-1,3-endoglucanase genes are transcriptionally upregulated after cold-acclimation and not at 20°C. Other β-1,3-endoglucanases may be transcribed at 20°C, or at 20°C and 4°C, but not at 4°C alone. RT-PCR will test whether β-1,3-endoglucanases have distinct transcription patterns after exposure to cold, ABA, ethylene, MeJa, drought, pathogen infection or wounding. The transcription pattern should be distinct to each β-1,3-endoglucanase, but only cold-regulated β-1,3-endoglucanases should be induced by cold treatment. In addition, cold-regulated β-1,3-endoglucanases may be induced by hormones involved in cold-response such as ethylene but not by treatments unrelated to cold such as wounding.

The size of the winter rye gene family in winter rye is unknown, but because of their diverse roles, glucanases are expected to be present in all tissue types to some degree. Previous immunoblots determined that there is at least one CA β-1-3-endoglucanase in the winter rye

leaf apoplast, but there may be more within the cell. Immunoblots of two-dimensional Sodium Dodecyl Sulfate (SDS)-PAGE of CA apoplastic extract and whole tissue extracts of NA and CA crowns and leaves were performed to address this question. Immunoblots of 2D-PAGE separated proteins from CA and NA winter rye leaves and apoplast will aid in the discovery of additional β-1,3-endoglucanases not seen on traditional 1D-PAGE blots. It is important to know how many glucanases are involved in winter rye cold defense in order to fully understand their roles in cold tolerance.

Finally, hydrogen peroxide, a common stress induced signaling molecule, is known to be produced in plant leaves in response to cold (Okuda *et al.,* 1991; Hung *et al.,* 2005). There is some evidence to suggest that treatment with hydrogen peroxide improves cold tolerance in plants (Prasad *et al.,* 1994) but there is no evidence that hydrogen peroxide is involved in AFP regulation. This thesis will determine if treatment with hydrogen peroxide results in AFP excretion into the leaf apoplast. This information would be beneficial in determining if hydrogen peroxide is involved in a signaling pathway for AFP expression.

2 Materials and Methods

2.1 Chemical Sources

All general chemicals used for experiments described in this thesis can be found, along with the names of the supplier in Table 2-1. Chemicals that were used for specific purposes or those supplied in a kit are mentioned within the text along side the supplier.

2.2 Plant germination, care and tissue harvesting

Seeds of winter rye (*Secale cereale* L. cv Musketeer) were surfaced sterilized for 5 minutes using a 0.3% (v/v) sodium hypochlorite solution and then rinsed 10 times with deionized water. Except for floral organ collection, seeds were sown in 15cm pots containing Pro-mix BX potting soil (Premier Horticulture, Riviere de Loup, Quebec, Canada) at a density of 5g per pot. These were germinated at 20/16°C (day/night) with a 16-hour day length and an average Photosynthetic Photon Flux Density (PPFD) of 300 $\mu mol.m^{-2}.s^{-1}$ for 1 week. Non-Acclimated (NA) plants and those designated for dehydration and wounding experiments were grown under the same conditions for an additional two weeks prior to harvest. CA plants were transferred to a chamber at 5°C/4°C (day/night) with a day length of 16 hours, and an average PPFD of 300 $\mu mol.m^{-2}.s^{-1}$ for an additional 7 weeks. CA plants grown for 7 weeks in these conditions are similar in physiological age to NA plants grown at 20/16°C for 3 weeks (Krol *et al.,* 1984 ; Griffith & McIntyre, 1993). Plants that were designated for hormone treatment or pathogen infection were grown for 1 week prior to treatment because if these treatments are applied to older plants, the larger leaves become weighed down by the amount of liquid that accumulates on the leaf surface causing erratic leaf growth. This erratic leaf growth leads to

Table 2-1: Chemical Sources.

The general chemicals used in this thesis are listed below. The name of the chemical supplier, and location of the supplier's office is also listed.

Chemical	Source	Location
1,4-diothreitol (DTT)	Roche Applied Science	Mannheim, Germany
2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)	BioShop Canada	Burlington, ON, Canada
2-β-mercaptoethanol	Sigma-Aldrich	St. Louis, MO, USA
3-(N-Morpholino) propanesulfonic acid (MOPS)	BioShop Canada	Burlington, ON, Canada
3-[3-(cholamidopropropyl)-1-propanesulfonate (CHAPS)	BioShop Canada	Burlington, ON, Canada
40% acrylamide/bis-acrylamide	BioShop Canada	Burlington, ON, Canada
agarose biotechnology grade	BioShop Canada	Burlington, ON, Canada
ammonium acetate	Sigma-Aldrich	St. Louis, MO, USA
ammonium persulfate (APS)	Sigma-Aldrich	St. Louis, MO, USA
ascorbic acid	Fisher Scientific	Burlington, ON, Canada
bromophenol blue	Sigma-Aldrich	St. Louis, MO, USA
calcium chloride	Fisher Scientific	Burlington, ON, Canada
Chloroform	Fisher Scientific	Ottawa, ON, Canada
deoxyribonucleotides (dNTPs)	Fermentas	Burlington, ON, Canada
diethylpyrocarbonate (DEPC)	BioShop Canada	Burlington, ON, Canada
Ethephon	PhytoTechnology Labs	Shawnee Mission, KS, USA
ethidium bromide	VWR	Mississauga, ON, Canada
ethylenediaminetetraacetic acid (EDTA)	BioShop Canada	Burlington, ON, Canada
Formaldehyde	Fisher Scientific	Ottawa, ON, Canada
Formamide	Fisher Scientific	Ottawa, ON, Canada
Glycerol	BioShop Canada	Burlington, ON, Canada
hexadecyltrimethylammoniumbromide (CTAB)	J.T. Baker	Phillipsberg, NJ, USA
hypochloric acid	Sigma-Aldrich	St. Louis, MO, USA
hyrogen peroxide	BioShop Canada	Burlington, ON, Canada
methyl Jasmonate	Sigma-Aldrich	St. Louis, MO, USA
N,N,N',N'-Tetramethylethylenediamine (TEMED)	BioShop Canada	Burlington, ON, Canada
Phenol	Fisher Scientific	Ottawa, ON, Canada
phosphoric acid	Sigma-Aldrich	St. Louis, MO, USA
Polyvinylpyrrolidone	Sigma-Aldrich	St. Louis, MO, USA
Polyvinylalcohol	Sigma-Aldrich	St. Louis, MO, USA
sodium acetate	Sigma-Aldrich	St. Louis, MO, USA
sodium chloride	BioShop Canada	Burlington, ON, Canada
sodium dodecyl sulfate (SDS)	BioShop Canada	Burlington, ON, Canada
sodium hypochlorite (Javex ™)	Collgate-Palmolive	Toronto, ON, Canada
Sucrose	Fisher Scientific	Burlington, ON, Canada
Thiourea	BioShop Canada	Burlington, ON, Canada
trichloroacetic acid	Fisher Scientific	Ottawa, ON, Canada
Triton-X™ 100	BioShop Canada	Burlington, ON, Canada
Tween™ 20	ICI Americas Inc.	Wilmington, DE, USA
Urea	BioShop Canada	Burlington, ON, Canada
xylene cyanol	Sigma-Aldrich	St. Louis, MO, USA

over crowding in growth chambers and damages leaves causing unwanted wound responses. Plants were maintained in controlled environment chambers (Model E-15, Conviron, Winnipeg, MB, Canada).

To collect floral organs for RNA extraction, seeds of winter rye (*Secale cereale* L. cv Musketeer) were surfaced sterilized as above then evenly sown in 40cm pots containing Pro-mix BX potting soil at a density of 10 seeds per pot. These were germinated and grown in a greenhouse until mature flowers appeared (approximately 12 weeks).

All plants were watered as needed and treated with $1mg \cdot mL^{-1}$ 20-20-20 (20% nitrogen, 20% potassium, 20% phosphorus) solution (Plant Products, Brampton ON, Canada) once a week.

2.3 Treatment of Winter Rye

During all hormonal treatments, experimental and control plants were kept in different growth chambers set at similar growth conditions to eliminate any contaminating volatile transcription signals. Each treatment was performed in triplicate.

2.3.1 Treatment with Ethephon

For ethephon (2-chloroethylphosphoric acid) treatment, leaves of 1-week-old NA plants were sprayed with 10mM ethephon and 0.005% (v/v) Tween 20 daily for 4 days at the beginning of the dark period. Control plants were sprayed daily with 2mM hypocholoric acid, 2mM phosphoric acid, and 0.005% Tween 20.

2.3.2 Treatment with Methyl Jasmonate

For MeJa treatment, leaves of 1-week-old NA plants were sprayed to run off with 15μM MeJa and 0.005% (v/v) Tween 20 daily for 4 days at the beginning of the dark period.

Control plants were sprayed with a 0.005% (v/v) Tween 20 solution. All spraying was performed in a fume hood. Plants were transported to the hood, sprayed, covered in a dark plastic garbage bag to prevent evaporation and light-induced breakdown of the MeJa and then transported back to the growth chamber where the plastic bag was removed. Care was taken not to transport control and experimental plants on the same cart and to use a clean pair of gloves while handling plants in order to ensure control plants did not come into contact with the MeJa.

2.3.2 Treatment with hydrogen peroxide

For hydrogen peroxide treatment, leaves of 1-week-old NA plants were sprayed to run off with 200mM hydrogen peroxide and 0.005% (v/v) Tween 20 daily for 4 days at the beginning of the dark period. Control plants were sprayed with a 0.005% (v/v) Tween 20 solution. All spraying was performed in a fume hood. Plants were transported to the hood, sprayed, covered in a dark plastic garbage bag to prevent evaporation and light-induced breakdown of the hydrogen peroxide and then transported back to the growth chamber where the plastic bag was removed. Care was taken not to transport control and experimental plants on the same cart and to use a clean pair of gloves while handling plants in order to ensure control plants did not come into contact with the hydrogen peroxide.

2.3.4 Wounding of Winter Rye

Wounding treatment was carried out on 3-week-old NA plants by nicking individual winter rye leaves at 1cm intervals using a sharp double sided razor blade. Unwounded NA plants were used as a control. The experiment was performed in triplicate.

2.3.5 Pathogen Infection of Winter Rye with *Microdochium nivale*

Mycelial inoculation of winter rye plants by the North American strain of *Microdochium nivale*, was carried out on 1-week-old NA plants. *M. nivale* that had been grown in wheat bran, dried, ground and diluted in uninoculated wheat bran was provided by Dr. Tom Hsiang, Professor, Dept. Environmental Biology, University of Guelph. The mycelia were kept sealed in a re-sealable sandwich bag at 4°C until use. To inoculate the plant, leaves were first sprayed lightly with MilliQ water to aid the wheat bran inoculum's adherence to the leaves. *M. nivale* was introduced to the plants by sprinkling the inocculum on moistened leaves at an approximate concentration of 0.01g per square centimeter of plant tissue, which averages approximately 8.3g per pot of winter rye. Pots were wrapped in clear plastic lawn waste bags and placed in a dark refrigerator set at 4°C for 3 days, this facilitated mycelial growth and entrance into plant leaves. After the 3 day period, the plants were removed from the refrigerator, sealed in clean, fresh, plastic bags and placed in a growth chamber under normal growing conditions (day length of 16 hours, 20°C day, 16°C night with an average PPFD of 300 $\mu mol.m^{-2}.s^{-1}$). Control plants were sprinkled with sterile wheat bran and were grown under identical conditions. The experiment ran for a total of eight days and was performed in triplicate.

2.3.6 Dehydration and Rehydration

In order to determine the point at which winter rye becomes irreversibly damaged by drought stress, 10 pots of 3-week-old winter rye were placed in a growth chamber and watered to run-off. Pots were checked twice a day for evidence of wilting. When wilting first occurred, two pots were watered to run-off, left for 2 hours and re-checked for reversal of wilting. If

wilting was reversed the next set of two pots were watered the following day and checked for recovery. The experiment stopped when plants would no longer recover after re-watering.

In order to determine Relative Water Content (RWC), a 5-10cm section from each leaf was cut with a pair of scissors and placed basal side down in a pre-weighed air-tight, oven-proof vial. The Fresh Weight (FW) of the tissue was determined by weighing the vial containing the leaf section, and subtracting the weight of the vial. After determining the mass of the sample, 2cm of MilliQ water were added to the bottom of each vial and the leaf sections were left to sit at room temperature for 4 hours. After which the vial was emptied and the leaf section was gently surfaced dried to remove excess water. The leaf was then weighed to determine its Turgid Weight (TW). The section was once again placed in the pre-weighed glass vial without the cap, baked at 80°C overnight, quickly capped, then cooled to room temperature in a desiccator before determining its Dry Weight (DW). The RWC of the leaf was calculated using the equation:

$$[TW-(DW/FW-DW)] \times 100\% = RWC$$

Six pots of 2-week-old winter rye plants were divided into 2 groups, a control group and the dehydration/rehydration group. At the onset of the experiment, all pots in both groups were well watered to run-off. After 1 hour the RWC of 3 plants from each pot was determined, and the average RWC was assumed to represent the RWC of the growth. From this point, the control group was watered every day to run-off while the dehydration/rehydration was not. All pots were kept under normal growth conditions for the entire experiment (day length of 16 hours, 20°C day, 16°C night with an average PPFD of 300$\mu mol.m^{-2}.s^{-1}$). The RWC of both groups was determined 24 hours prior to the point of irreversible drought damage. It was at this time that tissue for RNA and protein extraction was harvested from both groups for the

dehydration and control samples. Both groups of plants were watered to run-off every day for 3 days at normal growth conditions. The relative water content of the plants was again determined and tissue from rehydrated samples of each group was harvested for RNA and protein extraction for the rehydration samples.

2.4 Genomic DNA Extraction

Genomic DNA was extracted using a modified version of the protocol described by Rogers & Bendich (1988). Approximately 1g of 1-week-old winter rye was quickly cut into sections roughly 3cm long and immediately placed in a mortar containing liquid nitrogen. The leaves were ground into a fine powder and then transferred to a 1.5mL microcentrifuge tube containing 450µl of 2 x hexadecyltrimethylammonium bromide extraction solution (2% hexadecyltrimethylammonium bromide (w/v); 100mM Tris, pH 8.0; 20mM EDTA, pH 8.0; 1.4M NaCl; 1% polyvinylpyrrolidone (MW 40000) supplemented with 1µl of 2-β-mercaptoethanol . Contents were mixed by inversion and then incubated in a 70°C heat block for 30 minutes. This solution was centrifuged at 12,000xg, 4°C for 15 minutes and the top phase was removed and placed in a clean 1.5mL centrifuge tube. The bottom layer containing leaf debris and other unwanted material was discarded. To the aqueous phase 300µl saturated phenol and 300µl chloroform/isopropanol (24:1 v/v ratio) were added, and the contents mixed gently by inversion until an emulsion formed. This mixture was subsequently centrifuged at 12,000xg, 4°C for 10 minutes. The top aqueous layer was transferred to a clean 1.5mL microcentrifuge tube, and the bottom layer was discarded. Then, 600µl of chloroform were added to the retained aqueous phase. This solution was mixed by inversion and centrifuged at 12,000xg, 4°C for 10 minutes. The top aqueous phase was removed to a clean 1.5µl centrifuge tube, to which a 60% volume of cold 100% isopropanol was added along with a 10% volume

of 3M sodium acetate, pH 5.2. The solution was mixed by inversion, and then incubated at -80°C for 20 minutes. DNA was precipitated by centrifugation at 12,000xg, 4ºC, for 30 minutes. The aqueous layer was carefully poured off of the DNA pellet, and salt was removed by washing the pellet in 1mL 70% ethanol followed by centrifugation at 12,000xg, 4°C for 10 minutes. The aqueous phase was drawn off and the pellet was dried by incubating the samples at 37°C for approximately 10 minutes. Pellets were resuspended in 50µl PCR grade water (sterile, UV-treated). Tubes were incubated in a 50°C water bath for 5 minutes to ensure DNA was completely in solution. RNA contamination within the sample was removed by treating the sample with $2\mu g.\mu L^{-1}$ RNase A at 37°C for 30 minutes. DNA was re-pelleted and washed as previously described. The final pellet, was resuspended in 50µl PCR grade water. The DNA concentration of the sample was estimated through spectrophotometric analysis at wavelenghs of 260 and 280nm. The 260/280 ratios ranged from 1.8 to 1.9. The integrity of the DNA was assessed by electrophoresis of 5µl on a 0.8% (w/v) agarose gel containing ethidium bromide. DNA was visualized under UV light with a Fluorochem 8000 chemiluminesence and visible imaging system. Average recovery was 320µg DNA per gram of fresh leaf. All DNA stocks were stored at -20°C until needed.

2.5 RNA extraction

To harvest tissue for RNA, two 2-3cm tissue sections containing tissue from both the leaf and crown were cut quickly and placed into RNase-free 1.5mL microcentrifuge tubes, and flash frozen in liquid nitrogen. For plants treated with hormones, wounding and controls associated with these treatments, tissue was harvested for RNA extraction before the treatment and then 1, 2, 3, 6, 9, 12, 16, 24, 48 and 168 hours after the initial treatment. Tissue from cold-treated plants was harvested for RNA extraction at 0, 1, 2, 3, 6, 9, 12, 16, 24, 48 hours and 1

week, 3 weeks and 7 weeks after initial treatment. Tissue from non-acclimated plants was harvested for RNA extraction at 0, 1, 2, 3, 6, 9, 12, 16, 24, 48 hours, 1 week after initial treatment. The tissue from pathogen-infected tissue and uninfected control plants was harvested for RNA extraction immediately prior to infection and then again at 1, 3, 6 and 8 days after infection. All tissue was stored at -80°C until needed.

RNA was extracted from leaf and crown tissue of winter rye by TriPure™ (Roche, Mannheim, Germany) following the manufacturer's directions. All steps are carried out in a fume hood using RNAse free equipment. Approximately 50mg of leaf and crown tissue was added to a 1.5mL centrifuge tube, quickly ground to a fine powder using liquid nitrogen and a plastic pestle. To this, 1mL of TriPure isolation reagent was added, mixed by inversion and incubated at room temperature for 5 minutes to allow the dissociation of nucleoprotein complexes. Then, 200µl of chloroform were added to the solution, which was vigorously shaken for at least 15 seconds. The TriPure, chloroform, leaf solution was incubated at room temperature for 15 minutes and then centrifuged at 12,000xg, 4°C for 15 minutes. Three distinct phases were present in the centrifuged sample; the top aqueous layer contained RNA and was removed to a clean 1.5mL centrifuge tube. RNA was precipitated by the addition of 0.5mL of cold isopropanol, an incubation time of 20 minutes at -20°C and a subsequent centrifugation (12,000xg, 4°C for 10 minutes). The aqueous layer was discarded, and the RNA pellet was washed by the addition of 1mL of 75% absolute ethanol. The solution was gently inverted several times and then centrifuged at 12,000xg for 5 minutes. The aqueous layer was removed and the RNA pellet was air dried in the hood. The RNA pellet was resuspended in 30µl of diethylpyrocarbonate treated water and warmed at 50°C for 5 minutes to ensure the RNA was fully dissolved.

The concentration of the RNA sample was determined by reading its absorbance at 260nm and 280nm using a spectrophotometer equipped with a 96-well plate reader. Typical 260/280 ratios were in the range of 1.6 to 2.0. The quality of the RNA was analyzed on a 2% (w/v) agarose gel. Approximately 3µg of RNA were resuspended in 5µl of loading dye [19% (v/v) formaldehyde, 0.54% (v/v) formamide, 0.5mL formaldehyde gel-running buffer (5X formaldehyde gel-running buffer is 0.1 M 3-(N-Morpholino) propanesulfonic acid, pH 7.0, 40mM NaOAc, 5mM EDTA, pH 7.0), 50% (v/v) glycerol, 1mM EDTA, pH 8.0, 0.25 % (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 0.54% ethidium bromide] and denatured by heating at 70^{o}C for 10 minutes. The gel was electrophoresed 100mA for approximately 1 hour and the RNA was visualized under UV light with a Fluorochem 8000 chemiluminesence and visible imaging system. Typical RNA yield was 3µg RNA per milligram of leaf tissue. RNA was stored in water at –80°C until needed.

2.6 Gene Amplification Cloning and Sequencing

2.6.1 Genomic Walking

The Universal GenomeWalker™ Kit (BD Biosciences, Mountainview, CA, USA) was used to obtain genomic sequences in both directions along each glucanase gene. This was accomplished following manufacturer's instructions. In brief, four uncloned adapter-ligated "libraries" of winter rye DNA fragments were produced by digestion with one of the restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I) supplied in the kit. The fragments in each of these libraries were ligated to an oligonucleotide adapter (5′-GTAATACGACT CACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT- 3′) using T4 ligase. Two adapter-specific nested primers (AP1 and AP2) are provided with the kit while gene-specific

primers (GSP) (Table 2-2) were designed from available cDNA sequences, or from previous rounds of walking.

The 25µl PCR mix for each genome walking reaction is as follows:

16.0µl MilliQ PCR water
1.0µl AP1/AP2 [0.4µM]
1.0µl GSP1/GSP2 [0.4µM]
0.5µl 10mM dNTP mix
2.5µl 10 x Advantage cDNA Polymerase buffer (Clontech, CA, USA)
2.5µl 10x PCRx enhancer solution (Invitrogen, Carlsbad, CA, USA)
1.0µl library DNA [0.5 µg/µl]
0.5µl Advantage cDNA polymerase mix (Clonetech,CA, USA)

The PCR cycle for genomic walking is as follows:

94°C – 4 minutes

94°C -45 seconds 67°C – 1°C/cycle – 3 minutes	10x
94°C – 45 seconds 67°C – 3 minutes	32x

68°C – 5 minutes
72°C – 10 minutes

Table 2-2: Primers used for genomic walking and RT-PCR

The primers used in first strand synthesis and cDNA amplification reactions are listed. These primers were used for either forward or reverse walking, as indicated. An asterisk marks the primers used for both genomic walking and RT-PCR reactions.

Name of Primer	Use	Primer Sequence 5′ - 3′
AP1	The supplied primer for primary PCR designed against the oligonucleotide adapter from the genomic walking kit	GTAATACGACTCACTATAGGGC
AP2	The supplied nested primer for secondary PCR designed against the oligonucleotide adapter from the genomic walking kit.	ACTATAGGGCACGCGTGGT
GluR1a	Primary PCR of cold glucanase	ATGCCGTTGATGCCCTTGGACCTGTAA
GluR1b*	Secondary PCR of cold glucanase	GAGTTGCGGAGCGCGGAGAGGGCTTG
IntronR1a	Primary PCR based on intron sequence of scGlu6 obtained from first round of genomic walking	CCAGTGGTCCATTAGAAAACAACAGG
IntronR1b	Secondary PCR based on intron sequence of scGlu6 obtained from first round of genomic walking	TTTTGTTTGTAAGGATGCTAAGGATG
GluR1_promoter_a	Primary PCR for reverse walking from scGlu6 promoter	ACCACGCGTGCCCTATAGTAATCA
GluR1_promoter_b	Secondary PCR for reverse walking from scGlu6 promoter	ACCATCCTTTACCTTCTCGCACCA
GluR1 exon2 reverse*	Reverse walking to confirm exon 2 of scGlu6	ACGCACATTGTTCTGTACCCAGGA
Reverse walk R2*	Reverse walking to confirm scGlu9	TGTCGAGCAATGGCTAGAAAGA
Reverse walk R3*	Reverse walking to confirm scGlu10 sequence	GGCACGAGAGCAGATACATAG
Reverse walk R4*	Reverse walking to confirm scGlu11 sequence	ACGATGTTGCTTCCATGTTTG
Reverse walk R5*	Reverse walking to confirm scGlu12 sequence	GAATTGGGCTCAGCAAAGAAAGTCTATT
Promoter walk R2*	To obtain scGlu9 promoter sequence by genomic walking	CTCCCTATGTATCTGCTGCTG
Promoter walk R3*	To obtain scGlu10 promoter sequence by genomic walking	CCTATGTATCTGCTCTCGTGC
Promoter walk R5*	To obtain scGlu11 promoter sequence by genomic walking	CTAGCATACTCCTGTCTG
Promoter walk R6*	To obtain scGlu12 promoter sequence by genomic walking	GTATCCTCGTGCCGAATTC

Primary PCR was accomplished using the manufacturer's primer along with the GluR1a primer (Table 2-2) which was designed against a cDNA sequence obtained by another Griffith lab member. All primers were designed using Primer 3 which publicly available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Primary PCR products were cleaned using the GFX PCR and Gel band purification kit (Amersham, Buckinghamshire, UK). The purified product was diluted by a factor of 1/50 with MilliQ water and used in secondary PCRs using the nested primer provided in the kit along with GluR1b. The main PCR products were purified, cloned and sequenced as per Section 2.6.2.

Sequence obtained from this first round of walking provided the information to design two more nested primers, IntronR1a and IntronR1b. These were used to walk further along the glucanase coding regions and farther upstream in the promoter. Subsequent cloning and sequencing (as described in Section 2.6.2) resulted in five distinct glucanase sequences, only one of which belonged to scGlu9. These unexpected sequences were used to design non-nested primers to walk both upstream and through their promoters, and downstream, through the coding sequence and intron region of the corresponding coding regions.

The primers designed to walk through each promoter were designated: forward walk R2, forward walk R3, forward walk R4 and forward walk R5. The primers designed to walk backwards to obtain the coding sequence for each gene were designated: reverse walk R2, reverse walk R3, reverse walk R4 and reverse walk R5. These were used along with the nested primer provided in the kit. The template used for both forward and reverse walking was the diluted secondary PCR products from the last round of nested PCR. Major bands from each PCR reaction were purified, cloned and sequenced as per Section 2.6.2.

2.6.2 Cloning

Secondary PCR products were analyzed and resolved on 1% (w/v) agarose gel. Gel bands thought to contain the product of interest were excised from the gel with a razor blade and purified using the GFX DNA gel band purification kit (GE Biosciences, Buckinghamshire, UK). The purified DNA was then cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) according to manufacturer's instructions. Ligation products were examined on a 1% (w/v) agarose gel and transformed into competent DH5α *E. coli* cells. Positive recombinant clones were authenticated by screening on LB agar plates containing ampicillin, isopropyl-beta-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-b-D-galactoside and by colony PCR. Positive colonies were cultured overnight in LB broth at 37ºC and plasmids were extracted using the Fast Plasmid Mini Plasmid Purification Kit (Eppendorf, Hamberg, Germany). Plasmids containing inserts of interest were randomly chosen from the positive recombinants and sequenced by MOBIX laboratories (McMaster University, Hamilton, Ontario, Canada) or by the Univeristy of Waterloo Sequencing Facility (University of Waterloo, Waterloo, Ontario, Canada) using T7 and SP6 primers.

2.6.3 Sequence data analysis

Analysis of sequence and prediction of amino acid sequences was performed using the program BioEdit (ver 7.0.5.3; Hal, 1999). Additional β-1,3-endoglucanse sequences not obtained from this thesis research were obtained from the GenBank database (NIH, Bethesda, MD, USA) using the default settings of the program BLAST (Altschul *et al.,* 1994). This program is publicly available at http://www.ncbi.nlm.nih.gov/BLAST/.

Due to the strong sequence similarities between amino acid glucanase sequences, multiple sequence alignments of gene and amino acid sequences were performed using the Clustal-W alignment algorithm (Thompson *et al.,*1994) which was implemented using BioEdit.

2.6.4 Promoter analysis

Analysis of the cis-elements within each β-1,3-glucanase promoter was carried out by searching the online database of plant cis-acting regulatory DNA elements known as PLACE, (Higo *et al.,* 1999) which is publicly available at on the internet at the web address http://www.dna.affrc.go.jp/htdocs/ (as of November, 2006).

Due to the length of the cis-elements, there is a degree of probability that some of the cis-elements found by the databases are not true cis-elements, and that the sequence matches only by chance. Published articles describing each cis-element were examined to determine the criteria necessary for each element to be involved in gene expression. Only cis-elements found to meet all necessary published criteria are reported in this thesis.

2.7 RT-PCR

2.7.1 cDNA Synthesis

All reagents used for creating cDNA were from Fermentas (Burlington, ON, Canada) unless otherwise noted. To remove contaminating DNA, approximately 2 µg of RNA was placed into a 1.5mL microcentrifuge tube along with 2µl of DNase I (RNase Free) and 2µL 10x DNase Buffer. The final volume was brought to 20uL using MilliQ water that was made RNase free by UV light treatment. The mixture was incubated for 30 minutes at 37°C and the reaction was stopped by adding 2µl of the EDTA stop solution provided with the DNase,

followed by heating at 65°C for 10 minutes. From this stock of DNA-free RNA, 10µl was used to create cDNA, the rest (approximately 12µL) was stored at -80°C for later use.

The 10µl aliquot of RNA was mixed with 1 µl of oligo dT primer ($20U \cdot \mu l^{-1}$) and heated at 70°C for 10 minutes after which the tubes were cooled on ice. Then, 4µl of 5X RT buffer, 2µl of 10mM dNTPs and 0.5µl of RNase inhibitor ($40U \cdot \mu l^{-1}$) were added to the reaction which was subsequently placed in a 37°C water bath for 5 minutes. Finally, 2ul of MuLV-RT ($20U \cdot ul^{-1}$) was added and the tubes continued to incubate at 37°C for 1 hour. Finally, the reaction was stopped by heating the tube to 70°C for 10 minutes. Samples were stored at -20°C until needed.

2.7.2 RT-PCR conditions

The primers used for RT-PCR are identical to those used for reverse genomic walking (Table 2-2). The reverse primers used were designated gluR1 exon2 reverse, reverse walk R2 through R5, along with the forward primer GluR1a. Specific forward primers for all genes could not be designed due to the high sequence similarity between all five genes. The primers 5′ –CGTGATCGATGAATGCTACC-3′ and 5′ -GGGTTTGTTGCACGTATTA-′ 3 were used to amplify a 199bp stretch of the Arabidopsis 18S ribosomal RNA, this was used for the internal standard in all RT-PCR reactions.

In order to determine the exponential range for product amplification, each set of primers was used to prepare a master mix which contained equal concentrations of each of the 56 cDNA samples to be tested. This master mix was divided equally into 10 thin walled 0.2µl PCR reaction tubes. These were placed in a thermocycler programmed with the following cycle parameters:

94°C – 4 minutes

94°C – 45 seconds 56°C – 45 seconds 72°C – 45 seconds	40x

Tubes were removed from the thermocycler after cycles 15,19, 22, 25, 29, 32, 34, 36, 38 and 40. From these tubes, 10µl of each reaction was electrophoresed on a 3% (w/v) agarose gel containing ethidium bromide; the resulting DNA bands were visualized under UV light with a Fluorochem 8000 chemiluminesence and visible imaging system. Flourochem 8000 software was used to determine the spot density of each of the bands which were plotted against cycle number to determine the number of cycles that would put product amplification in the mid logarithmic range for each primer set.

The 25µl mix for each RT-PCR reaction is as follows:

18.5µl MilliQ PCR water
1.0µl UTR specific primer or 18S specific primer 1
1.0µl gluR1 exon 2 reverse of 18S specific primer 2
0.5µl 10mM dNTP mix
2.5µl 10 x Taq polymerase buffer
(500mM KCl, 100mM Tris HCl pH 9.0, 20mM $MgCl_2$ pH 8.)
0.5µl cDNA
1µl Taq DNA polymerase (Roche, Mannheim, Germany)

These were placed in a thermocycler programmed with the following cycle parameters:

94°C – 4 minutes

94°C – 45 seconds 56°C – 45 seconds 72°C – 45 seconds	25 cycles for 18S primers 30 cycles for scGlu9, 10 and 12 primers 35 cycles for scGlu6 and 11 primers

72°C – 5 minutes

Each cDNA sample was used in 6 RT-PCR reactions: one for each of the 5 glucanses and another for the 18S standards. Exactly 10µl of each reaction which was electrophoresed on a 3% (w/v) agarose gel and visualized as previously described.

2.8 SDS-PAGE and Immunoblotting

2.8.1 Whole leaf and crown tissue protein extraction for 1D-gel electrophoresis

For untreated non-acclimated plants and those for 4 days treated with MeJa and their associated controls, protein was extracted 1 week after initial treatment. CA leaf extracts were prepared from plants that were grown at 4°C for 7 weeks. One gram frozen leaf material was ground to a powder with a mortar and pestle; the tissue was kept frozen by periodic addition of liquid nitrogen. Additional liquid nitrogen was added to the mortar followed immediately by 2.5mL of tissue extraction medium (0.1M Tris base pH 8.8, 10mM EDTA, 0.9M sucrose and 0.4% β-mercaptoethanol which was added just prior to use). Grinding continued until the mixture of leaf tissue and tissue extraction medium resembled a fine powder. This was transferred to a sterile 15mL plastic centrifuge tube containing 2.5mL of Tris pH 8.8 buffered phenol. The solution was thoroughly mixed and then sonicated once for 30 seconds at output level 5 (Sonic Dismembrator model 100, Fischer Scientific, Fair Lawn, USA). The mixture was agitated for 30 minutes at 4°C and subsequently centrifuged (8000xg , 4°C for 10 minutes). The phenol (top) phase was removed to a clean, sterile 15mL plastic centrifuge tube. To precipitate the protein, 5 volumes of 0.1M ammonium acetate in 100% methanol were added to the tube, which was then vortexed and incubated overnight. The precipitate was collected by centrifugation (8000xg , 4°C for 10 minutes) and the pellet was completely resuspended in 600µl of 0.1M ammonium acetate in 100% methanol. The suspension was transferred to a microcentrifuge tube containing an additional 600µl of ammonium acetate in

methanol. To ensure complete suspension, the solution was sonicated 10 times for 5 second intervals output level 5 (Sonic Dismembrator model 100, Fischer Scientific, Fair Lawn, USA). The suspension was kept on ice during sonication to keep the sample cool. The protein was precipitated at -20°C for 2 hours and then collected by centrifugation (8000xg, 4°C for 5 minutes). The washing process in ammonium acetate in methanol was repeated twice, followed by a wash with 80% acetone and then in cold 70% ethanol. The final pellet was dried at room temperature and resuspended in a minimum volume of 50mM Tris Buffer, pH 8.8 ranging from 100 to 200µl.

2.8.2 Apoplastic protein extraction

Apoplastic protein was only extracted from winter rye plants that had been CA for 7 weeks, from 3-week-old NA plants and from plants treated with ethephon, MeJa and hydrogen peroxide. The procedure for apoplastic protein extraction was performed according to the method of Hon *et al.,* (1994). Leaves were cut into 2cm sections, washed three times with deionized water, and then vacuum-infiltrated with cold extraction buffer (20mM ascorbic acid, 20mM calcium chloride) for 30 minutes using a DuoSeal vacuum pump (model 1402; Welch Scientific Company, Skokie, USA). The brittle leaves were carefully dried with paper towels, and centrifuged at 2000xg (GSA rotor, Sorvall, Mississauga, Canada) for 15 minutes. Infiltrate was concentrated by spinning for 10 minutes at 5000xg in Amicon Ultra-15 centrifugal filter device with a molecular weight cut off of 10kDa (Millipore, Carrigtwohill Ireland). Concentrated apoplastic extract was stored at -20°C until needed.

2.8.3 Detection of ß-1-3-endoglucanases using 1D immunoblotting

Approximately 5µg of protein from whole tissue extracts and 10µg of apoplastic extract were separated on a SDS-polyacrylamide (12% w/v) gel. The gel was equilibrated in TBS-T (48mM Tris base, 39mM glycine, 0.05% Tween-20 , pH 9.2) for 20 minutes and transferred to Polyvinylidene Fluoride (PVDF) western blotting membrane (Roche, Mannheim, Germany) using a Trans-Blot SD Semi-dry transfer cell and a Semi-dry western transfer buffer (5.82g Tris, 2.93g glycine, 200mL methanol, 3.75mL of 10% SDS, 680mL water). The blots were transferred at 20V for 45 minutes. The PVDF membrane was blocked with 1% Poly(vinyl alcohol) (MW 30,000 – 70,000) for 30 seconds at room temperature, then quickly rinsed 3 times, 10 seconds each in water, followed by 2 washes of 10 seconds in TBS-T followed by 3 more washes in TBS-T each of 10 minutes. All washes are performed at room temperature. The blot was incubated at room temperature, with gentle agitation for 1 hour in 1:3000 anti-β-1-3-endoglucanase antibody (Hon *et al.,* 1995). The blot was washed three times in TBS-T and then incubated at room temperature for 1 hour in a 1:10000 dilution of peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson Immunoresearch, West Grove, PA, USA) in diluted in 5% (w/v) fat-free milk powder in TBS-T. The blot was washed for 10 minutes 3 times in

TBS-T. The antibody binding was detected by chemiluminesence by incubating the membrane in ECL Western Blotting Reagent System (Amersham, Buckinghamshire, UK) as per the manufacturer's instructions, after which the membrane was exposed to photographic film (X-omat AR, Kodak, Chicago IL, USA).

2.8.4 Whole leaf and crown Protein Extraction for 2D-electrophoresis

Protein was extracted from whole leaf and crown tissue using the method outlined in Section 2.8.1 – with the following changes. The final pellet was dried at room temperature and

resuspended in a minimum volume (around 100µl) of IEF Sample Buffer (8M urea, 2M thiourea, 2% CHAPS, 2% Triton X-100). Salt was removed from samples using an Ettan Sample Preparation Mini Dialysis Kit, 8kDA cut-off, 250µl volume (GE Biosciences, Buckinghamshire, England). Samples were dialyzed overnight at 4°C against IEF Sample buffer and stored at -20°C until needed.

2.8.5 First Dimension IEF electrophoresis

Protein samples for 2D-electrophoretic analysis were thawed at room temperature for 1 hour to ensure that urea present in the buffer was fully dissolved. Approximately 10µg of each CA and NA protein sample, or 2µg of CA apoplastic protein sample were added to Destreak Rehydration Solution (GE Biosciences, Buckinghamshire, England) containing 0.05% IPG Buffer pH 3-10NL (GE Biosciences, Buckinghamshire, England) to a final volume of 120µl. The solution was placed in the 7cm ceramic DryStip strip holder and a 7cm Immobiline DryStrip pH 3-10 NL (GE Biosciences, Buckinghamshire, England) was laid in the solution and overlaid with drystrip solution (GE Biosciences, Buckinghamshire, England) to prevent evaporation. The holder was placed on the GE Biosciences (Buckinghamshire, England) IPGphor focusing system and cycled as follows:

Step and Hold – 30 m, 150V
Step and Hold – 30 m, 300V
Step and Hold – 60m, 600V
Gradient – 2h, 1000V
Gradient – 1h, 3500V
Step and Hold 1h, 3500V

Samples were maintained at a constant 50V until the strips were removed from the apparatus.

After focusing, the strips were immediately placed inside individual sterile 15mL Falcon tubes (BD biosciences, San Jose, USA) and carried on dry ice to a -80°C freezer where they were stored until use.

2.8.6 Second Dimension electrophoresis

The strips were removed from the -80°C freezer. Each strip was placed in 10mL SDS equilibration buffer solution (50mM Tris-HCl pH 8.8, 6M urea, 30% (v/v) glycerol, 2% SDS (w/v), 0.0002% bromophenol blue) supplemented with 100mg freshly prepared 1,4-dithiothreitol (Roche Diagnostics, Mannheim Germany). The strips were incubated in this solution for 10 minutes at room temperature with gentle shaking. The strips were then placed in clean 15mL falcon tubes containing 10mL SDS equilibration buffer solution supplemented with 250mg freshly prepared iodoacetamide (BioShop, Burlington, ON, Canada). The strips were incubated in this solution at room temperature, with gentle shaking for 10 minutes. Strips were then quickly washed in Milli Q water (2 seconds) to remove excess buffer and placed on top of a 1mm thick 12% SDS-polyacrylamide separating gel that was overlaid with 1% agarose (w/v) in running buffer (g L^{-1} Tris base, 14.4g L^{-1} glycine, and 1g L^{-1} SDS). The agarose overlay contained a trace of bromophenol blue to allow the progress of electrophoresis to be visualized. A pre-stained molecular weight marker (20 to 118kDA, Fermentas) was loaded on the gel to help assess protein size. Electrophoresis was performed in a Bio-Rad Mini-Protein III Dual Slab Cell Apparatus [50 mV for 1 hour, then at 100mV until bromophenol blue dye front reached the end of the gel, approximately 2 hours].

2.8.7 Visualization of 2D-PAGE using Ruby Red SYPRO Stain

All fixing, staining and destaining steps were performed in a round, 750mL Rubbermaid Servin' saver container, as recommended by the stain supplier. After electrophoresis, the gel was rinsed briefly in MilliQ water (2 seconds) and then added to a fixing solution of 50% (v/v) methanol, 10% (v/v) trichloroacetic acid and placed on an orbital shaker, at a gentle rotation speed, for 3 hours. The gel was rinsed of fixing solution by washing 3 times with MilliQ followed by the addition of 50mL of SYPRO Ruby protein gel stain (Invitrogen Molecular Probes, Eugene, USA). As SYPRO Ruby is light sensitive, the container containing the gel and the stain was tightly wrapped in aluminum foil before it was placed on an orbital shaker at room temperature over night. The gel was destained for 1 hour with a 10% (v/v) methanol, 7% (v/v) acetic acid solution and then rinsed 3 times for 5 minutes with MilliQ water. The gel was visualized under UV light with a Fluorochem 8000 chemiluminesence and visible imaging system.

2.8.9 Detection of ß-1,3-endoglucanases using 2D immunoblotting

A 2D SDS-PAGE gel of whole tissue protein extract from NA and CA winter rye as well as apoplastic protein extract from CA winter rye was performed as described in section 2.86. Detection of β-1,3-endoglucanases was accomplished using the protocol described in section 2.8.3.

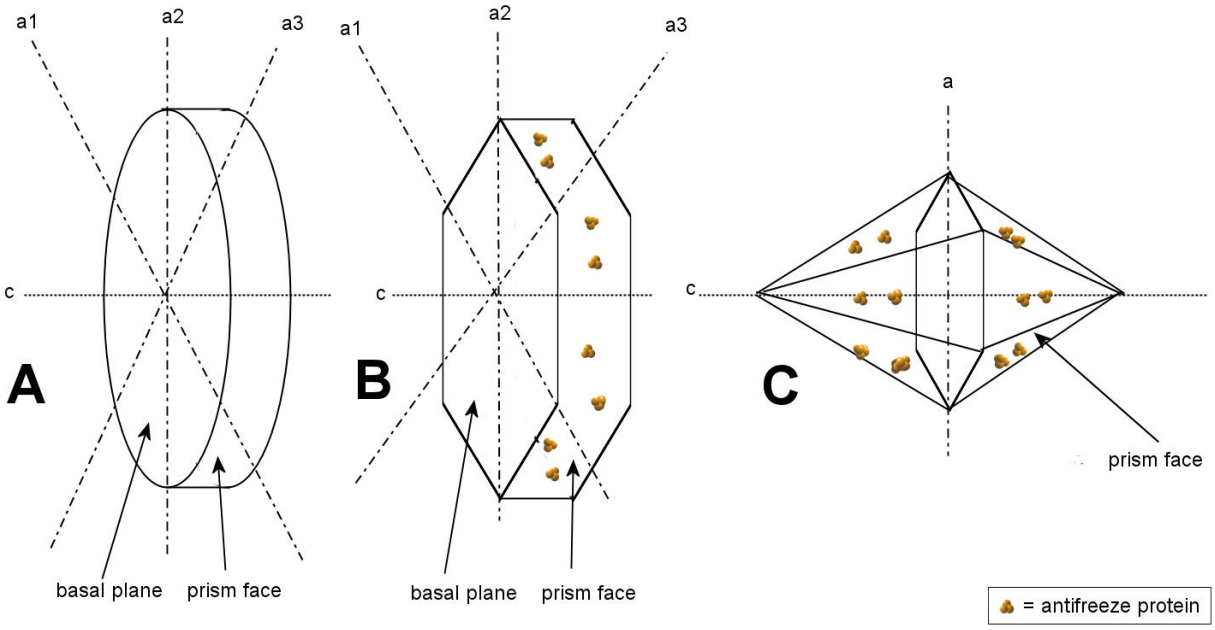
2.9 Antifreeze Activity Assay

To assay for antifreeze activity, a Clifton nanoliter osmometer (Clifton Technical Physics, Harford, NY, USA) and a phase contrast photomicroscope (Olympus BHT, Carsen Medical and Scientific Co. Ltd, Ontario, Canada) were used following the method first

described by DeVries (1986). Antifreeze activity is determined qualitatively by observing the morphology of ice crystal growth in a given solution (Figure 1-1). Ice crystals grown in the absence of AFPs grow normal to the c axis producing spherical crystals. If AFPs are present, ice crystal formation along the a axis is inhibited and so ice crystals form hexagonal shapes. The amount of hexagonal structure formed is proportional to the amount of AFP in the solution such that a solution low in AFP will produce crystals shaped as simple hexagons, while solutions high in AFP will produce hexagonal bipyramids along the c axis.

Figure 2-1: Morphology and growth performance of ice crystals in the presence of different concentrations of antifreeze proteins.

(A) In solutions without AFPs, new water molecules adsorb onto the prism face of existing ice crystals. Therefore, crystals increase in width (a-axes) but not in height (c-axes). (B) In solutions with low antifreeze activity, AFPs adsorb onto the prism faces of the ice crystal and limits, but does not prevent growth along the a-axis. This results in an ice crystal with a hexagonal shape. (C) When there is a high concentration of AFPs, the adsorption to the prism face inhibits growth along the a-axis, making it energetically favourable for water to bind to the basal plane. This causes crystal growth along the c-axis generating bipyramids that are hexagonal in cross-section.


a1
a2
a3
c
A
basal plane
prism face
a1
a2
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B
basal plane
prism face
a
c
C
prism face
= antifreeze protein

3 Results

The β-1,3-endoglucanase gene family of winter rye is poorly characterized at the molecular level. At the onset of this project only one winter rye cDNA sequence was available. Since that time seven additional β-1,3-endoglucanase cDNAs, all displaying high amino acid sequence conservation (95-98%) have been fully or partially sequenced (Yaish *et al.,* 2006). This project aims to compare the genetic structure of genes coding for β-1,3-endoglucanases with and without antifreeze activity. In order to accomplish this goal it was first important not only to determine the conditions that cause winter rye to produce β-1,3-endoglucanases but also to determine whether winter rye produces more than one β-1,3-endoglucanase after cold-acclimation.

3.1 Results of plant treatments

3.1.1 Treatment with Ethephon

Ten mM ethephon or the ethephon spray control (2mM hypocholoric acid, 2mM phosphoric acid, and 0.005% Tween 20) was sprayed onto the foliage of 1-week old winter rye plants for a period of 4 days. The appearance of control plants and ethephon-treated plants remained physically similar throughout the experiment with the exception that after 4 days the leaves of ethephon-treated plants appeared to have a slightly decreased levels of anthocyanins. That is, they had a brighter green appearance than the control plants. These are described below.

3.1.2 Treatment with Methyl Jasmonate

One-week old winter rye plants were sprayed daily for 4 days with 5mL of 15μM MeJa or a 0.05% Tween 20 spray control. After 1 day, a few of the plants sprayed with MeJa began turning a pale purple. In addition, the leaves of all treated plants began to curl. After two days, the tips of each plant had started to yellow and most plants had gained a purple tinge. After 4 days of treatment, some experimental plants had turned dark purple and the tip of each plant had died back around 0.5cm. None of these changes occurred in the control plants.

3.1.3 Treatment with hydrogen peroxide

Two-hundred mM hydrogen peroxide or a 0.005% Tween 20 spray control was applied onto the foliage of 1-week old winter rye plants. The only observable physical difference between experimental and spray control plants involved leaf length. The leaves of plants sprayed with hydrogen peroxide were 2 ± 0.2cm longer then those of the control group. This average was determined by measuring the leaf length of 50 randomly chosen plants. This was repeated once for each of the three replicates.

3.1.4 Wounding of Winter Rye

For the wounding experiments, leaves of three-week old winter rye plants were scored at 1cm intervals with a double-sided razor blade. Control plants were not damaged. The plants were followed for 2 days. Wounded leaves of experimental plants became flaccid 6 hours after wounding and did not become turgid before the end of the experiment. Wounded smaller leaves, that is those that were less than 0.4cm wide, died before the end of the experiment. Thus, the leaves sampled for glucanase expression were greater than 1cm wide.

3.1.5 Pathogen Infection of Winter Rye with *Microdochium nivale*

To study the effect of pathogen infection on winter rye, wheat bran laced with *M.nivale* was sprinkled at a density of $0.01g \cdot cm^{-2}$ on the leaves of 1-week old winter rye. The leaf surface area of a 15cm pot of winter rye containing approximately 110 plants was estimated to be $828cm^2$. This was calculated by averaging the surface area of 10 randomly selected leaves and multiplying this area by the average number of winter rye plants per 15cm pot. Plants were then wrapped in plastic bags and placed in a refrigerator at 4°C for 3 days to facilitate mycelial growth before transfer to a growth chamber set to optimal winter rye growth conditions. The experiment ran for a total of eight days and was performed in triplicate.

In all replicates, mycelial growth was not visible to the human eye until day 4. By day 8, mycelial growth had created a white blanket across the soil and surrounded the winter rye crowns with a web. Control plants, which were sprinkled with sterile wheat bran had no visible signs of infection.

3.1.6 Dehydration and Rehydration

Although wilting is a traditional sign of dehydration, this visible indication of water loss does not occur at similar stages for all plant species. Plants that are dehydration intolerant, such as the water lily, will wilt within minutes if removed from a saturated environment (Salisbury and Ross, 1991). Succulents, on the other hand may not wilt until deprived of water for months and even years (Salisbury and Ross, 1991).

The point at which each plant begins experiencing drought stress also differs, and this point is generally considered to start before any visible signs of drought stress, such as wilting, become evident. Drought stress was quantified by determining the RWC of rye plants experiencing a water deficit. This measure, allows one to estimate how much water is in a fully

hydrated plant tissue and then determine to what extent a plant is dehydrated. In the case of the analysis of winter rye, it was necessary to determine how long it took winter rye to lose a fatal amount of water. This point was reached 4 days after onset of drought conditions. Next, the RWC was determined for well-watered plants and water stressed plants that displayed the effects of drought (wilting). This experiment was performed with 3 sets of plants, with each set consisting 6 pots (3 experimental and 3 control). The plants were watered to run-off and then the RWC of one plant from each pot was determined. The experiment continued for 4 days, during this time the experimental pots of winter rye were not watered, while the control pots were watered to run-off. At this point the RWC of 150 random plants from each set (50 plants per pot) was taken and compared. At the onset of the experiment, the winter rye had a RWC of 94.8 ± 0.98% After four days, water stressed plants had a RWC of 82 ± 1% while control pots had a RWC of 96 ± 2%.

3.2 Estimated antifreeze activity of winter rye apoplastic extract after treatment with cold, ethephon, hydrogen peroxide and MeJa

The effect of cold, MeJa and ethephon treatements on antifreeze activity were tested and compared. Previous studies determined the length of cold-treatment and the concentration of hormones necessary to produce apoplastic extracts with maxium levels of AFPs (Yu *et al.,* 2001; Merz, 2004). As a preliminary study, plants were treated with 200mM of hydrogen peroxide for 4 days. The apoplast from treated plants and their spray controls were extracted, dialyzed against dH_2O, concentrated to $1mg \cdot mL^{-1}$ and tested for antifreeze activity. A solution of 0.005% Tween-20 was used as the spray control for both MeJa and hydrogen peroxide treatment. An additional ethephon spray control (2mM hypocholoric acid, 2mM phosphoric acid, and 0.005% Tween-20) for antifreeze activity. The apoplastic extract of 3-week-old NA

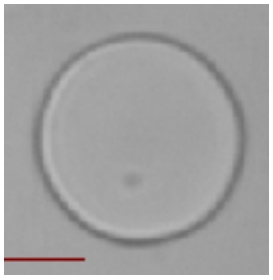
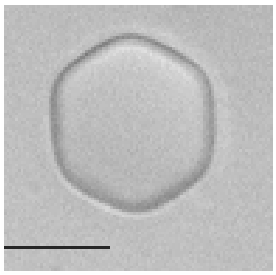
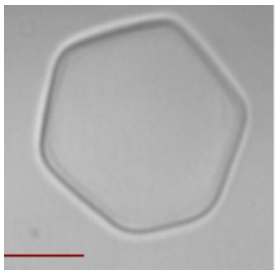
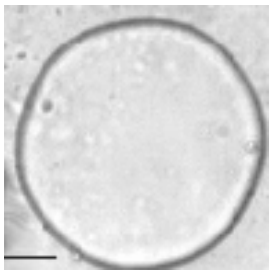
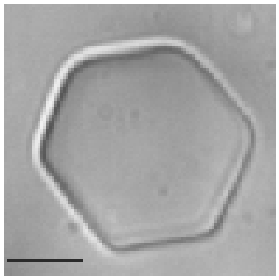
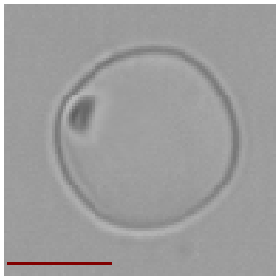
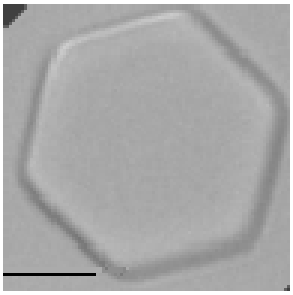
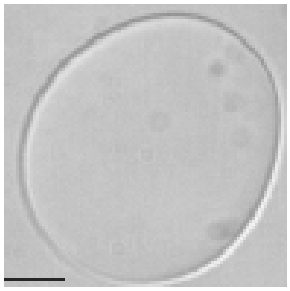
winter rye plants, which are at an equivalent growth stage to 7-week old cold-treated plants, were used as a cold-treatment control while MilliQ water was used as the as a negative control.

In order to compare the antifreeze activity between the samples, the salt and protein concentration of each extract were equilibrated by dialyzing each sample against water and adjusting the protein concentration of each extract to $1mg \cdot mL^{-1}$. Aliquots of approximately 10nL of each sample were analyzed for antifreeze activity on a Clifton nanoliter osmometer. As expected, the apoplastic extracts from NA plants, both sets of spray controls plants, and the MilliQ water control formed disc-shaped, circular ice crystals. This growth along the a-axis but not along the c-axis indicates that these samples have no antifreeze activity (Figure 3-1). Using the same assay, apoplastic extracts from MeJa, ethephon, hydrogen peroxide and cold exposed plants all displayed antifreeze activity, but to different degrees. The apoplastic extract from MeJa-treated plants had very weak for antifreeze activity, producing ice crystals with a rounded hexagonal shape and no growth along the c-axis. Extracts from plants treated with hydrogen peroxide, ethephon, or cold produced ice crystals with increased antifreeze activity over the apoplastic extract from MeJa-treated plants. Ice crystals were hexagonal in shape, and had sharp angular edges. These samples had apparently equivalent levels of antifreeze activity.

Figure 3-1: Estimation of antifreeze activity in apoplastic extracts of NA winter rye leaves treated daily with cold, methyl jasmonate, ethephon or hydrogen peroxide.

Plants were sprayed with 5mL of the appropriate hormone solution or spray control daily. The apoplastic proteins were extracted after 4 days of treatment. Each extract was dialyzed against dH_2O and concentrated to $1mg \cdot mL^{-1}$. Each assay was performed on an

approximate volume of 10nL. The ice crystals formed in the control extracts and the negative water control did not display antifreeze activity. Treatment with MeJa yielded apoplastic extract with the lowest amount of antifreeze activity. Ice crystals formed in the MeJa sample were so low that the ice crystal was hexagonal in shape, but the edges still had a slightly rounded appearance. The plants treated with hydrogen peroxide, ethephon and cold displayed higher antifreeze activity then that seen from MeJa produced extract, as evidenced by the presence of crystals with hexagonal shapes with linear edges. A: Apoplastic extract from the spray control, sprayed daily with 5mL of 0.005% Tween 20 in dH_2O. B: Apoplastic extract from plants treated with 15μM MeJa + 0.005% Tween 20. C: Apoplastic extract from plants treated daily with 5mL of 200mM hydrogen peroxide in 0.005% Tween 20 D: Apoplastic extract of plants treated with the ethephon spray control (2mM hypochoric acid, 2mM phosphoric acid and 0.005% Tween 20). E: Apoplastic extract of plants treated with 10mM ethephon plus 0.005% Tween 20 daily. F: Apoplastic extract from 2-week old NA winter rye plants G: Apoplastic extract of plants cold-treated for 7 weeks H: dH_2O control. Bar = 10μm

A 	B 	C 
D 	E 	F 
G 	H 	

3.3 A survey of the cold-induced β-1,3-endoglucanases of winter rye

There have been no tests to determine the β-1,3-glucanase content within whole leaf tissue of treated and untreated plants. Cold-acclimation may lead to the presence of glucanases within the cell that may contribute to the cold-tolerance of winter rye. In addition, it is unknown whether the apoplastic extracts of MeJa treated plants contain β-1,3-endoglucanases. The presence of these proteins within the apoplast would suggest that these glucanases could contribute to the observed antifreeze activity. In order to clarify both issues, analysis of whole leaf tissue and apoplastic extracts from NA, CA and MeJa treated plants were examined by 1D SDS-PAGE followed by immunoblotting with anti-glucanase antibody. Protein analysis and subsequent immunoblotting with anti-glucanase antibody is the conventional method to test for the presence of glucanse within the apoplastic extract of winter rye.

Proteins of identical or nearly identical sizes can fail to be differentiated using 1D-SDS PAGE analysis. A single stained protein band may contain any number of proteins that cannot be separated by size alone. Two-dimensional SDS-PAGE analysis separates proteins based on size and charge. Thus, 2D SDS-PAGE is a powerful tool for protein composition studies. It may be possible that there are additional proteins, including additional glucanases, within the apoplast of CA and NA winter rye that have eluded detection by the previous one dimensional SDS PAGE studies. To examine this possibility, that apoplastic extracts CA and NA winter rye were examined by 2D-SDS PAGE followed by immunoblotting with an anti β-1,3-endoglucanase antibody. This method was also used to closely examine the glucanase content within whole leaf extracts of identically treated plants. These were compared and contrasted to the results of the corresponding 1D SDS-PAGE analysis of the same samples.

3.3.1 1D-SDS PAGE gel and immunoblotting results

Samples were analyzed by 1D-SDS PAGE were prepared from untreated and cold-treated winter rye plants. Whole leaf extracts of all treatments were analyzed at a concentration of 10µg/lane while apoplastic extracts that contain far fewer polypeptides were loaded at a concentration of 2µg/lane. Duplicate gels were prepared for each experiment; one gel was stained with colloidal coomassie blue to visualize the proteins while the other was used in immunoblotting analysis.

For the most part, the Colloidal Coomassie Blue staining revealed a banding pattern (Figure 3-2) characteristic of previously described whole leaf and apoplastic protein extracts (data not shown, see Yu *et al.,* 2001). In contrast, the protein collected from whole leaf tissue that had had its apoplast removed was missing the expected AFP protein bands and had poor resolution. The protein extraction from apoplastic free leaf tissue was repeated several times with the same result.

Immunoblots using β-1,3-endoglucanse specific antibodies showed that all NA samples, including controls, have low levels of the 32 kDa glucanase (Figure 3-3). This was expected, as glucanases are not only important in stress responses but are also required to build and maintain cell wall structure. When compared to the controls, CA whole leaf and apoplastic extracts have increased β-1,3-endoglucanase concentrations. The results between CA whole tissue and CA apoplast cannot be directly correlated as the amount of protein in each lane differs due to the nature of the extracts. There was a noticeable absence of the 35kDa glucanase within these samples. It is possible that the concentration of 35kDa polypeptide is too low to be detected in NA whole leaf protein extract and that the large signal of the 32kDa glucanse in proteins taken from CA plants masks the presence of the 35kDa protein. A 35kDa protein band

is present in the 1D Coomassie stained SDS-PAGE of these extracts, but the extract is known to contain chitinases of a similar (36kDa) size; thus, the Coomassie stained gel cannot be used to positively identify this glucanase as the 36kDa chitinase will not appear as a separate band from the 35kDa glucanase band.

The colloidal coomassie stained gel of proteins extracted from MeJa treated winter rye (Figure 3-4) showed a banding pattern characteristic of apoplastic extracts from CA winter rye. Similarly, immunoblot analysis of these proteins using an antibody against β-1,3-endoglucanses gave results similar to those expected from apoplastic extract from CA treated plants (Figure 3-5). Both whole leaf extract and apoplastic extracts from MeJa-treated leaves contain β-1,3-endoglucanases. The glucanase signal appears stronger in the apoplastic extracts then in the whole tissue extract, but semi-quantitative comparisons cannot be made due to differences in protein concentration and composition. The immunoblots provide evidence that the apoplast of MeJa-treated winter rye contains β-1,3-endoglucanses. These may contribute to the observed antifreeze activity (refer to Section 3.2).

Unlike the whole leaf tissue extract from CA plants, immunoblot analysis of whole leaf protein from MeJa-treated plants contained glucanases of 32kDa and 35kDa. The 35kDa glucanase is present at a much lower concentration than the 32kDa glucanase. Apoplastic protein extracts do not appear to contain glucanases of both sizes, but the strong positive signal from the 32kDa glucanse may mask the weak positive signal of the 35kDa glucanse. The control sample also has the potential to contain glucanases of both sizes,

Figure 3-2: 1D SDS-PAGE of proteins extracted from NA and CA winter rye plants along with their controls.

Protein samples from NA controls were taken from 1-week old plants while the protein samples from CA plants were taken from plants grown for 1 week at normal conditions and then transferred to a growth chamber set at 4˚C for an additional 7 weeks. Samples visualized using colloidal Coomassie blue. Whole leaf protein from NA and CA plants present similar polypeptide banding patterns. Proteins within the CA whole leaf extract appear to have degraded and is poorly resolved. The banding pattern in the CA apoplastic extract is typical of this sample type. CA apoplastic extract (A) 10µg whole leaf protein from NA plants; (B)10µg whole leaf protein from CA plants; (C) 10µg whole leaf protein from CA plants with apoplast extracted; (D) 2µg CA apoplastic extract. (MW) Molecular weight marker.

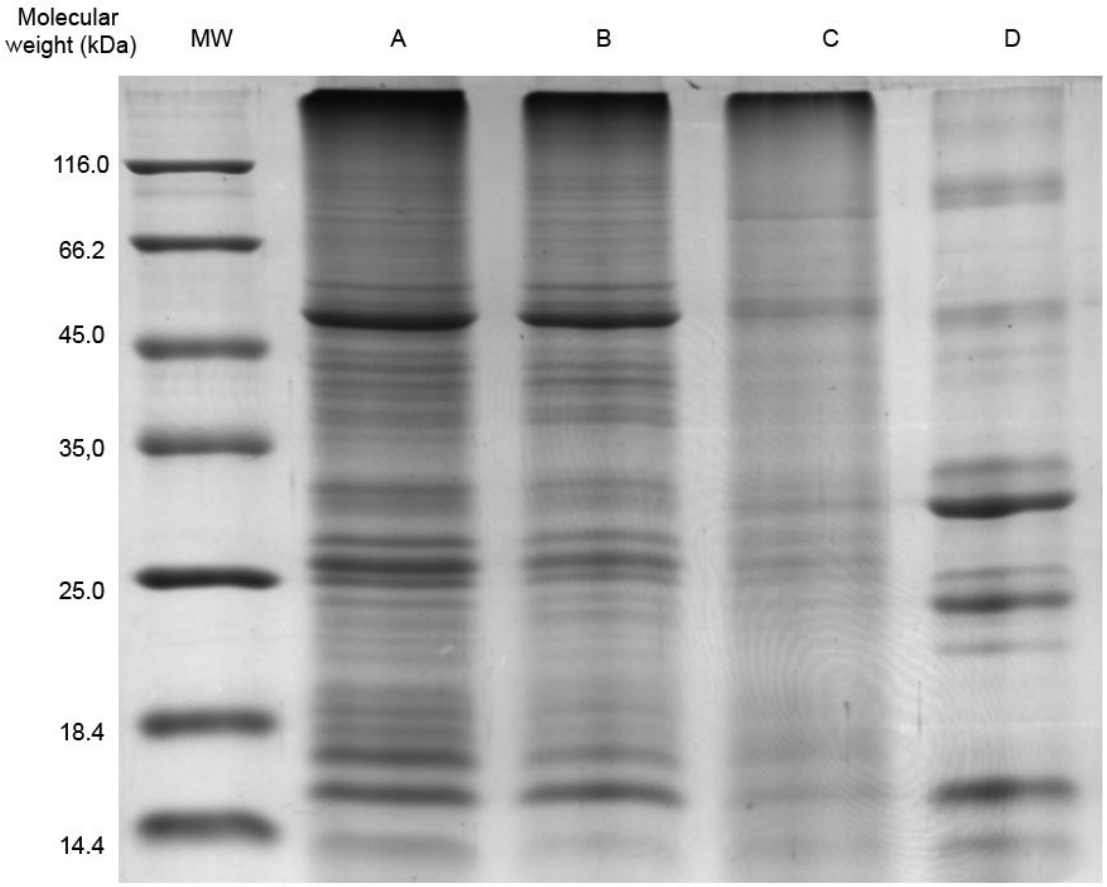

Molecular weight (kDa)
MW
A
B
C
D
116.0
66.2
45.0
35,0
25.0
18.4
14.4

Figure 3-3: 1D immunoblotting for β-1,3-endoglucanases in proteins extracted from NA and CA winter rye.

The protein sample from NA plants was taken from plants grown for 2 weeks at normal conditions. The protein samples from CA plants were taken from plants grown for 1 week at normal conditions and then transferred to a growth chamber set at 4˚C for an additional 7 weeks. Weak glucanse signals are present in the NA whole leaf extract and the whole leaf extract with apoplastic proteins removed. A stronger glucanase signal is evident in whole leaf extract from CA winter rye plants. The glucanase signal is strongest in the apoplastic extract. (A) 10μg of whole leaf tissue protein extract of NA winter rye plants; (B) 10μg of leaf tissue protein with apoplastic proteins removed from CA winter rye plants; (C) 10 μg of whole leaf tissue protein from CA winter rye; (D) 2μg apoplastic extract from CA winter rye plants. Image is taken from area corresponding to the region between the 35 and 25kDa MW markers. The exposure time for this blot was 10 seconds.

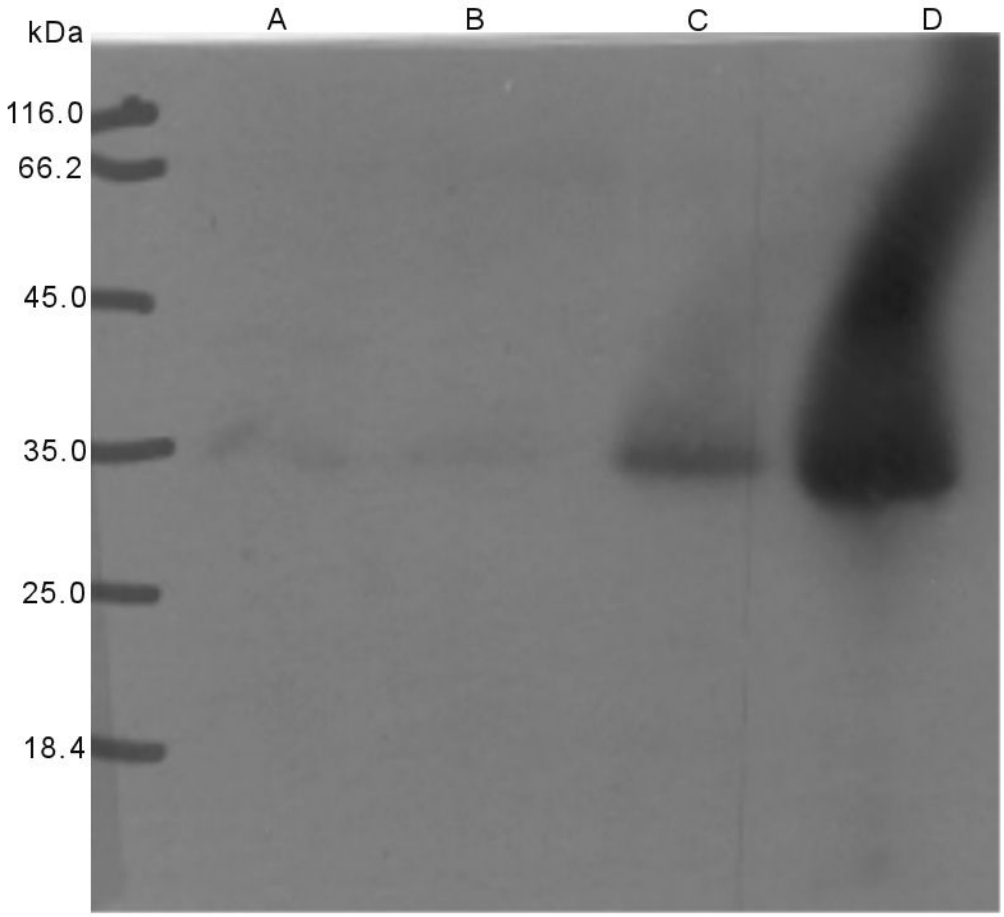

kDa
A
B
C
D
116.0
66.2
45.0
35.0
25.0
18.4

Figure 3-4: 1D SDS-PAGE of proteins extracted from winter rye treated with MeJa.

One-week old winter rye plants were treated with 5ml of 15mM MeJa daily, for 4 days. The spray control plants were treated daily for 4 days with 0.005% of Tween 20. Proteins were visualized using colloidal coomassie blue. Whole leaf extracts presented a protein profile typical of all winter rye leaf protein extracts while the polypeptide banding pattern within the apoplastic extract appeared similar to the banding pattern of CA winter rye. (A) 10µg whole tissue protein from MeJa-treated plants; (B) 2µg apoplastic extract from MeJa-treated plants; (C) 10µg whole tissue protein from spray control plants; (MW) MW marker.

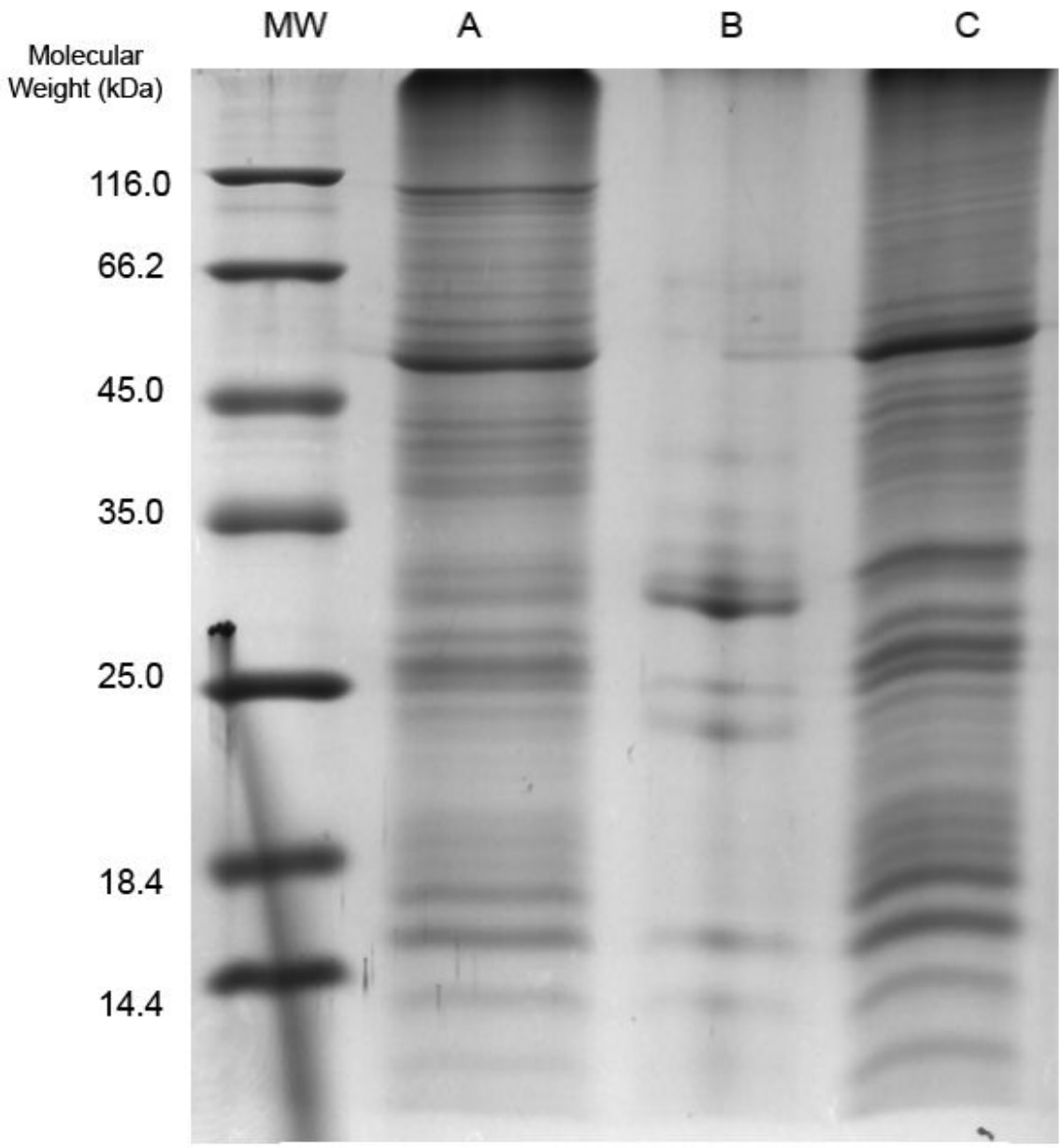
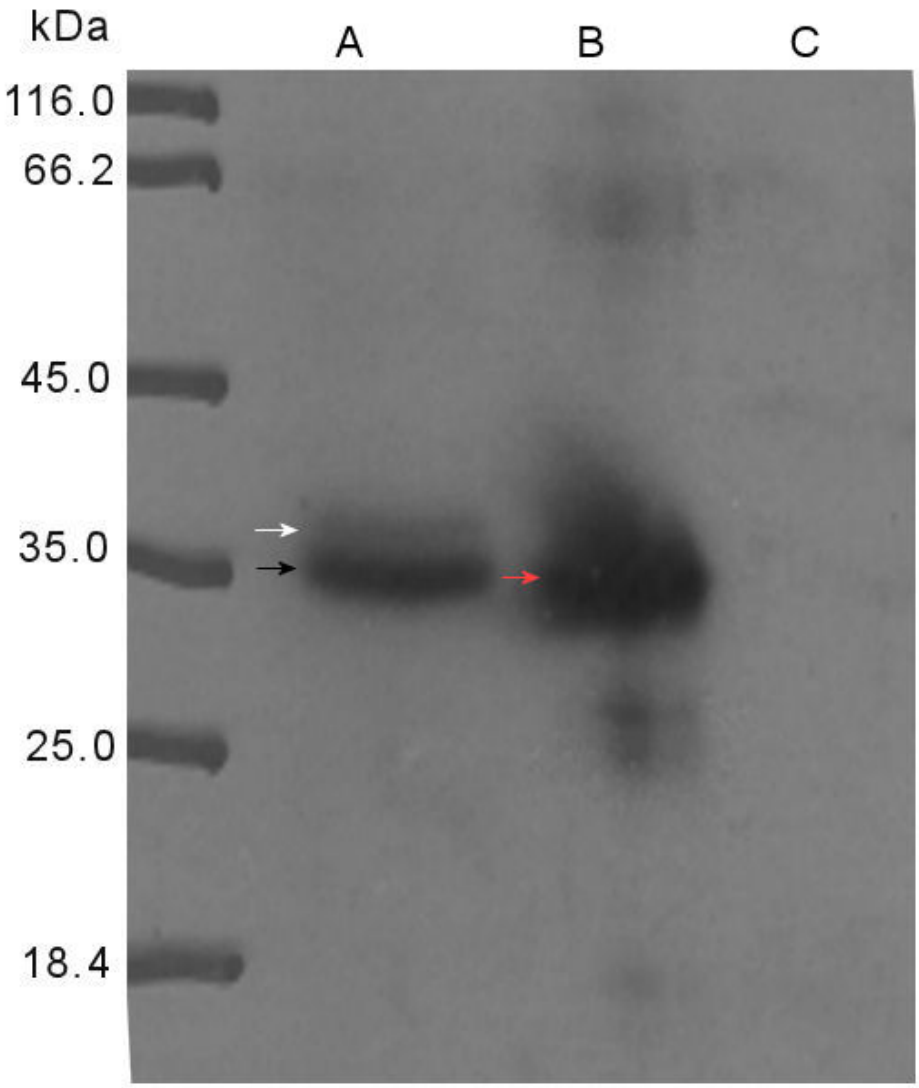

MW
A
B
C
Molecular Weight (kDa)
116.0
66.2
45.0
35.0
25.0
18.4
14.4

Figure 3-5: 1D immunoblotting analysis for B-1,3-endoglucanases of proteins extracted from winter rye treated with MeJa.

One-week old winter rye plants were treated with 5ml of 15mM MeJa daily, for 4 days. The spray control plants were treated daily for 4 days with 0.005% Tween 20. Proteins were run on a 12% (v/v) acrylamide gel and then transferred using a semi-dry transfer to PVDF. Samples were blotted using an antibody against β-1,3-endoglucanases and the signal was detected using chemiluminescence and x-ray film. Whole leaf protein from MeJa treated plants contained two glucanses, a 35 kDa glucanse (white arrow) and a more concentrated 32kDa glucanse (black arrow). A single glucanse of 32kDa is present in the apoplastic extract (red arrow). The strong signal may mask the presence of a 35kDa glucanase. (A) 10µg whole tissue protein from MeJa-treated plants; (B) 2µg apoplastic extract from MeJa-treated plants; (C) 10µg whole tissue protein extract from spray control plants. Image is taken from area corresponding to the region between the 35 and 25kDa MW markers. The exposure time for this blot was 7sec.


kDa
A
B
C
116.0
66.2
45.0
35.0
25.0
18.4

but the concentration of PR related proteins is expected to be lower than those produced by CA or hormone treated plants and may be present at concentrations too low to detect.

3.3.2 2D SDS-PAGE gel and immunoblotting results

Unlike the 1D-SDS PAGE gels and blots previously presented, the 2D versions give a fuller picture of the β-1,3-endoglucanases produced by winter rye as proteins were separated both by size and by charge instead of by size alone. For each SDS-PAGE gel, two IEF strips were loaded with 10μg of NA or CA whole leaf protein or 0.2μg of CA apoplastic protein. The extremely small sample concentration for the apoplastic protein is necessary, as the Ruby Red Sypro staining used for 2D gels is far more sensitive than that for 1D gels. Loading excess protein onto 2D gels results in gels with low resolution and high background, and immunoblots with high background and positive signals so strong that weaker positive signals are masked. The potential to mask weak signals by over abundant proteins is also a problem in over-loaded gels. Each replicate required 2 gels of each protein sample. One gel was stained with Sypro Ruby Red while the other was used in immunoblotting analysis. Samples used for 2D analysis included whole tissue protein extracts from NA and CA along with CA and NA apoplastic extracts.

Sypro Ruby Red staining of whole tissue protein extracts (Figure 3-6) resulted in gels with seemingly similar protein staining patterns. It was difficult to see upregulation of proteins after cold acclimation, but down regulation of a number of proteins after cold acclimation was obvious. Although the majority of winter rye proteins under both conditions appear to be acidic, alkaline proteins are not absent from the protein samples. The 55kDa large subunit of rubisco is present on both gels, and is indicated by an arrow. Due to the high concentration of this abundant plant protein, exposure time of the gel to UV light was limited to 1 second so that

the rubisco signal did not overwhelm and hide the signals of nearby proteins. Unfortunately, this short exposure time hid the signal of proteins present at low concentration.

Apoplastic extract from CA and NA winter rye plants were also separated using 2D SDS-PAGE (Figure 3-7). Previous studies of winter rye apoplastic extract (Hon et. al., 1995) revealed the presence of 13 proteins in NA winter rye apoplast and 7 in CA winter rye apoplast.

The proteins expected in the apoplast of NA winter rye are 144, 97, 36, 35, 34, 33, 32, 28, 26, 25, 16 (x 2) and 14kDa in size (Hon *et al.,* 1995). Due to the concentration of the acrylamide gel, and the length of time the gel was electrophoresed, only proteins of 18.4kDa or larger were visible. It is therefore not unexpected that the 2 16 and the 14kDa proteins are not represented on the gel. In addition, the 2D analysis does not confirm the presence of a 144kDa protein but it does reveal 9 other proteins. One protein is of the expected 97kDa size, and 8 proteins are in the 25 to 40kDa in size range.

Previous studies revealed that the CA apoplast contained 7 minor and 6 major proteins (Griffith *et al.,* 1992). 2D analysis of the CA winter rye reveal 25 proteins or protein complexes within the apoplast of winter rye. The β-1,3-endgoglucanases in CA winter rye apoplast are stable between pH 3-7 (Stressmann *et al.,* 2004) and are 32 and 35kDa in size. The chitinases known to be in the apoplast of winter rye are similar in both size and pH stability, and cannot be distinguished from the glucanases on this gel without immunoblot analysis.

Immunoblots of proteins from both treated and untreated leaves confirm the presence of at least 3 β-1,3-endoglucanases (Figure 3-8). These 3 glucanases appear to range in size from 32kDa to 35kDa and all are acidic. The concentration of glucanase is higher in cold-treated plants than in untreated plants.

The number of β-1,3-endoglucanses in the leaf apoplast of NA and CA winter rye plants was established by immunoblot analysis. No glucanases were detected in the apoplast of untreated leaves despite the high sensitivity of the detection method and long exposure time (600x longer than the CA blot) (Figure 3-9). In contrast, strong positive signals for 5 glucanases, as well as another weak positive were seen after exposing the blot to film for only 2 seconds. The strong positive signal makes it difficult to determine the size of each glucanase, but they fall in the range of 32 to 35kDa. Four of the glucanases with the strongest signals as well as the glucanase with the weakest signal have separated into the acidic range of the IPG strip and lie between pH 3.2 and and pH 6. Another unexpected glucanase signal is strongly alkaline with a pH nearing 9.5. All of these proteins are visible on the Sypro Ruby Red stained 2D gel, although the staining of the alkaline glucanase is very weak and appears to be background noise. This suggests that there may be additional proteins in the apoplast that are present at low concentrations in comparison to the major polypeptides that have been detected previously by 1D analysis and less sensitive staining methods. If the exposure time is increased the background of the gel and the bright signal from the major polypeptides within the sample blur the rest of the gel making it impossible to tell if there are additional polypeptides.

Figure 3-6: 2D SDS-PAGE analysis of whole leaf protein extract from NA and CA winter rye plants.

Ten μg of whole leaf protein extract from NA and CA winter rye plants were electrophoresed on separate IEF drystrips (pH 3-10NL) and then separated by SDS-PAGE on a 12% (w/v) gel. Proteins were stained overnight using Sypro Ruby Red visualized by a 1-second exposure to UV light. Both gels were processed in a similar manner. The large 55kDa subunit of Rubisco is indicated with an arrow. Red-dashed circles surround some proteins that are present in lower concentrations after cold acclimation. . .

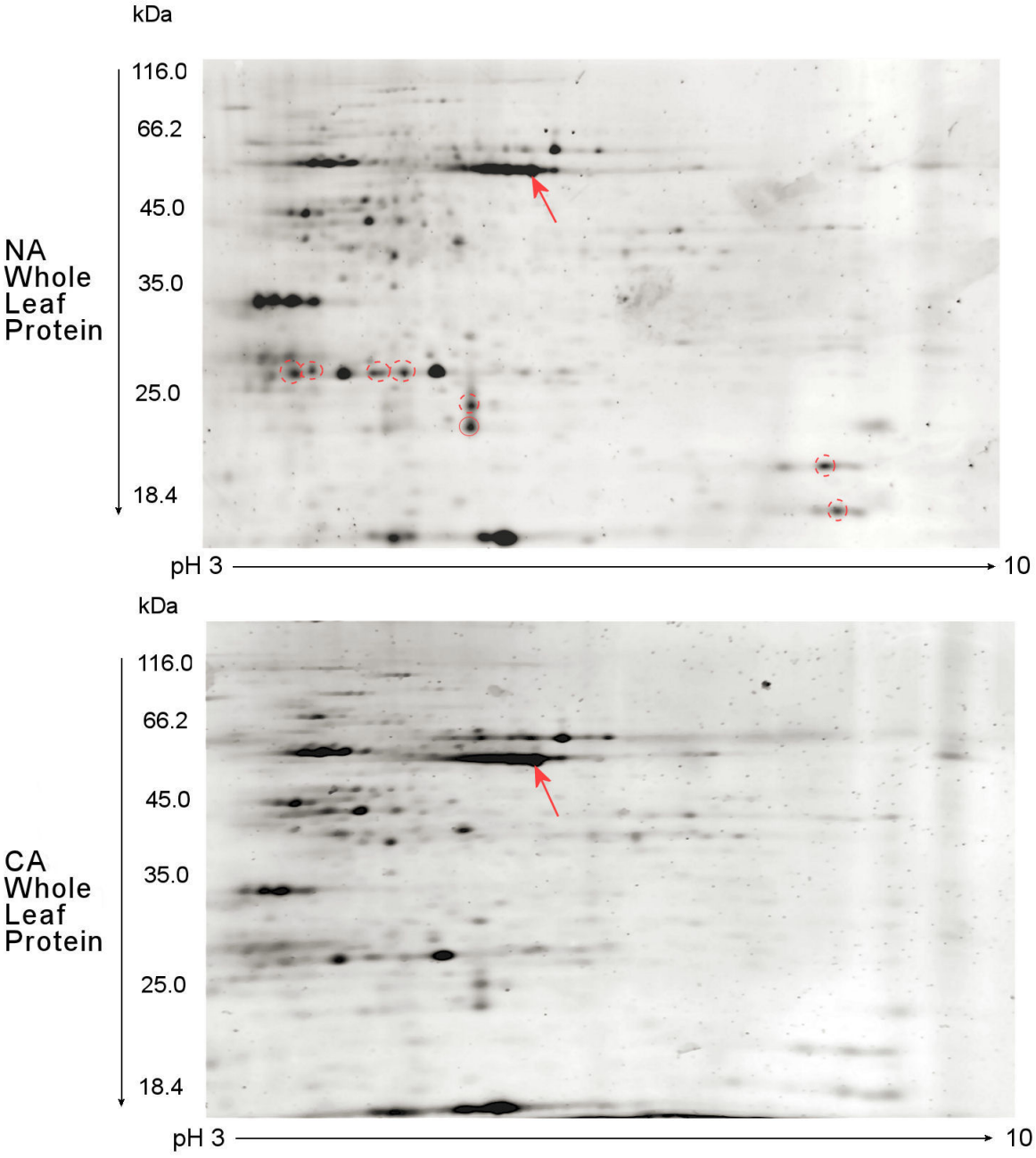

kDa
116.0
66.2
45.0
35.0
25.0
18.4
NA Whole Leaf Protein
pH 3
10
kDa
116.0
66.2
45.0
35.0
25.0
18.4
CA Whole Leaf Protein
pH 3
10

Figure 3-7: 2D SDS-PAGE analysis of apoplastic protein extracts from NA and CA winter rye plants.

The apoplast of winter rye leaves recovered from 2-week-old NA plants and from CA plants grown for 1 week at normal conditions and then transferred to a growth chamber set at 4˚C for an additional 7 weeks. After focusing on IEF pH 3-10NL dry strips, proteins were separated by size by electrophoresis on 12% (w/v) polyacrylamide gels. Proteins were stained overnight using Sypro Ruby Red and visualized by exposure to UV light. The apparent profile between the extracts was remarkably different. All polypeptides found in NA sample were absent from the CA extract. Separated NA apoplastic extract was exposed for 1s while the gel containing CA apoplastc extract was exposed for 2.5 seconds.

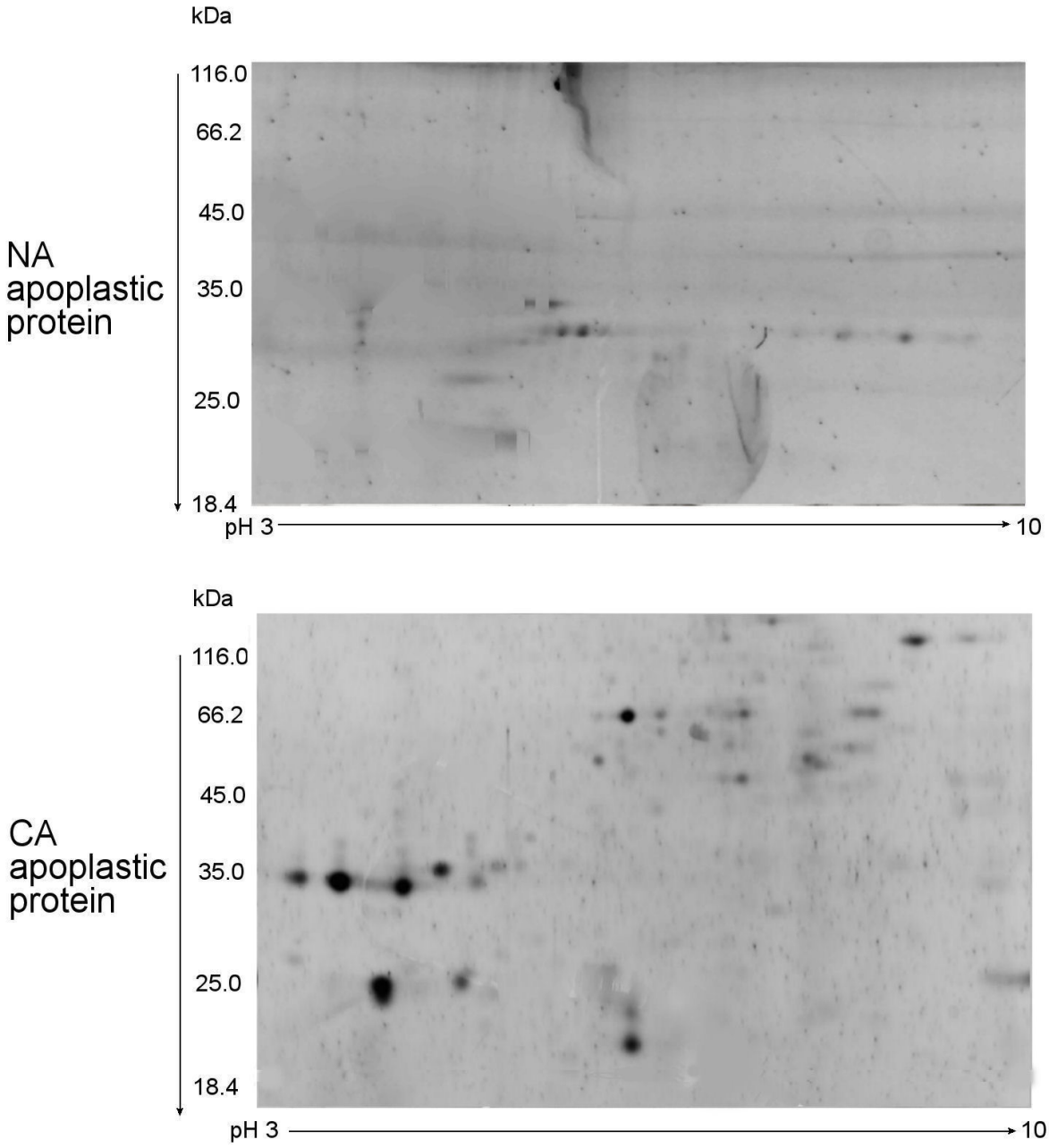
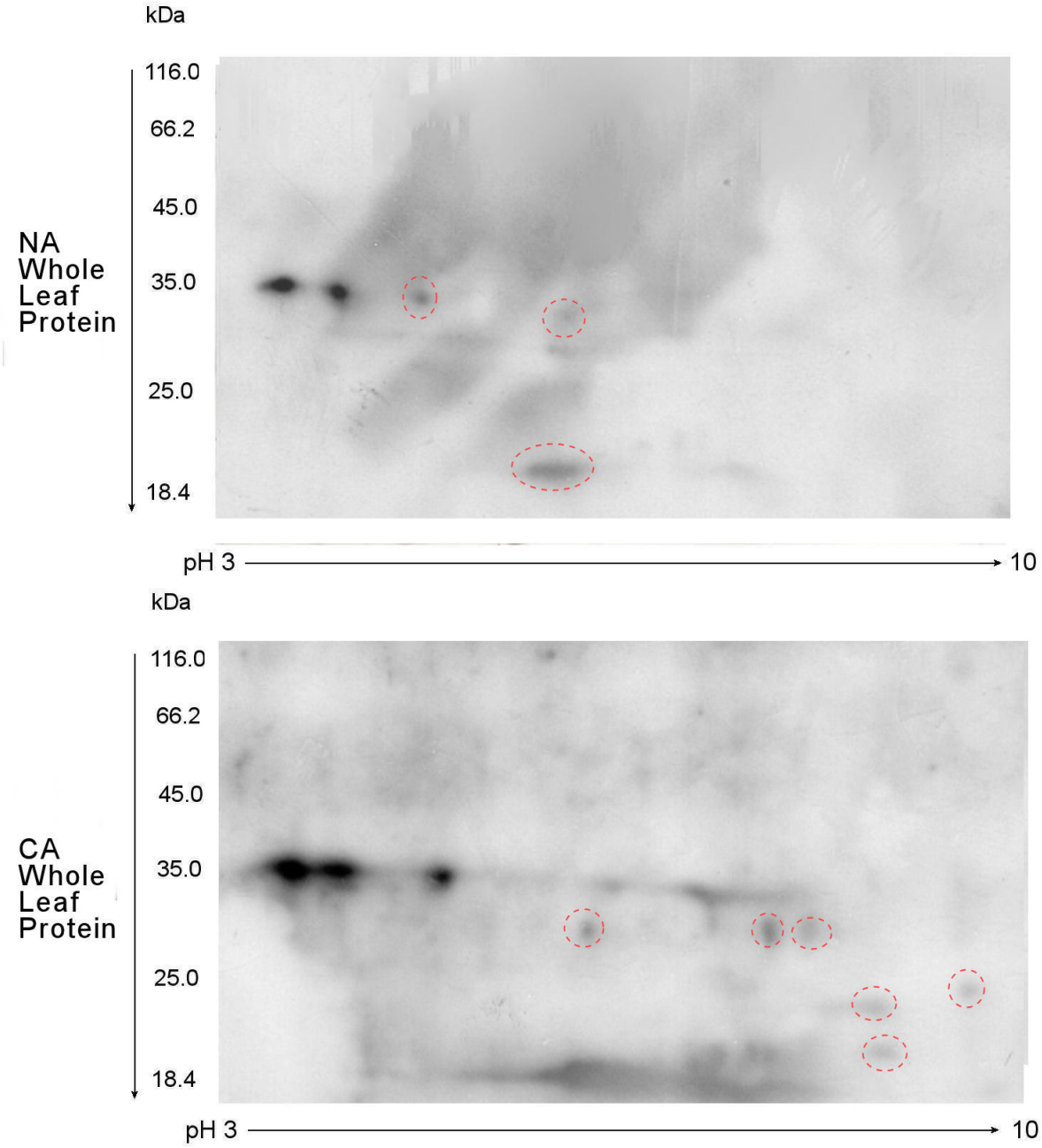

kDa
116.0
66.2
45.0
35.0
25.0
18.4
NA
apoplastic
protein
pH 3
10
kDa
116.0
66.2
45.0
35.0
25.0
18.4
CA
apoplastic
protein
pH 3
10

Figure 3-8: 2D SDS-PAGE of NA and CA whole leaf tissue extract, transferred to a PVDF membrane and immunobloted with antibody against β-1,3-endoglucanses.

Whole tissue protein of winter rye leaves was taken from 2-week-old NA plants and from CA plants grown for 1 week at normal conditions and then transferred to a growth chamber set at 4˚C for an additional 7 weeks. Each extract was dialyzed against 50mM Tris buffer pH 7.0 prior to isoelectric focusing of 10μg of protein on IEF drystrips (pH 3-10NL). After focusing, proteins were separated by size by electrophoresed as before. Protein was transferred to PVDF membranes using a semi-dray transfer and were blotted using an antibody against β-1,3-endoglucanases and the signal was detected using chemiluminescence and X-ray film. The whole leaf extract from NA winter rye appeared to contain 2 glucanases while the CA extract appeared to contain 3. There are a number of weak glucanase signals apparent on the gel which may or may not be glucanses, these are highlighted with a dotted-red circle. The exposure time for each blot was 10 seconds.


kDa
116.0
66.2
45.0
35.0
25.0
18.4
NA
Whole
Leaf
Protein
pH 3
10
kDa
116.0
66.2
45.0
35.0
25.0
18.4
CA
Whole
Leaf
Protein
pH 3
10

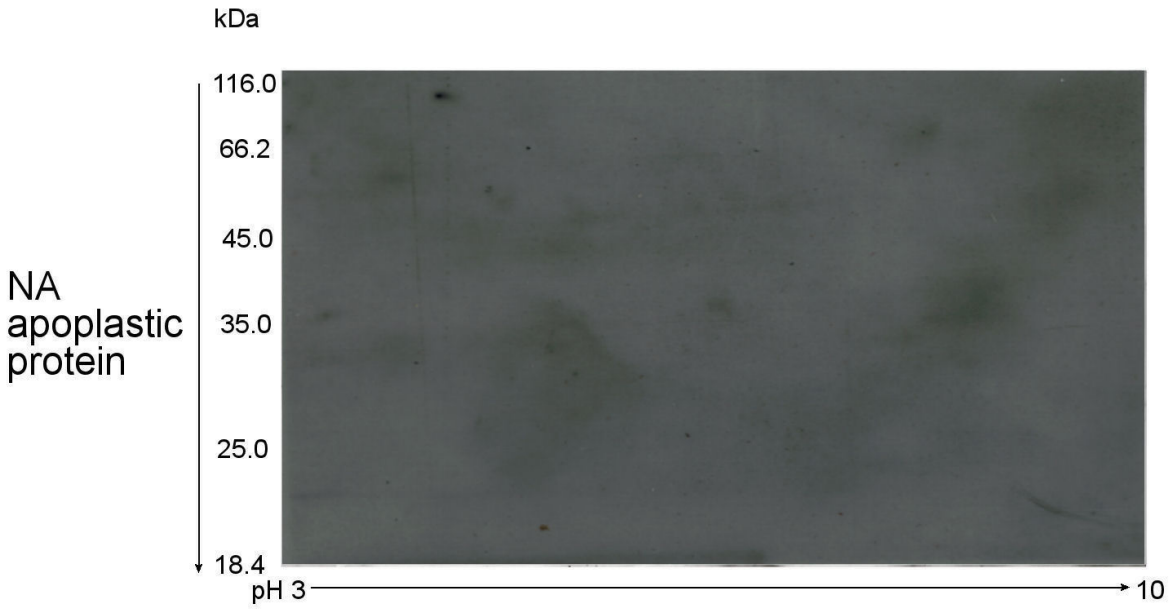
3.4 Isolation and Comparison of Glucanase Genes and Promoters in Winter Rye

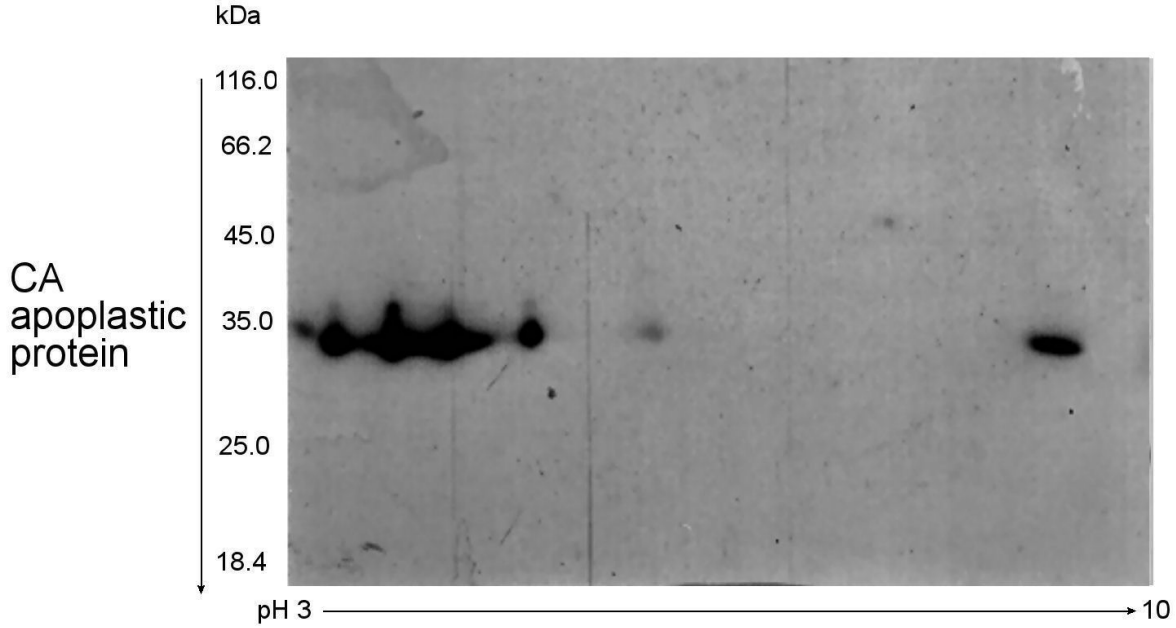
One goal of this project was to isolate and compare the genes that code for glucanases with and without antifreeze activity, this includeed isolating the corresponding promoters of the gene of interest. Promoters are highly variable in length and sequence as the non-regulatory regions are less evolutionarily conserved as compared to coding regions. This is especially true in large gene families such as the glucanases where duplication events would have allowed each new copy to evolve separately from the parent gene, allowing family members to change both in function and regulation. It is therefore impossible to use conventional PCR to isolate gene promoter regions as a gene specific upstream 5′ primer cannot be designed.

In order to obtain promoter regions, a PCR-based technique known as genomic walking was developed. This involves taking four different aliquots of genomic DNA and cutting each aliquot with a different restriction enzyme to create four collections of DNA fragments. The fragments in each aliquot are then ligated to an oligonucleotide adapter of known sequence, against which PCR primers are designed. Each collection of adapter ligated fragments can now be used as a template in a PCR reaction where only one gene specific region, either a 5′ or 3′ sequence is known. For this project, the commercially available Universal Genomic Walking Kit by Clonetech was used. This kit included the rare cutting restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I required to make a total of four unique collection of winter rye DNA fragments, and an oligonucleotide adapter to which a set of nested PCR primers was designed. Adapter-specific and gene-specific nested primers were used in successive PCR reactions eliminating much of the non-specific PCR products commonly produced in traditional genomic walking experiments.

Figure 3-9: 2D SDS-PAGE analysis of apoplastic protein extract from NA and CA winter rye plants.

The apoplastic proteins of winter rye leaves was taken from 2-week-old NA plants and from CA plants grown for 1 week at normal conditions and then transferred to a growth chamber set at 4 ° C for an additional 7 weeks. Each extract was dialyzed against 50mM Tris buffer pH 7.0 prior to isoelectric focusing of 0.2µg of protein on IEF drystrips (pH 3-10NL). After focusing, proteins were separated by size by electrophoresis on acrylamide gels. Protein was transferred to PVDF membranes using a semi-dray transfer and were blotted using an antibody against β-1,3-endoglucanases and the signal was detected using chemiluminescence and x-ray film. The exposure time for the NA apoplastic blot is 10 minutes, the exposure time for the CA apoplastic blot was 2 seconds.


kDa
116.0
66.2
45.0
35.0
25.0
18.4
NA
apoplastic
protein
pH 3
10


kDa
116.0
66.2
45.0
35.0
25.0
18.4
CA
apoplastic
protein
pH 3
10

The initial set of gene-specific nested primers were designed against a glucanase cDNA sequence obtained from a cold-acclimated winter rye cDNA library. This cDNA was designated scGlu9. The binding site of these nested primers, which were designated GSP1 and GSP2 (Table 2-2) were 3′ of the predicted intron splice site as predicted by the online splice program GeneSplicer (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html). PCRs using the *Dra*I library with this primer set 1.5 kb; a major PCR product of 2.4 kb was recovered from the *Stu*I library and two predominant PCR products of approximately 1kb and 800bp were isolated using the *Pvu*II library. The *Eco*RV library produced a small 500bp clone which was considered too small for further analysis.

The gene products of the *Dra*I, *Stu*I and *Pvu*II libraries were cloned and sequenced. Sequence alignment between the sequence obtained from the *Stu*I fragment and the known glucanase cDNA sequence revealed the strong sequence similarity to β-1,3-endoglucanases from barley (92%) and wheat (96%). The sequence revealed the presence of a 421bp intron that separated two exons of 81bp and 900bp. A 1.6kb fragment of the promoter region was also obtained, but could not be used for promoter analysis as it contained many sequencing errors. In order to confirm and obtain a longer fragment of this promoter sequence, another primer set (IntronR1a and IntronR1b) was designed against the intron with the assumption that this region was gene specific. Using the DNA 'libraries' as templates, primary PCRs were carried out using IntronR1a and the supplied primer AP1 . The products of these PCR reaction were then diluted 50-fold and used in a secondary PCR reaction with the nested primers, IntronR1b and AP2. A 1.5kb band was recovered in the *Stu*I library. This was cloned into the pGEM T-Easy vector. After transformation and plasmid isolation the plasmid DNA from positive transformants was sent for sequencing.

Sequence alignment revealed that the IntronR1/R2 primer set amplified 5 different β-1,3-endoglucanases. One of these glucanases was identified as scGlu9, the other 4 sequences (which were subsequently designated scGlu10, scGlu11, scGlu12 and scGlu13) appeared to be different genes with strong sequence nucleotide similarity (96-98%) to each other.

In order to fully understand the differences between each of these glucanases a complete DNA sequence, including promoter and coding regions for each was required. Nested primers were designed from the 3' UTR sequence of each gene. One set of nested primers was designed for reverse walking through the promoter, the other set was needed to walk through the coding and intron regions. Each PCR was performed three times and the largest products from each PCR were sent for sequencing. Products verified as glucanases were used for subsequent sequence analysis.

3.4.1 Sequence Comparison between the Five isolated β-1,3endoglucanases and glucanases from other related plant species

Five full length β-1,3-endoglucanases from winter rye were obtained, including at least 600bp upstream of the transcription start site in each case. These were designated scGlu9 through scGlu13. As 8 full or partial cDNA sequences for winter rye glucanases had been recently published (Yaish *et al.,* 2006) new glucanase sequences were analyzed to ensure they represented unique genes. Sequence analysis determined that 4 of the 5 glucanase sequences were unique while scGlu13 was identical to scGlu6 (accession number AM101310). These sequences were identical save for two fact that the 5'UTR region obtained through genomic walking was truncated and that the second exon of scGlu6 contained two ambiguous nucleotides (Figure 3-10). These two nucleotides are conserved in all of the other glucanases as either a pair of cytosines or a pair of thymines. Based on the fact that there were no additional

differences between scGlu13 and scGlu6, it was concluded that scGlu13 was the same gene as scGlu6 and that the two ambiguous nucleotides were a pair of thymines. scGlu13 was redesignated scGlu6 to reflect these considerations.

The start and stop codons for each glucanase gene were determined by sequence alignments with known glucanase sequences. The start codon (ATG) and the surrounding nucleotides were conserved amongst the glucanases, as was the stop codon (TAA). In addition to the predicted stop codon, which was determined based on known glucanse amino acid sequences there were two subsequent stop codons in close proximity to the noted stop codon. The presence of these additional stop codons suggests a possibility of additional open reading frames.

Figure 3-10: Alignment of the UTR and coding regions of the scGlu6 gene obtained through genomic walking, and the previously published scGlu6 cDNA sequence.

The coding sequence of the scGlu6 gene obtained through genomic walking was compared to the published sequence (Gen Bank accession number AM181310) by aligning the sequences using Clustal X. The 3′ UTR of scGlu6 obtained through genomic walking is 21bp shorter than the cDNA sequence. The coding region of each sequence is identical except that the cDNA sequence contains two ambiguous nucleotides at position 644 and 645 (underlined). These ambiguous nucleotides correspond to two thymines in the genomic walking sequence. Due to the high sequence similarities between the sequences, it was assumed that they represent the same gene. Start (ATG) and stop (TAA) codons are indicated by a box.


Glu-6 emb.AM181310 1 TCGTGCCGAA TTCGGCACGA GGATACATAG GGAGCTAAGC TAGCAGCAAT GGCTAGAAAC CATGTTGCTT 70
scGlu6 1 TCGTGCCGAA TTCGGCACGA GGATACATAG GGAGCTAAGC TAGCAGCAAT GGCTAGAAAC CATGTTGCTT 70
Glu-6 emb.AM181310 71 CCATGTTTGC CGTTGCTCTC TTCATTGGAA CGTTCGCTGC TGTTCCTACC AGTGTGCAAT CCATCCGCGT 140
scGlu6 71 CCATGTTTGC CGTTGCTCTC TTCATTGGAA CGTTCGCTGC TGTTCCTACC AGTGTGCAAT CCATCCGCGT 140
Glu-6 emb.AM181310 141 CTGTTACGGC GTGATCGGCA CCAACCTTCC CTCCCGAACC GACGTGGTGC AGCTTTACAG GTCCAAGGGC 210
scGlu6 141 CTGTTACGGC GTGATCGGCA CCAACCTTCC CTCCCGAACC GACGTGGTGC AGCTTTACAG GTCCAAGGGC 210
Glu-6 emb.AM181310 211 ATCAACGGCA TGCGCATCTA CTTCGCCGAA GGGCAAGCCC TCTCCCGCCT CCGCAACTCC GGCATCAGCC 280
scGlu6 211 ATCAACGGCA TGCGCATCTA CTTCGCCGAA GGGCAAGCCC TCTCCCGCCT CCGCAACTCC GGCATCAGCC 280
Glu-6 emb.AM181310 281 TCATCCTCGA CATCGGTAAC GACCAGCTCG CCAACATCGC CGCCAGCACC TCCAACGCGG CGTCCTGGGT 350
scGlu6 281 TCATCCTCGA CATCGGTAAC GACCAGCTCG CCAACATCGC CGCCAGCACC TCCAACGCGG CGTCCTGGGT 350
Glu-6 emb.AM181310 351 ACAGAACAAT GTGCGTCCCT ACTACCCGGC CGTGAACATC AAGTACATCG CTGCCGGCAA CGAGGTCCTG 420
scGlu6 351 ACAGAACAAT GTGCGTCCCT ACTACCCGGC CGTGAACATC AAGTACATCG CTGCCGGCAA CGAGGTCCTG 420
Glu-6 emb.AM181310 421 GGAGGCGCCA CGCAGAGCAT CGTCCCTGCC ATGCGGAACC TCAACGCGGC CCTCTCCGCC GCCGGCCTCG 490
scGlu6 421 GGAGGCGCCA CGCAGAGCAT CGTCCCTGCC ATGCGGAACC TCAACGCGGC CCTCTCCGCC GCCGGCCTCG 490
Glu-6 emb.AM181310 491 GCGCCATCAA GGTGTCCACC TCGATCCGGT TCGACGCGGT GGCCAACTCA TTCCCACCCT CCGCCGGCGT 560
scGlu6 491 GCGCCATCAA GGTGTCCACC TCGATCCGGT TCGACGCGGT GGCCAACTCA TTCCCACCCT CCGCCGGCGT 560
Glu-6 emb.AM181310 561 GTTCGCGCAG TCGTACATGA CGGACGTGGC CCGGCTCCTG GCGAGCACCG GCGCGCCCCT GCTGGCCAAC 630
scGlu6 561 GTTCGCGCAG TCGTACATGA CGGACGTGGC CCGGCTCCTG GCGAGCACCG GCGCGCCCCT GCTGGCCAAC 630
Glu-6 emb.AM181310 631 GTGTACCCCT ACTTNNCGTA CCGCGACAAC CCGCGCGACA TCAGCCTCAA CTACGCGACG TTCCAGCCAG 700
scGlu6 631 GTGTACCCCT ACTTTTCGTA CCGCGACAAC CCGCGCGACA TCAGCCTCAA CTACGCGACG TTCCAGCCAG 700
Glu-6 emb.AM181310 701 GCACCACTGT GCGTGACCAG AACAACGGGC TGACCTACAC TTGCCTCTTC GACGCCATGG TGGACGCCGT 770
scGlu6 701 GCACCACTGT GCGTGACCAG AACAACGGGC TGACCTACAC TTGCCTCTTC GACGCCATGG TGGACGCCGT 770
Glu-6 emb.AM181310 771 GTATGCGGCA CTGGAGAAGG CCGGCGCGCC GGGCGTGAAG GTGGTGATTT CCGAGAGCGG GTGGCCGTCG 840
scGlu6 771 GTATGCGGCA CTGGAGAAGG CCGGCGCGCC GGGCGTGAAG GTGGTGATTT CCGAGAGCGG GTGGCCGTCG 840
Glu-6 emb.AM181310 841 GCGGGCGGGT TTGCGGCGTC GCCGGACAAC GCGCGGACGT ACAACCAGGG GCTGATCAAC CACGTCGGCG 910
scGlu6 841 GCGGGCGGGT TTGCGGCGTC GCCGGACAAC GCGCGGACGT ACAACCAGGG GCTGATCAAC CACGTCGGCG 910
Glu-6 emb.AM181310 911 GGGGCACGCC CAAGAAGCGG GAGGCGCTGG AGACGTACAT CTTCGCCATG TTCAACGAGA ACCAGAAGAC 980
scGlu6 911 GGGGCACGCC CAAGAAGCGG GAGGCGCTGG AGACGTACAT CTTCGCCATG TTCAACGAGA ACCAGAAGAC 980
Glu-6 emb.AM181310 981 CGGGGATCCG ACGGAGAGGA GCTTCGGGCT GTTCAACCCG GACAAGTCGC CGGCCTACGC CATCCAGTTC 1050
scGlu6 981 CGGGGATCCG ACGGAGAGGA GCTTCGGGCT GTTCAACCCG GACAAGTCGC CGGCCTACGC CATCCAGTTC 1050
Glu-6 emb.AM181310 1051 TAACTGGCTA CCTACATCTT CTTCATCCTA AATAAATAAG CTGCATGTAA TGCGGCATCC AAGTGTAATA 1120
scGlu6 1051 TAACTGGCTA CCTACATCTT CTTCATCCTA AATAAATAAG CTGCATGTAA TGCGGCATCC AAGTGTAATA 1120
Glu-6 emb.AM181310 1121 TGTAGATTCA TCAAGGGAAG AGTGCAACCA TGCATGCGTT AACTTCCTGG CGATG 1175
scGlu6 1121 TGTAGATTCA TCAAGGGAAG AGTGCAACCA TGCA 1154

The coding sequence of each of the 5 glucanase genes is 1041bp long. The coding sequence is composed of 2; exons, the first exon codes for a signal peptide and is 81bp in length and the second exon, which codes for the functional enzyme is 960bp. The exons are separated by an intron that varies in size among the glucanase genes. The intron of scGlu6 (Figure 3-11) is 598bp long, scGlu9 has an intron of 421bp (Figure 3-12), the intron of scGlu10 is 162bp in length (Figure 3-13), scGlu11 has an intron of 592bp (Figure 3-14) and scGlu12 has an intron of 550bp (Figure 3-15). The first 40bp of each intron share 96-99% nucleotide sequence identity. There is no apparent sequence conservation amongst the remaining intron sequences. The region upstream of the ATG codon of each gene also shares high sequence similarity (96-98%) for the first 40bp, after which this region becomes highly variable between glucanases. The received upstream regions, including the UTR and promoter of each gene were 659bp, 890bp, 941bp, 1238bp and 603bp for scGlu6, scGlu9, scGlu10, scGlu11 and scGlu12, respectively. A longer upstream region (1.2kb) for scGlu9 was sequenced, but Blast analysis established that the sequence beginning 659bp upstream of the start of translation 87% identical to the barley gene hv1LRR2 (GenBank accession number AF108010). This indicates that this sequence belongs to an adjacent gene and is not part of the promoter of scGlu9.

The core promoter contains all of the cis-acting regulatory elements required to initiate transcription. The TATA region is generally within 50bp of the start site of transcription (Smale and Kadonaga, 2003) and was predicted by searches on the PLACE database. The TATA box of each glucanase is identical, or nearly identical, to one of the known TATA box signals for rice, CTATAAATAC.

Figure 3-11: The full length genomic sequence of scGlu6

The full-length sequence of scGlu6 was obtained through genomic walking. The coding sequence of the gene is 1041bp in length; this includes the 81bp exon 1, which codes for a signal peptide that is presumably cleaved from the protein after transport. The longer exon 2 is 960bp in length and codes for the functional enzyme. The exons are separated by a 598bp intron. The region upstream of the transcription start site, including the UTR and promoter is 659bp in length. Boxes represent the start (ATG) and stop (TAA) codons. The intron is the region located between the arrows. The TATA box within the proximal promoter is underlined.


1 CTTGACGTTT CTGTCGTGCT CTTGTACAAT TGGCTAATCT ACTTAGGATG CACCGCTGCG CTTCAGACTT AGCTGAAATA TATATAGCAA GTAACAAGAA GTAGCTCAAG AAATCGTTTC 120
121 CGAATGCTGG CTATATATAG AGAGGATCAC AGAAAGCCGG TGGATGGTAC CGTGGCATGG ATGTTTGGCC CGGGACCAAC CAGCATGGAT ACGGTCTTCA CGGAACGTCG CGTAGTTGAG 240
241 GCTGATGTCG CGCGGGTTGT CGCGGTACGC TAAATTAAGG GGTACACGTT TGGCCATCAA GGGGCGCGCC CGTTGCTTCG CCAGGAAACC GGGCCCACGT TCCGTAATGT ACCAATTGCC 360
361 GCGAATTCAC TAGTGATTGT GGTGAGCATG GCGCGGTTTG TGTATTTTAT TACGGATCCC CTGTCAAGTT TTCTACGGCC GACCATCAGC ACTCCCCCTG TATGTAGAAG AATCCTCGGG 480
481 TAGCACTAAC TCCCTATGCA ATTAATTAAT TAATTAAGTT TTTATATGTT ACAAATTAAA ACCAATGTTG GGCCTTGCTG GAGGATTTTT ATTCAAAGGA GAACCGTCAA GGCTATAATA 600
601 ATCCCGTCGT GCCGAATTCG GCACGAGGTA CATAGGGGAG CTAAGCTAGC AGCAATGGCT AGAAACCATG TTGCTTCCAT GTTTGCCGTT GCTCTCTTCA TTGGAACGTT CGCTGCTGTT 720
721 CCTACCATTG ACGTTTCTGT CGTGCTCTTG TACAATTGGC TAATCTACTT AGGATGCACC GCTGCGCTTC AGACTTAGCT GAAATATATA TAGCAAGTAA CAAGAAGTAG CTCAAGAAAT 840
841 CGTTTCCGAA TGCTGGCTAT ATATAGAGAG GATCACAGAA AGCCGGTGGA TGGTACCGTG GCATGGATGT TTGGCCCGGG ACCAACCAGC ATGGATACGG TCTTCACGGA ACGTCGCGTA 960
961 GTTGAGGCTG ATGTCGCGCG GGTTGTCGCG GTACGCTAAA TTAAGGGGTA CACGTTTGGC CATCAAGGGG CGCGCCCGTT GCTTCGCCAG GAAACCGGGC CCACGTTCCG TAATGTACCA 1080
1081 ATTGCCGCGA ATTCACTAGT GATTGTGGTG AGCATGGCGC GGTTTGTGTA TTTTATTACG GATCCCCTGT CAAGTTTTCT ACGGCCGACC ATCAGCACTC CCCCTGTATG TAGAAGAATC 1200
1201 CTCGGGTAGC ACTAACTCCC TATGCAATTA ATTAATTAAT TAAGTTTTTA TATGTTACAA ATTAAAACCA ATGTTGGGCC TTGCTGGAGG ATTTTTATTC AAAGGAGAAC CGTCAAGGGG 1320
1321 TCCCCATACA CCAAACCGCG CCGFGTGTGC AATCCATCCG CGTCTGTTAC GGCGTGATCG GCACCAACCT TCCCTCCCGA ACCGACGTGG TGCAGCTTTA CAGGTCCAAG GGCATCAACG 1440
1441 GCATGCGCAT CTACTTCGCC GAAGGGCAAG CCCTCTCCCG CCTCCGCAAC TCCGGCATCA GCCTCATCCT CGACATCGGT AACGACCAGC TCGCCAACAT CGCCGCCAGC ACCTCCAACG 1560
1561 CGGCGTCCTG GGTACAGAAC AATGTGCGTC CCTACTACCC GGCCGTGAAC ATCAAGTACA TCGCTGCCGG CAACGAGGTC CTGGGAGGCG CCACGCAGAG CATCGTCCCT GCCATGCGGA 1680
1681 ACCTCAACGC GGCCCTCTCC GCCGCCGGCC TCGGCGCCAT CAAGGTGTCC ACCTCGATCC GGTTCGACGC GGTGGCCAAC TCATTCCCAC CCTCCGCCGG CGTGTTCGCG CAGTCGTACA 1800
1801 TGACGGACGT GGCCCGGCTC CTGGCGAGCA CCGGCGCGCC CCTGCTGGCC AACGTGTACC CCTACTTTTC GTACCGCGAC AACCCGCGCG ACATCAGCCT CAACTACGCG ACGTTCCAGC 1920
1921 CAGGCACCAC TGTGCGTGAC CAGAACAACG GGCTGACCTA CACTTGCCTC TTCGACGCCA TGGTGGACGC CGTGTATGCG GCACTGGAGA AGGCCGGCGC GCCGGGCGTG AAGGTGGTGA 2040
2041 TTTCCGAGAG CGGGTGGCCG TCGGCGGGCG GGTTTGCGGC GTCGCCGGAC AACGCGCGGA CGTACAACCA GGGGCTGATC AACCACGTCG GCGGGGGCAC GCCCAAGAAG CGGGAGGCGC 2160
2161 TGGAGACGTA CATCTTCGCC ATGTTCAACG AGAACCAGAA GACCGGGGAT CCGACGGAGA GGAGCTTCGG GCTGTTCAAC CCGGACAAGT CGCCGGCCTA CGCCATCCAG TTCTAACTGG 2280
2281 CTACCTACAT CTTCTTCATC CTAAATAAAT AAGCTGCATG TAATGCGGCA TCCAAGTGTA ATATGTAGAT TCATCAAGGG AAGAGTGCAA CCATGCA 2377

Figure 3-12:The full length genomic sequence of scGlu9

The full-length sequence of scGlu9 was obtained through genomic walking. The coding sequence of the gene is 1041bp in length; this includes the 81bp exon 1, which codes for a signal peptide that is cleaved from the protein after transport. The longer exon 2 is 960bp in length and codes for the functional enzyme. The exons are separated by a 421bp intron. The region upstream of the transcription start site, including the UTR and promoter is 890bp in length. Boxes represent the start (ATG) and stop (TAA) codons. The intron is the region located between the arrows. The TATA box within the proximal promoter is underlined.


1 TAGATTTATT TATTTTCGAG TTTTAAAACT TTTATTTTTA TTTTTTGAGG ACGGTTAGAC TTATTGGCTG TGTGCATCGA GCTATGCAGA GGCCGAGCAT TCTTTTGATG TGTTTGTATC 120
121 AACTCGATAT ATCAATTGAA TGGAATGTCC TTTACCGAAA AAAGCTAGTT AGGTTGGTCG ACCGTCTTTT ATAGGCGGAA GGGTCACCAC CAACCAAGGA CATACATGCA CTTACTTGAG 240
241 TCGATTGAGC ATTGCATCAT CATATATATT GATATGAGTG GCGGCACAAC GCAATGCAAC ATCATCGATC ATGCTGACCT ATTCACTCAT CCCCATCATC ATGCGCAAAG TTGCAAACCC 360
361 TCTAGTGTTA GTATTGCACG AGAAGTTAGT TGATGACCAT ACATGGGATG CATGGACTAG GACGCAACGA GGAAGTAGGT GTTACACATC CGGCTACATC ATTTTCTTAC CTGCTTCGAA 480
481 TCGACCGGGA GACAAAGACA CACCGCTCTC TGACGAGGAT TCTGTGCTGA CGGACCAGGC TTGACGGGGT GTCCCGTGCC TTGAATGGAT GAAGACATCT ATAGTGGTAG CACGTACTAG 600
601 CTGCTTGATC CATGCTCATT AAATGGTAGC ATGTGCAATT GGGCTCAGCA AACAAAGTCT ATTGATATTT GCCTCACCAA GCATCCATAC CAGCTTCCTG TGAACACCTC GAGCTGGGCA 720
721 GCAATATGCA ATTGGGCAAT AAGTGTAGCT GTTGCTGTAT GTGTTGATCC AAACAATTCT AGTCCAAGAT CTGAGTGAGT CGCCACCTAT AATAATCTAT AGTAATCGAA TTCCCGCGGC 840
841 CCTCGTGCCG AATTCGGCAC GAGGATACAT AGGGAGCTAA GCTAGCAGCA ATGGCTAGAA ACCATGTTGC TTCCATGTTT GCCGTTGCTC TCTTCATTGG AACGTTCGCT GCTGTTCCTA 960
961 CCATTGACGT TCTTCGTGCT CTCTGTACAA TATAGGCTAA TAGCTACTTA CGCTGGGGAG GGGAAGGGGA GGGGCTCTGG GGAAGAATGT TTGGGGGCAA AGAGGGGAAA ACAATGGAGT 1080
1081 TACCAAAGCA GCCCTTTTCC GTTCATGTGG CAGGGGTAAA ATAGGAAATA TCGCAGGGCT AAACTTTCGG GCGCGACATT AACAAATTCC AATCTACCAC ATAACCACGG GCACGGCTTT 1200
1201 CGAAAGATCA TAACCCTGCA GGGGTGTCCC AACTTAGCCC ATCACATGCT CTCATGATCA GCAGAGGTAA AAATAATAAT ACCCCTTATC TTAGGGACAA TCCGATCAGA CTCGGAATCC 1320
1321 CGGTGCACAA GACATTTTGA CAATGGTAAA ACAAGTCCAG CAAGACCTCC CGGCGTGCTG ACACCCTGAT AATAGCCACG CGTATCTCGT CTCAGGCGAT GGGCGATCCT ACGGGAGCCA 1440
1441 GATACCTTGA GTTTCCCCGT GACGGCCCCG CATAACCCCC GTTGTGTGCA ATCCATCGGC GTCTGTTACG GCGTGATCGG CAACAACCTC CCCTCCCGGA GCGACGTGGT GCAGCTTTAC 1560
1561 AGGTCCAAGG GCATCAACGG CATGCGCATC TACTTCGCCG AAGGGCAAGC CCTCTCCCCG CTCCGCAACT CCGGCATCAG CCTCATCCTC GACATCGGTA ACGACCAGCT CGCCAACATC 1680
1681 GCCGCCAGCA CCTCCAACGC GGCGTCCTGG GTACAGAACA ATGTGCGTCC CTACTACCCG GCCGTGAACA TCAAGTACAT CGTGCCGGGC AACGAGGTCC TGGGAGGCGC CACGCAGAGC 1800
1801 ATCGTCCCTG CCATGCGGAA CCTCAACGCG GCCCTCTCCG CCGCCGGCCT CGGCGCCATC AAGGTGTCCA CCTCGATCCG GTTCGACGCG GTGGCCAACT CATTCCCACC CTCCGCCGGC 1920
1921 GTGTTCGCGC AGTCGTACAT GACGGACGTG GCCCGGCTCC TGGCGAGCAC CGGCGCGCCC CTGCTGGCCA ACGTGTACCC CTACTTTTCG TACCGCGACA ACCCGCGCGA CATCAGCCTC 2040
2041 AACTACGCGA CGTTCCAGCC AGGCACCACT GTGCGTGACC AGAACAACGG GCTGACCTAC ACTTGCCTCT TCGACGCCAT GGTGGACGCC GTGTATGCGG CACTGGAGAA GGCCGGCGCG 2160
2161 CCGGGCGTGA AGGTGGTGAT TTCCGAGAGC GGGTGGCCGT CGGCGGGCGG GTTTGCGGCG TCGCCGGACA ACGCGCGGAC GTACAACCAG GGGCTGATCA ACCACGTCGG CGGGGGCACG 2280
2281 CCCAAGAAGC GGGAGGCGCT GGAGACGTAC ATCTTCGCCA TGTTCAACGA GAACCAGAAG ACCGGGGATC CGACGGAGAG GAGCTTCGGG CTGTTCAACC CGGACAAGTC GCCGGCCTAC 2400
2401 GCCATCCAGT TCTAACTGGC TACCTACATC TTCTTCATCC TAAATAAATA AGCTGCACGT AATGCGGCAT CCAAGTGTAA TATGTAGATA CATCAAGGGA AGAGTGCAAC CATGCAGC 2518

Figure 3-13: The full length genomic sequence of scGlu10.

The full-length sequence of scGlu10 was obtained through genomic walking. The coding sequence of the gene is 1041bp in length; this includes the 81bp exon 1, which codes for a signal peptide that is cleaved from the protein after transport. The longer exon 2 is 960bp in length and codes for the functional enzyme. The exons are separated by a 162bp intron. The region upstream of the transcription start site, including the UTR and promoter is 941bp in length. Boxes represent the start (ATG) and stop (TAA) codons. The intron is the region located between the arrows. The TATA box within the proximal promoter is underlined.


1 CGCCAGGGAG CAAGAGATCT CCCTCTAGGA GGAACAACTT GAGGCCGCTC TTCGCACCAA GGATGAAAGT CTGGAAGCTC TTGTGCAACA GCGCACCAAG GAGTTGGAGG ACAAGCATAG 120
121 AGCTGCACTG GATGCTTGTG CCGATGGCTA TGCTGCTCAG CTGAAGAAAC TTACTAACGA TCAGGTTGTC GCATCTGCCG TCAAGGATGG GCTTGATAAG CTGGTGGCGA AGTTGGGTGA 240
241 GGATCTCACA TGCAGCACCA AGGAGGCGGA GGTTCTCAAA GAGAAAACTC AGCAGGTCGA GACCCTCTTG GCTGATGTAC AATTGCATCT CTCCTCCAAG CCTCAGAGCC TTGAAACTGC 360
361 CAATGTCAGC ATCTCAGACT TGAAGGCAAG GATCAACATG CTGGAGCGTT CAACGGAATC ACTTGAGTCC TGTGAGCAGT TGTTGTCCAA GGATCTGGAG GCCTCCCAAC GTCACCGGCA 480
481 AGATGTAGAG GACAGGCTGA AGAATCATGT TGACATGGTC AAACTATGGA CCAAGAGCCT GATCGACATT GCCAAGTGCC TTGGTGACCA GGCTAAGGTG ATGGGCATGG ATGGCCCGGT 600
601 CTTCTCGATC GGCAGGCACG AGGTCCCCAG CGCCAAGCTA GGACTCTTCT TCAATGAGCT GATCGAGAAG CTCGAGGCGC ATGAAGAGGG GAGGGCGGGA CACTTTGCAC CGAGTCTCGG 720
721 AGGCTTGCCT AAGACGCCCT CTTCATGGTC CTGAGCAATT TCACTTGCCG CCATCGGGAC CTCGATCTCG ACGATGGATT CAGGGCGCCG CCAGCGGGTG CTGACGTTGT GGCTGCCAAG 840
841 GAGAAAGTCG TGCCCCGAGC CGACATGGTC CTTCAAGTTT GAACCGCTCC GCCGAGCCGC CAGTCTAATT ATCGGTCCAC TGCTGCCCTG TAATGAATCA GAACATATAT TTTGCATTTA 960
961 GTTACTCTAT CTCTCATATT TGCGTGAAAC TGATATCTCT AGTCGGATTC TAGATGCGTG TTTATCCCTT TGAGCTCTAT ATGTTTGCAC TATATAATCC GAAACCTATG TATGTTTTGG 1080
1081 ATGCATCAGC AGCAGATACA TAGGGAGCTA AGCTAGCAGC AATGGCTAGA AACGATGTTG CTTCCATGTT TGCCGTTGCT CTCTTCATTG GAGCGTTCGC TGCTGTTCCT ACGATCGACC 1200
1201 ATAACCTGAA TTTTACCCAT AAGAACATTG TTTATTTATT ATTAAATAAA ATGGTTCATA ATAGTGTGCC ATGTATAATT TTACAATAGT GCCGTGCTAT TTTTCTTTTT TTAATCATCT 1320
1321 CAAGACAAAT GTATCACAGG CAAACACGCA TTTCAGTGTG CAATCCATCG GCGTCTGTTA CGGCGTGATC GGCAACAACC TCCCCTCCCG GAGCGACGTG GTGCAGCTTT ACAGGTCCAA 1440
1441 GGGCATCAAC GGCATGCGCA TCTACTTCGC CGACGGGCAA GCCCTCTCCG CGCTCCGCAA CTCCGGCATC AGCCTCATCC TCGACATCGG TAACGACCAG CTCGCCAACA TCGCCGCCAG 1560
1561 CACCTCCAAC GCGGCGTCCT GGGTACAGAA CAATGTGCGT CCCTACTACC CGGCCGTGAA CATCAAGTAC ATCGCTGCCG GCAACGAGGT CCTGGGAGGC GCCACGCAGA GCATCGTCCC 1680
1681 TGCCATGCGG AACCTCAACG CGGCCCTCTC CGCCGCCGGC CTCGGCGCCA TCAAGGTGTC CACCTCGATC CGGTTCGACG CGGTGGCCAA CTCATTCCCA CCCTCCGCCG GCGTGTTCGC 1800
1801 GCAGTCGTAC ATGACGGACG TGGCCCGGCT CCTGGCGAGC ACCGGCGCGC CCCTGCTGGC CAACGTGTAC CCCTACTTCG CGTACCGCGA CAACCCGCGC GACATCAGCC TCAACTACGC 1920
1921 GACGTTCCAG CCAGGCACCA CTGTGCGTGA CCAGAACAAC GGGCTGACCT ACACTTGCCT CTTCGACGCC ATGGTGGACG CCGTGTATGC GGCACTGGAG AAGGCCGGTG CGCCGGGCGT 2040
2041 GAAGGTGGTG ATTTCCGAGA GCGGGTGGCC GTCGGCGGGC GGGTTTGCGG CGTCGCCGGA CAACGCGCGG ACGTACAACC AGGGGCTGAT CAACCACGTC GGCGGGGGCA CGCCCAAGAA 2160
2161 GCGGGAGGCG CTGGAGACGT ACAACTTCGC CATGTTCAAC GAGAACCAGA AGACCGGGGA TCCGACAGAG GGGAGCTTCG GGCTGTTCAA CCCGGACAAG TCGCCGGCCT ACGCCATCCA 2280
2281 GTTCTAACTG GCTACCTACA TCTTCTTCAT CCTAAATAAA TAAGCTGCAT GTAATGCGGC ATCCGAGTGT AATATGTAGA TAT 2363

Figure 3-14: The full length genomic sequence of scGlu11.

The full-length sequence of scGlu11 was obtained through genomic walking. The coding sequence of the gene is 1041bp in length; this includes the 81bp exon 1, which codes for a signal peptide that is cleaved from the protein after transport. The longer exon 2 is 960bp in length and codes for the functional glucanase. The exons are separated by a 592bp intron. The region upstream of the transcription start site, including the UTR and promoter is 1238bp in length. Boxes represent the start (ATG) and stop (TAA) codons. The intron is the region located between the arrows. The TATA box within the proximal promoter is underlined.


1 AAACTAGCAT GAATTGAATA AAAGTATGTT GGGACAGTCA ATATATTTTA TTCTGCCATG CATGCACATC TCTGGACCTA CCGGTACCTT TTAGCAGGGA ATACAAAATA TGGAATTCTA 120
121 TCATACCTGG TTATACATAT GGATGGGCCC TATTTATGCA CAGATCATAA AGCACGTGAA GGGTGGATGC TACGCCGGTA GCTCCCGGCT CCGGTAAAAA GGAGTTTGCG CTAAACTGGG 240
241 CCGTTCGGAT AACCAAGGTC CACCAACCCC ACCGGATATT CCTCAAAAAA AATTTTTAAC CAACCCCACC CGACGCAACC GCACCACTCT GCCCCCTCCC CCTCTGCTCT CGTCCCGGCG 360
361 AAACATGCTT CGCGGCGGCG TGGCCGGCGC TTGAATCGCT GGTGACGGAG CGATGGAAGA AGCCGGGCTC GAGTTCTTAC TTGATGCCGG TGACCGGTAA GTGGATCTCC ACACCTCGGA 480
481 AATTTCCATT TTTCATGAAT CTGAGGTCAT GGCCGGGATG AGTGAGCTAA TTTGATGAGT GGCCTGCCAC CCATGGCGGA ATATGATATG TAGGTTTCTC CTAAGGGAAG AAAGCGAGAT 600
601 CTTGGAAGGC AAGATGAACG TAATGATGCT TCTGGGTGCG GGCAGCATAT TGGAGGAGAT ACAGCTGCTG AGCCAACTGC AGCGAACTAC GGCGGCGAGA AATATCGCCC ACTACATCAG 720
721 CGCAATCTAC TGACAGGGTT AACCCAGAGG CACTTGGGTG GACAAAAAGG GTCGTATGTT TCCTTAATCA TGTCAATATA CATATTTTCT GATGCAAGTA GGAGTTTTGC AGTCTGCACG 840
841 CTGGACCAAA GACATACTGC AAACCTGAAT GCTGTTGCTG TGATCATTTG CTGAAAGATT AAAAAAAAAT GCCGTCTTTG AAATTTCTGG CACTTATGAC ATTTATTATA TAATACATGT 960
961 CTAGGAAATA GGATGGCAAT ATATTCGTAA CTTACTGAAA GTGAAATGTT TCCTTATCAT GACAATAACA TATTTTCTGA ATGCAAGTAG GTAGTTTTGC AGTTGACACG CCTAGACGAA 1080
1081 AAGCACATAG CTGCAAAAAC TTTTGATGTT GTCGAGAAGA ACCATAGGCA GGGTGGTCAC AATAAACCCT GAAATGTTTG CATTAATTTA TAATAAATCG ACTTGCTATA ATAATCGGGG 1200
1201 GCACGAGAGC AGATACATAG GGAGCTAAGC TAGCAGCAAT GGCTAGAAAC GATGTTGCTT CCATGTTTGC CGTTGCTCTC TTCATTGGAG CGTTCGCTGC TGTTCCTACG ACTACATGCA 1320
1321 TGCGGATAAG AGTGGCATCC GGCCGTTTAG CTGTCGTAGG CGTTGACGTG AATGACTATG CTGGCGTGTC CTTTACTATT AATTAATATG TTTGATTTTG TATTACTAAA TGTCATACGA 1440
1441 ATTATTCAAC AAGAATTTCT TTGCACTGCA TTTTGAAAAT AATTGTATCC ACTCAAATTT CTTAAAAATA ACTCTTCCTT GCTTTCGGTG GCGCAACCAA AAATATTACG TACATATTTT 1560
1561 ACATTATTCT ATTTTTTAAG ACAAAAATAT TTAATTTATT CGAATAGGCA TGACACTCCA ATCCTTCGTT TTTCTGTTTC TGCCATCTTT AGCATCCTTA CGAACAAAAG AGACAATAAA 1680
1681 TTTTTCTTCG AAATTGCCCA CCAAATTAAT TTTGCATAAT AGTACTAGCA CATTACAAAA TTCCATCAAC AAAATAGATA AATTATCCAG TGGTCCATTA AAAAACAGCA TGATCGAACT 1800
1801 CCATGCATGA ATTTGCTTTG CCCCGGTAAC CATGCCGACA GTAGGTTAGG TGCATGCATG CTAAGTAGAT AGAAAATAGT ACAAGAGCAC GAGAGAAACG TCAACCACGT GTGTGCAATC 1920
1921 CATCGGCGTC TGTTACGGCG TGATCGGCAA CAACCTCCCC TCCCGGAGCG ACGTGGTGCA GCTTTACAGG TCCAAGGGCA TCAACGGCAT GCGCATCTAC TTCGCCGACG GGCAAGCCCT 2040
2041 CTCCGCGCTC CGCAACTCCG GCATCAGCCT CATCCTCGAC ATCGGTAACG ACCAGCTCGC CAACATCGCC GCCAGCACCT CCAACGCGGC GTCCTGGGTA CAGAACAATG TGCGTCCCTA 2160
2161 CTACCCGGCC GTGAACATCA AGTACATCGC TGCCGGCAAC GAGGTCCTGG GAGGCGCCAC GCAGAGCATC GTCCCTGCCA TGCGGAACCT CAACGCGGCC CTCTCCGCCG CCGGCCTCGG 2280
2281 CGCCATCAAG GTGTCCACCT CGATCCGGTT CGACGCGGTG GCCAACTCAT TCCCACCCTC CGCCGGCGTG TTCGCGCAGT CGTACATGAC GGACGTGGCC CGGCTCCTGG CGAGCACCGG 2400
2401 CGCGCCCCTG CTGGCCAACG TGTACCCCTA CTTCGCGTAC CGCGACAACC CGCGCGACAT CAGCCTCAAC TACGCGACGT TCCAGCCAGG CACCACTGTG CGTGACCAGA ACAACGGGCT 2520
2521 GACCTACACT TGCCTCTTCG ACGCCATGGT GGACGCCGTG TATGCGGCAC TGGAGAAGGC CGGCGCGCCG GGCGTGAAGG TGGTGATTTC CGAAAGCGGG TGGCCGTCGG CGGGCGGGTT 2640
2641 TGCGGCGTCG CCGGACAACG CGCGGACGTA CACCAAGGGG CTGGTCAACC ACGTCGGCGG GGGCACGCCC AAGAAGCGGG AGGCGCTGGA GACGTACATC TTCGCCATGT TCAACGAGAA 2760
2761 CCAGAAGACC GGGGGTCCGA CGGAGGGGAG CTTCGGGCTG TTCAACCCGG ACAAGTCGCC GGCCTACGCC ATCCAGTTCT AACTGGCTAC CTACATCTTC TTCATCCTAA ATAAATAAGC 2880
2881 TGCATGTAAT GCGGCATCCG AGTGTAATAT GTAGATATC 2919

Figure 3-15: The full length genomic sequence of scGlu12.

The full-length sequence of scGlu11 was obtained through genomic walking. The coding sequence of the gene is 1041bp in length; this includes the 81bp exon 1, which codes for a signal peptide that is cleaved from the protein after transport. The longer exon 2 is 960bp in length and codes for the functional glucanase. The exons are separated by a 550bp intron. The region upstream of the transcription start site, including the UTR and promoter is 603bp in length. Boxes represent the start (ATG) and stop (TAA) codons. The intron is the region located between the arrows. The TATA box within the proximal promoter is underlined.


1 GTGCCAACAT CAACTGCGAC GACAGAGAGC CGTTCGGACC AGCTCTACCA GTGAGGGGGA TTTTGACTAC GGACCGGATC AAATCTTCCT TAATATTGCT GTTCAGCCCT CTTCCAGTAA 120
121 TTTAATCCGT GCACTTAACA TTTTGTACAA AACTAGGAGA ATGATGGGCA CAACCGCAAA CCCATGGACA TGGCACCTCT CATCTACATG GCATCCATGA GGAAGAATTG GATGGTGCGA 240
241 GAAGCGCTAA AGGATGGTGC TTGGTTTAAG ATGAAGATAA CGCCTAGTTT CTCTATAGAG CATTTTAGGC AATTTCTCGG GCTTTGGGCC ATCATCCGGG ATTTTCACCT ACAGAAGGAT 360
361 ATTGACGATG ACATTGTGTG GAAGCATTCT ACTAGTGGCC TCTACACAAC GGAGTCCGCC TACAAAGTGT AACTTTTTTA TTCATCCGCT CTCCCTTGGA GCATGCAATG TGGAGAATCT 480
481 GGGCTCCACC CAAGGTCAAG TTCTTTGCTT GGCGAGCCAT CCAAAATAGA GTTTAGACCG CGAATAGATT GGCCAAGCGC GGTTGGCCTA ACTGTGGCTC GTGCCCCTTA ATGCATGCAT 600
601 GAGAATGAAT CGGTTGACCA TCTCTTCTTC AAAGTCGGTT CATCATTCAC CTATGGGGCT TGCTATAATA ATCACGCAGC TCATTCATGT TGGCAGATAC AGACAGGAGC TATGCTAGCA 720
721 GCAATGGCCA GAAACGATGT TGCTTCCATG TTTGCCGTTG CTCTCTTCAT TGGAGCGTTG CCTGCTGCTC CTACGACGTG GTTGACGTTT CTCTCGTGCT CTTGTACTAT TTTCTATCTA 840
841 CTTTGCATGC ATGCACCTAA CCTACTGTCG GCATGGTTAC CGGGGCAAAG CAAATTCATG CATGGAGTTC GATCATGCTG TTTTTTAATG GACCACTGGA TAATTTATCT ATTTTGTTGA 960
961 TGGAATTTTG TAATGTGCTA GTACTATTAT GCAAAATTAA TTTGGTGGGC AATTTCGAAG AAAAATTTAT TGTCTCTTTT GTTCGTAAGG ATGCTAAAGA TGGCAGAAAC AGAAAAACGA 1080
1081 AGGATTGGAG TGTCATGCCT ATTCGAATAA ATTAAATATT TTTGTCTTAA AAAATAGAAT AATGTAAAAT ATGTACGTAA TATTTTTGGT TGCGCCACCG AAAGCAAGGA AGAGTTATTT 1200
1201 TTAAGAAATT TGAGTGGATA CAATTATTTT CAAAATGCAG TGCAAAGAAA TTCTTGTTGA ATAATTCGTA TGACATTTAG TAATACAAAA TCAAACATAT TAATTAATAG TAAACCGGAT 1320
1321 GCCACTCTTA TCCGCATGCA TGTAGGTGTA CAATCCATCG GCGTCTGCTA CGGCGTGATC GGCAACAACC TCCCGTCCCG GAGCGACGTG GTGCAGCTTT ACAGGTCCAA GGGCATCAAC 1440
1441 GGCATGCGCA TCTACTTCGC CGACGGGCAG GCCCTCTCCG GGCTCCGCAA CTCCGGCATC GGCCTCATCC TCGACATCGG TAACGACCAG CTCGCCAACA TCGCCGCCAG CACCTCCAAC 1560
1561 GCGGCGTCCT GGGTACAGAA CAATGTGCGT CCCTACTATC CGGCCGTGAA CATCAAGTAC ATCGCTGCCG GCAACGAGGT CCTGGGAGGC GCCACGCAGA GCATCGTCCC TGCCATGCGG 1680
1681 AACCTCAACG CGGCCCTCTC CGCCGCCGGC CTCGGCGCCA TCAAGGTGTC CACCTCGATC CGGTTCGACG CGGTGGCCAA CTCCTTCCCG CCCTCCGCCG GCGTGTTCGC GCAGTCGTAC 1800
1801 ATGACGGACG TGGCCCGGCT CCTGGCGAGC ACCGGCGCGC CCCTGCTGGC CAACGTGTAC CCCCTACCTT CGTACCGCGA CAACCCGCGC GACATCAGCC TCAACTACGC GACGTTCCAG 1920
1921 CCGGGCACCA CCGTGCGTGA CCAGAACAAC GGGCTGACCT ACACTTGCCT CTTCGACGCC ATGGTGGACG CCGTGTACGC GGCGCTGGAG AAGGCCGGCG CGCCGGGCGT GAAGGTGGTG 2040
2041 ATTTCCGAGA GCGGGTGGCC GTCGGCGGGC GGGTTTGCGG CGTCGCCGGA CAACGCGCGG ACGTACAACC AGGGGCTGAT CAACCACGTC GGCGGGGGCA CGCCCAAGAA GCGGGAGGCG 2160
2161 CTGGAGACCT ACATCTTCGC CATGTTCAAC GAGAACCAGA AGACCGGGGA TCCGACGGAG AGGAGCTTCG GGCTGTTCAA CCCGGACAAG TCGCCGGCCT ACGCCATCCA GTTCTAACTG 2280
2281 GCTACCTACA TCTTCTTCAT CCTAAATAAA TAAGCTGCAC GTAATGCGGC ATCGAAGTGT AATATGTAGA TTCATCCAGG GAAGAGTGCA ACCATGCATG 2380

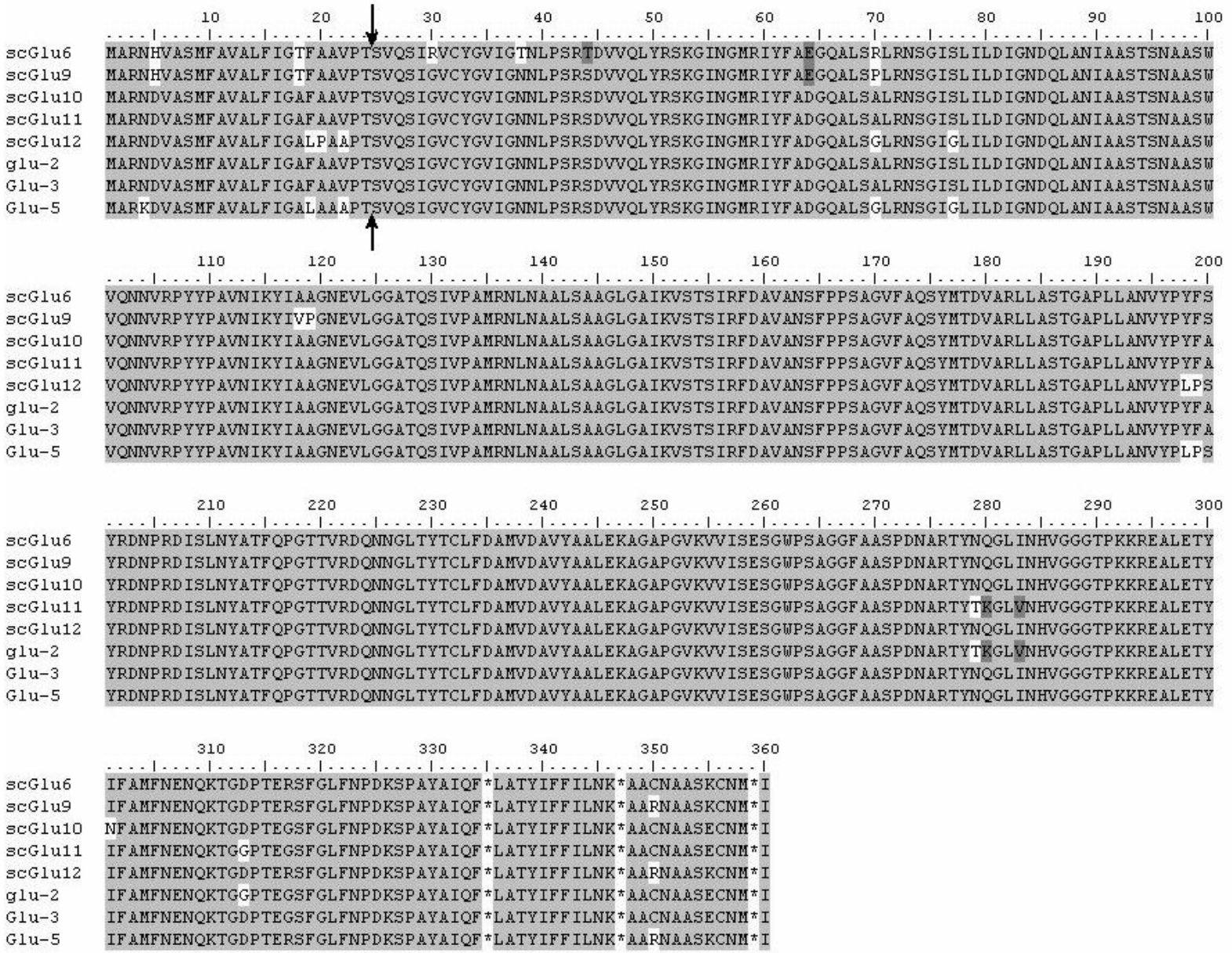
The coding region of each gene was used to deduce the virtual amino acid sequence of each glucanase (Figure 3-16). These were aligned with the deduced amino acids sequence of three other known glucanase amino acid sequences. These glucanases were originally published without the 'sc' prefix used throughout this thesis. This prefix has been added to the names of these glucanases throughout this thesis for clarity and consistency. These glucanases were identified as scGlu2 (GenBank accession number AM181306), scGlu3 (GenBank accession number AM181307) and scGlu5 (GenBank accession number AM181309).

The amino acid alignment revealed that the first exon, which codes for the signal peptide, is not as highly conserved as might be anticipated. Although there was no difference in the predicted sequences of the first exons of scGlu10, scGlu11, scGlu2, and scGlu3, amino acid subsitutions were found in scGlu5 (Phe19Leu, Val22Ala), scGlu6 (Asp5His, Ala18Thr), scGlu9 (Asp5His, Ala18Thr, Phe19Leu) and scGlu12 (Ala21Pro).

Additionally, there were differences in the sequences in the second exon, including 14 sites of substitution within this 320 amino acid stretch; these sequence changes are shown in Figure 3-16 as unshaded regions.

Figure 3-16: Alignment of the deduced amino acid sequence of scGlu6, 9, 10, 11 and 12 with comparison to deduced amino acids sequence of published glucanase sequences.

Based on sequence alignments and predicted intron spice site, the coding sequences for scGlu6, 9, 10, 11 and 12 were deduced and used to predict the amino acid sequence of the gene product. The deduced amino acid sequences previously characterized (GenBank accession numbers AM181305, AM181307 and AM181309) were included in this comparison. All sequences were aligned using Clustal X. Amino acid identities are indicated by grey boxes and possible stop codons are indicated with an asterisk. The sequence identity amongst the glucanases was 94% or higher.


scGlu6 MARNHVASMFAVALFIGTFAAVPTSVQSIRVCYGVIGTNLPSRTDVVQLYRSKGINGMRIYFAEGQALSRLRNSGISLILDIGNDQLANIAASTSNAASW
scGlu9 MARNHVASMFAVALFIGTFAAVPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFAEGQALSPLRNSGISLILDIGNDQLANIAASTSNAASW
scGlu10 MARNDVASMFAVALFIGAFAAVPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSALRNSGISLILDIGNDQLANIAASTSNAASW
scGlu11 MARNDVASMFAVALFIGAFAAVPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSALRNSGISLILDIGNDQLANIAASTSNAASW
scGlu12 MARNDVASMFAVALFIGALPAAPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSGLRNSGIGLILDIGNDQLANIAASTSNAASW
glu-2 MARNDVASMFAVALFIGAFAAVPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSALRNSGISLILDIGNDQLANIAASTSNAASW
Glu-3 MARNDVASMFAVALFIGAFAAVPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSALRNSGISLILDIGNDQLANIAASTSNAASW
Glu-5 MARKDVASMFAVALFIGALAAAPTSVQSIGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSGLRNSGIGLILDIGNDQLANIAASTSNAASW
scGlu6 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPYFS
scGlu9 VQNNVRPYYPAVNIKYIVPGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPYFS
scGlu10 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPYFA
scGlu11 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPYFA
scGlu12 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPLPS
glu-2 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPYFA
Glu-3 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPYFA
Glu-5 VQNNVRPYYPAVNIKYIAAGNEVLGGATQSIVPAMRNLNAALSAAGLGAIKVSTSIRFDAVANSFPPSAGVFAQSYMTDVARLLASTGAPLLANVYPLPS
scGlu6 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYNQGLINHVGGGTPKKREALETY
scGlu9 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYNQGLINHVGGGTPKKREALETY
scGlu10 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYNQGLINHVGGGTPKKREALETY
scGlu11 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYTKGLVNHVGGGTPKKREALETY
scGlu12 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYNQGLINHVGGGTPKKREALETY
glu-2 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYTKGLVNHVGGGTPKKREALETY
Glu-3 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYNQGLINHVGGGTPKKREALETY
Glu-5 YRDNPRDISLNYATFQPGTTVRDQNNGLTYTCLFDAMVDAVYAALEKAGAPGVKVVISESGWPSAGGFAASPDNARTYNQGLINHVGGGTPKKREALETY
scGlu6 IFAMFNENQKTGDPTERSFGLFNPDKSPAYAIQF*LATYIFFILNK*AACNAASKCNM*I
scGlu9 IFAMFNENQKTGDPTERSFGLFNPDKSPAYAIQF*LATYIFFILNK*AARNAASKCNM*I
scGlu10 NFAMFNENQKTGDPTEGSFGLFNPDKSPAYAIQF*LATYIFFILNK*AACNAASECNM*I
scGlu11 IFAMFNENQKTGGPTEGSFGLFNPDKSPAYAIQF*LATYIFFILNK*AACNAASECNM*I
scGlu12 IFAMFNENQKTGDPTERSFGLFNPDKSPAYAIQF*LATYIFFILNK*AARNAASKCNM*I
glu-2 IFAMFNENQKTGGPTEGSFGLFNPDKSPAYAIQF*LATYIFFILNK*AACNAASECNM*I
Glu-3 IFAMFNENQKTGDPTEGSFGLFNPDKSPAYAIQF*LATYIFFILNK*AACNAASECNM*I
Glu-5 IFAMFNENQKTGDPTERSFGLFNPDKSPAYAIQF*LATYIFFILNK*AARNAASKCNM*I

3.4.2 Promoter Analysis

The full-length upstream region of each glucanse was analyzed using the online database of cis-acting regulatory regions known as PLACE – a database of cis-acting regulatory DNA elements. By convention, this is the region upstream of the start site of transcription. Since the lengths of the UTR was not determined experimentally, they were each estimated by subtracting the length of the coding region from the recovered cDNA sequence. The predicted 5′ UTRs varied in length. Positions upstream of the start site of transcription, which considered to occur at the start codon (ATG), are referred to as negative numbers counting back from +1 where +1 is the A in ATG (i.e. the nucleotide at position -200 would be 200 base pairs upstream of ATG).

A long list of putative cis-acting regulatory regions for each promoter region was recovered from the PLACE analysis of each sequence. These were reviewed and only those that matched the following criteria are discussed within this thesis. Each element had to be located a specific distance from the assumed start of transcription. Transcription factors bind to these motifs and interact with transcriptional machinery. If the element is too close or too far from the start of transcription, factors that interact with that element may not be able to perform their specified functions. Although many papers give an exact location for each cis-element, this number was not strictly conserved and elements were still accepted if they fell within 50bp of the suggested location. Based on previous studies, some elements must be present in multiple copies which must be spaced at specific intervals in order to interact with trans-acting factors. If such a criterion existed for an element, it was adopted.

In addition to physical qualities, putative cis-acting elements were also screened for function, for instance glucanases are known to be involved in cell wall maintenance, seed and

floral organ development, and in response to dehydration, pathogen infection, wounding, and cold. Light regulation may also be important as cold acclimation is enhanced if plants are exposed to short days and long nights. Cis-elements known to be related to any of these functions were possible candidates for glucanase expression. Functions that are not known to be related to glucanases, such as Finally, only cis-elements determiend to be involved in the designated expression pattern based on experimentation were included in this study; elements that were only suggested by bioinformatic research were not considered.

Table 3-1 lists all of the putative cis-elements found within the promoters of the 5 glucanase genes. Putative cis-elements can be classified into a number of distinct categories. These include biotic or abiotics stresses, light regulation, metabolism and development.

Five different putative elements relating to cold were found within the promoters. At least one of these elements was found within the promoters of scGlu9, 10, 11 and 12. There were no cold-related elements within scGlu6. Both scGlu9 and scGlu10 contained 7 cold-related elements within their promoters.

The promoter scGlu6 did not contain any cold-related elements but did contain elements known to be related to disease resistance, metabolism, light regulation and most noticeably, cis-elements involved with development. In addition to cold-related elements, scGlu9 and 10 both contained elements relating to biotic and abiotic stress such as pathogen defense these promoters had fewer elements related to development than the other glucanse genes studied. The largest upstream sequence was acquired for scGlu11 and thus it contained the largest number putative cis-elements spanning all of the categories. Despite the large number of putative elements, the 3 elements involved in gene regulation by ABA are absent from this promoter.

Table 3-1: Name, function and position of cis-acting regulatory elements located on each of the 5 glucanase putative promoters.

The online data base of plant cis acting elements know as PLACE was used to search promoter sequences for putative cis-acting regulatory elements. Each putative cis-element was carefully screened, and only those that met all criteria necessary for promoter function were included in the final list. The name, function, distance from the start site of translation (start codon, ATG) and whether the cis-element was located on the + or – DNA strand is indicated. Most of the putative cis-elements can be classified into groups based on their involvement in biotic or abiotic stress, light regulation, metabolism and development. The elements specifically involved in regulation of cold-related genes are shaded in gray.

Name of element	Function	Distance from start codon and strand specificity of element within the promoter.					References
		scGlu6	scGlu9	scGlu10	scGlu11	scGlu12	
2S-SEED-PRO-Banapa	Regulates storage proteins		(-)110				Stalberg *et al.,* 1996
ABRE-related	Dehydration response.		(-)591		(+)174 (-)173		Simpson *et al.,* 2003; Nakashima *et al.,* 2006
ARF-AT	Auxin Responsiveness. Unregulated by ABA. Found in ABA insensitive genes.		(+)242 (+)517 (-)479 (-)489 (-)824				Menhauser *et al.,* 2004; Goda *et al.,* 2004; Hagen & Guilfoyle, 2004
ASF1-motif CamV	Involved in response to auxins or salicylic acid. May be relevant in light regulation. May be involved in biotic stress and disease resistance.		(+)511 (+)528 (+)542	(+)822 (-)199 (-)470	(+)403	(+)363	Despres *et al.,* 2003
BIHD1OS	Disease resistance.	(+)1047 (+)1145 (-)244		(+)364 (-)511	(+)791 (-)731 (-)937 (-)1020 (-)1064	(-)369	Song *et al.,* 2005
CANBNNAPA	Seed specific. Acts as a repressor and an activator.	(-)99 (-)164)	(+)120				Ellerstrom *et al.,* 1996
CAC GTG motif	Involved in regulation of pathogen defence, ABA and light regulated gene transcription.			(+/-)173			Chandrasekharan *et al.,* 2003; Hudson & Quail, 2003; Chakravarthy *et al.,* 2003
CBF hv	Cold response. Binding site of the CBF gene family.		(+)177 (+)480 (-)177 (-)240	(+)542 (+)859	(+)1177	(-)634	Xue, 2002; Svensson *et al.,* 2006
CIACADIAN-LELHC	Genes regulated by the circadian rhythm.		(+)297 (-)107	(+)320 (+)417 (-)179 (-)206		(-)367	Piechulla *et al.,* 1998
CPBCSPOR	Cytokinin-enhanced protein binding.	(+)398)					Fusda *et al.,* 2005
CRTDRE hv CBF2	CRT/DRE element. Binds to CBF2. Cold responsive element.		(+/-)177				Xue, 2003
DOFCOREZM	Carbon Metabolism.	(+)24 (+)59 (-)153 (-)203	(+)161 (+)347	(+)66 (+)278 (+)844 (-)703 (-)1028	(+)218 (+)561 (-)88	(+)249	Yangisawa & Schmidt, 1999; Yangisawa, 2000
DRE/CRT At	Dehydration and cold response. Found in the promoter of CBF3 and binds ICE1.			(+)859		(-)634	Diaz-Martin *et al.,* 2005; Suzuki *et al.,* 2005; Skinner *et al.,* 2005

Name of element	Function	Distance from start codon and strand specificity of element within the promoter.					References
		scGlu6	scGlu9	scGlu10	scGlu11	scGlu12	
DPBFCDC3	Embryo Specific. Induced by GA and IAA. Found in genes that do not respond to ABA.	(-)96	(-)362				Finkelstein & Lynch, 2000; Lopez-Molina & Chia, 2000
ELRE PCRP1	Elictor response element. Involved in pathogen-resistance.			(-)517		(+)614 (-)494	Chen & Chen, 2000; Rushton *et al.,* 2002; Laloi *et al.,* 2004
GAREAT	GA responsive element.	(+)34					Ogawa *et al.,* 2003
GATA box	Required for high level light regulated and tissue specific expression.	(+)32 (+)56 (+)685 (+)968 (-)158 (-)178 (-)196 (-)374 (-)451 (-)970	(+)126 (+)271 (+)664 (-)117 (-)130	(+)215 (+)992 (-)910 (-)968 (-)994 (-)1023	(+)248 (+)275 (+)565 (+)658 (-)119 (-)703 (-)1015	(+)276 (+)358	Lam & Chua, 1989; Gilmartin *et al.,* 1990; Benfey & Chua, 1990; Reyes et al, 2004; Rubio-Somoza *et al.,* 2006
GCC core	Involved in pathogen-response. Response to ethylene. Response to MeJa.		(-)280	(+)767 (+)807 (+)896	(-)374 (-)691		Brown *et al.,* 2003; Chakravarthy *et al.,* 2003
GT-1 consensus	For light regulated genes. May stabilize the TATA box.	(+)56 (+)105 (+)222 (-)293 (-)391 (-)577	(+)157 (-)12 (-)151 (-)461	(-)908 (-)1021	(+)213 (+)478 (+)964 (-)482 (-)804 (-)1032 (-)489	(-)341	Terzaghi & Chashmore, 1995; Villain *et al.,* 1996; Le Gourrierec *et al.,* 1999; Buschel *et al.,* 1999
GT-1 Cam4	pathogen and salt induced. Similar to GT-1 box.	(+) 105	(+)157				Park *et al.,* 2004
I box	Light regulation.	(+)32 (+)56		(+)215		(+)276	Terzaghi & Cashmore, 1995
LTRE1 hvBLt49	Low-temperature-responsive-element.		(+)155	(+)860 (+) 1059 (-)1002	(+)310	(-)634	Dunn *et al.,* 1998
MYB1-LePr	Involved in defense response.		(+)385				Chakravarthy *et al.,* 2003
MYB core	Element found in genes responsive to water stress.		(+)52 (+)752	(+)411 (-)437		(+) 560 (+)611 (-)11 (-)171 (-)407 (-)569	Lusher & Eiseman, 1990; Urao *et al.,* 1993; Solano *et al.,* 1995
MYBST-1	GA regulated expressoin	(+)31		(-)1023	(+)247 (+)274	(+)357	Baranowskij *et al.,* 1994
MYCAT-RD22	ABA and drought responsive element.	(-)161		(+)247			Abe *et al.,* 1997; Busk & Pages, 1998

Name of element	Function	Distance from start codon and strand specificity of element within the promoter.					
		scGlu6	scGlu9	scGlu10	scGlu11	scGlu12	References
MYC consensus AT	Dehydration and cold response. Found in the promoter of CBF3 and binds ICE1.	(+/-)801	(+/-)729	(+/-)762	(+/-)751 (+/-)885 (+/-)1060	(+/-)11	Abe *et al.,* 2003; Lee *et al.,* 2005; Hartman *et al.,* 2005
POL A Sig1	Signal for polyadenylation must be -2 to -30 upstream of ATG.		(-)5 (-)9 (-)31		(+)17		Loke *et al.,* 2005
PRE ATP-PRO DH	Found in genes that respond to hypoosmolarity.	(+/-)811	(+)325 (-)274		(-)518 (-)535		Satoh *et al.,* 2004; Welmeier *et al.,* 2006
TATCCA-Os AMY	Sugar and hormone regulation.	(-)30					Lu *et al.,* 1998; Lu *et al.,* 2002; Chen *et al.,* 2006
T-box GAPB	Light activated gene transcription	(+)152		(+)702		(-)423 (-)630	Chan *et al.,* 2001
WRKY71 Os	Pathogenesis-related. Represses GA pathways.	(+)244 (+)504 (-)765 (-)1048 (-)1146	(+)315 (+)394 (+)511 (+)528 (+)542 (-)203	(+)511 (+)565 (+)822 (-)200 (-)365 (-)471 (-)518	(+)410 (+)451 (+)731 (+)937 (+)1020 (+)1064 (-)37 (-)506 (-)792 (-)1136	(+)64 (+)363 (+)369 (+)615 (-)495	Eulgem *et al.,* 1999; Eulgem *et al.,* 2000; Zhang *et al.,* 2004; Xie *et al.,* 2005

3.5 RT-PCR Analysis

To test the validity of the cis-elements in directing gene expression, the transcript abundance of each gene was evaluated under several conditions. Semi-quantitive RT-PCR was used to monitor the level of glucanase mRNA produced by each gene after cold treatment, pathogen infection, wounding and treatment with the hormones MeJa and ethephon. A freshly pollinated winter rye flower was also tested for the presence of glucanase transcripts. cDNA was synthesized from total RNA extracted from a freshly pollinated winter rye flowers or leaves before treatment, and at specific time points after each treatment. Each cDNA pool was used in RT-PCR reactions using primers specific to scGlu6, 9, 10, 11 and 12 as well as primers against 18S rRNA as an internal control (Figures 3-17, 3-18, 3-19, 3-20, 3-21).

Based on these analyses scGlu9 was expressed three weeks after the onset of cold treatment, 8 days after infection with pink snow mould, in small concentrations after wounding, and after 2 days of ethephon. scGlu10 was also expressed after cold treatment, but expression was stronger than that of scGlu9 and observed after only 1 week of cold treatment. Transcripts of scGlu10 were still present after 3 weeks of cold treatment, but are present at a low concentration. scGlu10 was also weakly expressed after pathogen infection and dehydration. The last glucanase expressed under cold conditions was scGlu11, which were evident after 1-week cold treatment. These levels were still evident after 3 weeks of cold treatment. scGlu11 is also strongly expressed after wounding and treatments with MeJa and ethephon. Transcripts of scGlu11 are evident 2 days after MeJa treatment and only 12 hours after treatment with ethephon. The transcript of the final glucanase, scGlu12 is only seen 1 hour after treatment with MeJa. None of the cDNA pools tested positive for scGlu6 although the primers specific to scGlu6 were able to amplify this gene's sequence from genomic DNA.

Figure 3-17: Results of the semi-quantitative RT-PCR reactions performed to test expression of the five glucanases after cold exposure.

Each of the five glucanases were tested for expression in leaves after cold treatment. cDNA made from total RNA extracted from cold-treated and control plants before onset of treatment, after 1 day of treatment, 7 days of treatment and 3 weeks of treatment. These cDNAs were used as templates semi-quanitative RT-PCR reactions using primers specific to scGlu6, 9, 10, 11 and 12 as well as primers against 18S rRNA which was included as an internal control. Results show that scGlu9, 10 and 11 transcripts are expressed under cold conditions. scGlu9 mRNAs are present after 3 weeks, while expression of scGlu10 and scGlu11 can be detected after 7 days cold treatment. .

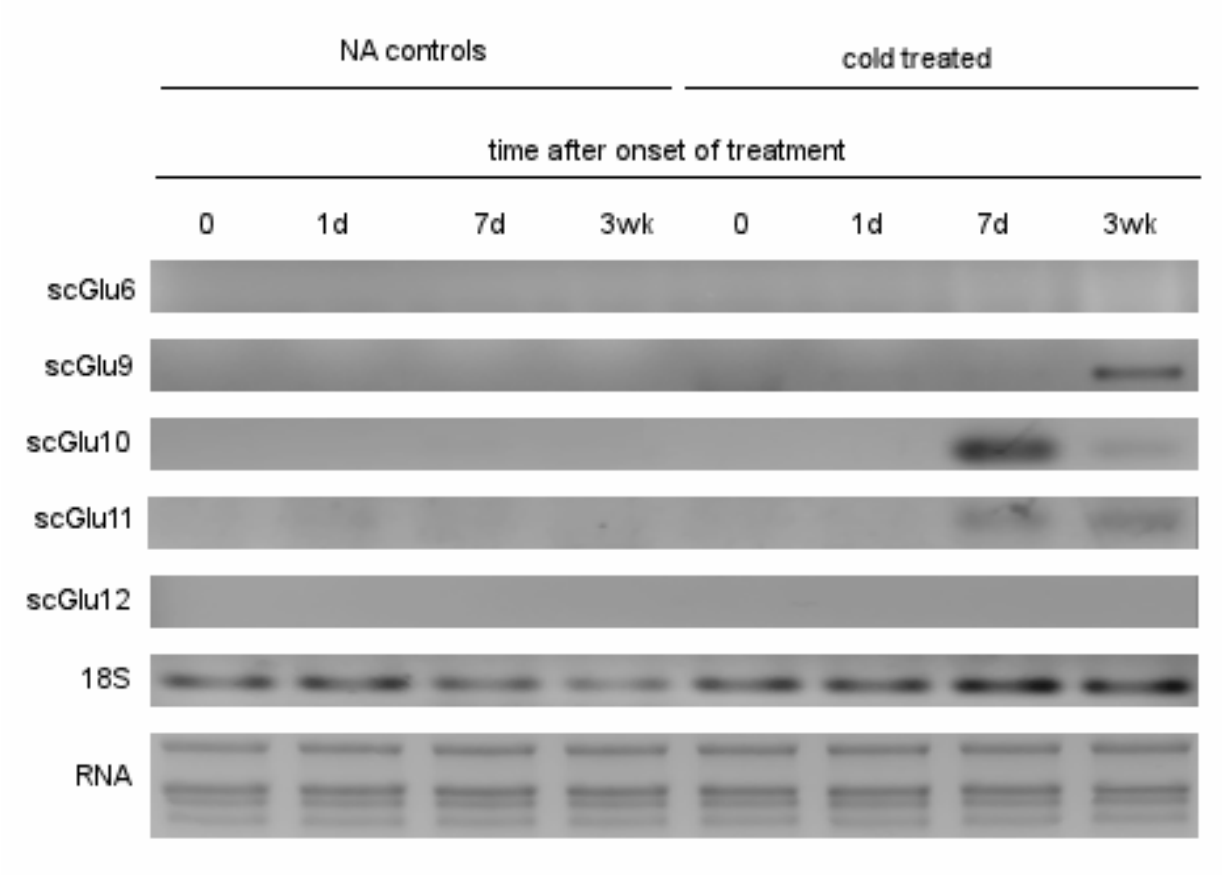

NA controls
cold treated
time after onset of treatment
0
1d
7d
3wk
0
1d
7d
3wk
scGlu6
scGlu9
scGlu10
scGlu11
scGlu12
18S
RNA

Figure 3-18: Results of the semi-quanitative RT-PCR reactions performed to test expression of the five glucanases after pathogen infection.

Each of the five glucanases were tested for expression in leaves after infection by the pink snow mold *M. nivale*. cDNAs made from total RNA extracted from plants prior to infection and after eight days of infection were prepared. These cDNAs were used as templates in RT-PCR reactions using primers specific to scGlu6, 9, 10, 11 and 12. Primers against 18S rRNA was included as an internal control. Results show that scGlu9 and scGlu10 transcripts are present expressed after pathogen infection.

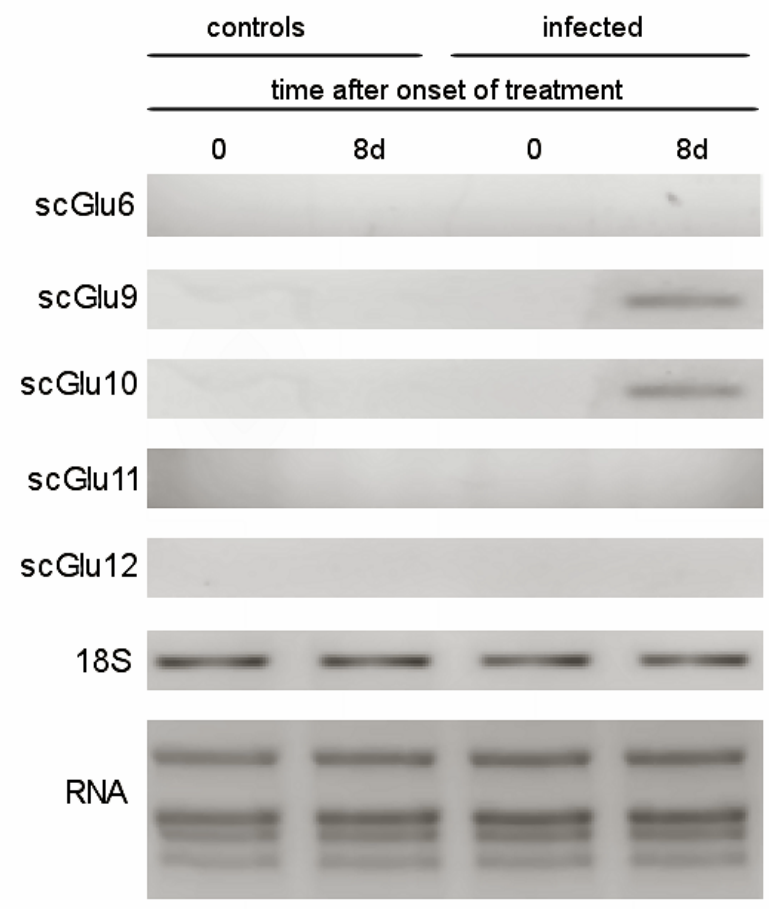

controls
infected
time after onset of treatment
0
8d
0
8d
scGlu6
scGlu9
scGlu10
scGlu11
scGlu12
18S
RNA

Figure 3-19: Results of the semi-quanitative RT-PCR reactions performed to test expression of the five glucanases in the pollinated flower and after wounding.

The transcript abundance of each of the five glucanases (scGlu6, scGlu9, scGlu10, scGlu11 and scGlu12) in pollinated flowers and wounded winter leaves was determined using semi-quantitative RT-PCR. cDNAs made from total RNAs extracted from pollinated floral organs and the leaves of plants before and 24 hours after wounding. These cDNAs were used in RT-PCR reactions using primers specific to scGlu6, 9, 10, 11 and 12. The level of 18S rRNA was tested an internal control. Transcripts of both scGlu9 and scGlu11 are expressed after wounding. scGlu11 is also weakly expressed in freshly fertilized flower organs.

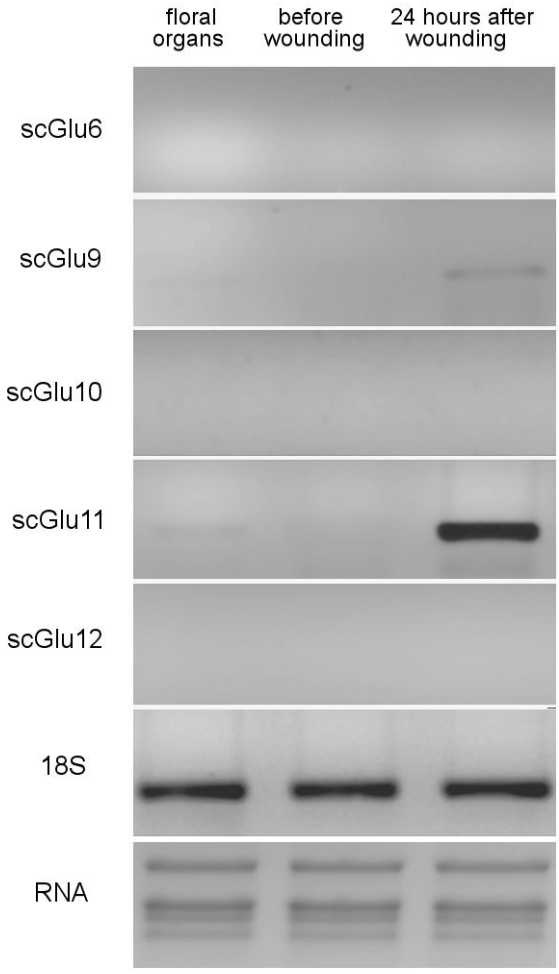

floral organs
before wounding
24 hours after wounding
scGlu6
scGlu9
scGlu10
scGlu11
scGlu12
18S
RNA

Figure 3-20: Results of the semi-quanitative RT-PCR reactions performed to test expression of the five glucanases after dehydration and rehydration.

RT-PCRs were performed in order to determine which of the five glucanases is expressed before, during and after dehydration. Using total that RNA was extracted from winter rye. The primers used in these reactions were specific to scGlu6, 9, 10, 11 and 12 and 18S rRNA. Results showed that scGlu10 mRNAs are present during and after dehydration.

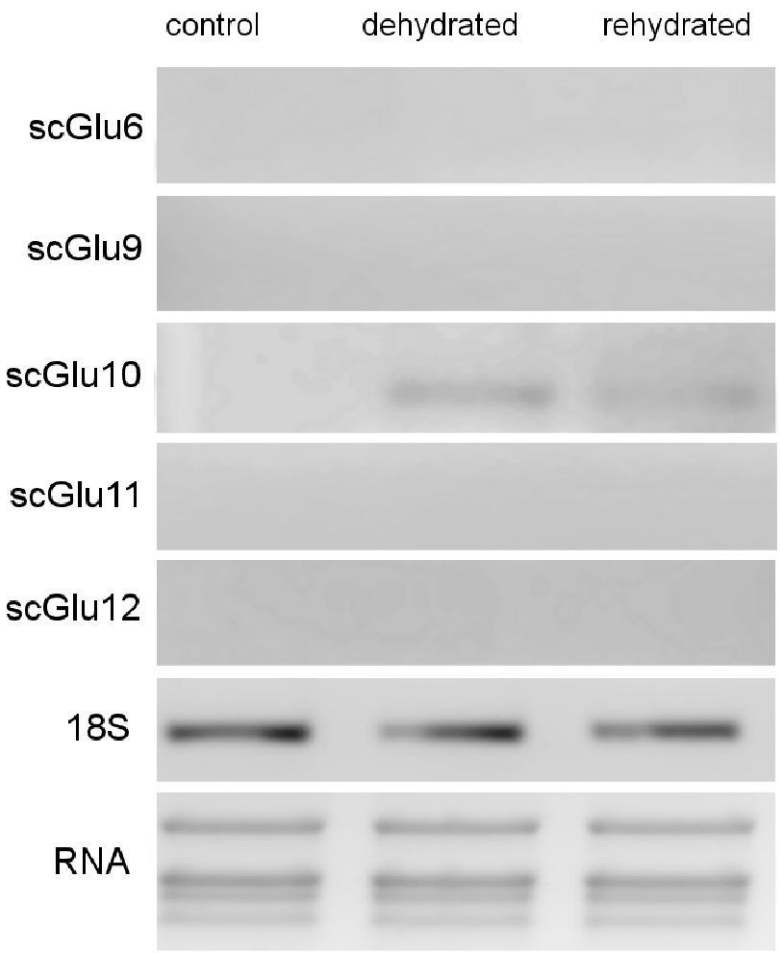
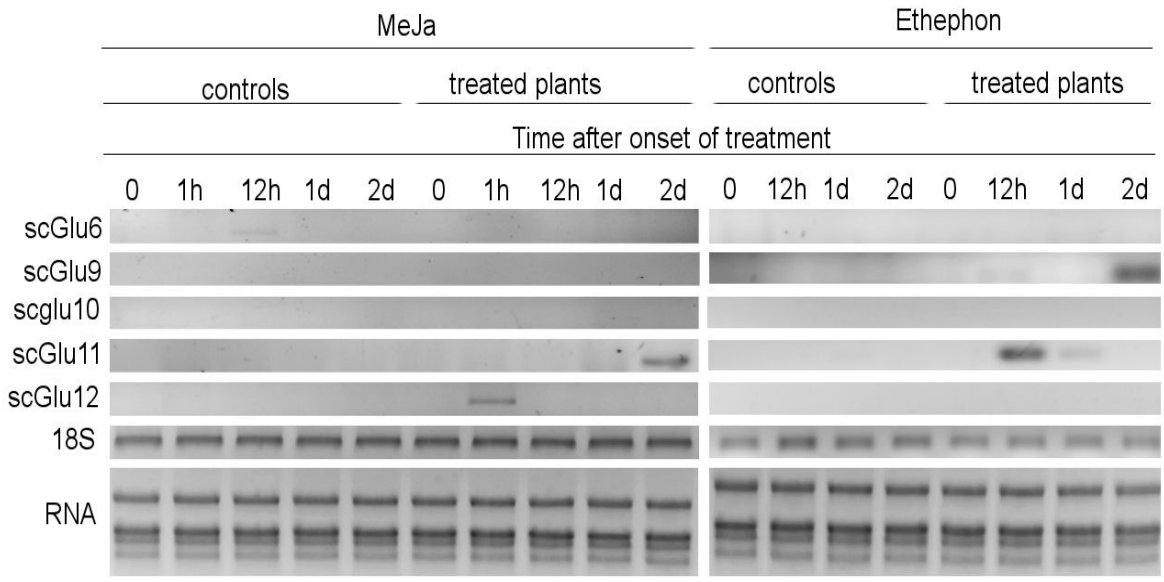

control
dehydrated
rehydrated
scGlu6
scGlu9
scGlu10
scGlu11
scGlu12
18S
RNA

Figure 3-21: Results of the semi-quanitative RT-PCR reactions performed to test transcript abundance of the five glucanase genes after treatment with 15mM MeJa and 10mM ethephon.

RT-PCRs were performed in order to determine which of the five glucanases is expressed after treatment with the phytohormone methyl jasmonate and the ethylene releasing chemical ethephon. Total RNA was extracted from winter rye leaves before, during and after dehydration and made into cDNA. This cDNA was used in RT-PCR using primers specific to scGlu6, 9, 10, 11 and 12 as well as primers against 18S rRNA. scGlu11 and scGlu12 were responsive to treatment with MeJa and scGlu9 and 11 to treatment with ethephon.


MeJa
Ethephon
controls
treated plants
controls
treated plants
Time after onset of treatment
0 1h 12h 1d 2d 0 1h 12h 1d 2d 0 12h 1d 2d 0 12h 1d 2d
scGlu6
scGlu9
scglu10
scGlu11
scGlu12
18S
RNA

4 Discussion

4.1 Treatments leading to the expression of antifreeze activity

Previous studies by Marilyn Griffith and co-workers determined that apoplastic extracts produced by winter rye plants after treatment with cold, drought or ethephon contain AFPs while extracts from plants treated with ABA, salicylic acid or pathogen infection do not (Hiilovaara-Teijo *et al.,* 1999; Yu *et al.,* 2001; Yu & Griffith, 2001). Other signaling molecules, such as MeJa and hydrogen peroxide are known to increase the levels of PR proteins and enhance cold tolerance. Preliminary immunological studies indicate that the apoplast of MeJa-treated winter rye contained at least one β-1,3-endoglucanase, chitinase and thaumatin-like protein (Merz, 2005). In addition, the apoplastic extract of these plants displayed weak antifreeze activity. Unfortunately, a thorough comparison between the level of antifreeze activity between plants treated with other signaling molecules and hormones was not conducted. Moreover, the effect of hydrogen peroxide on the apoplastic proteins in winter rye has never been tested.

Cold and ethephon treatments cause winter rye to excrete AFPs in to the apoplast (Hon *et al.,* 1994; Yeh *et al.,* 2000). This was again evident in these results as apoplastic extracts induced the formation of ice crystals with hexagonal shapes in the antifreeze assay (Figure 3-1). Although previous reports indicated that CA winter apoplast causes the formation of ice crystals with bipyramidal shapes, the concentration of apoplastic extracts used in the research reported in this thesis is too low to expect ice crystals to form these structures. Doubling the concentration to $2mg \cdot ml^{-1}$ lead to bipyramidal structures (results not shown) but this is approximately 4 times higher than native concentrations (Yu & Griffith, 1999).

The physical appearance of ethephon treated plants was compared to control plants and to plants treated with MeJa or hydrogen peroxide. Ethephon-treated plants appeared to have decreased anthocyanin within their leaves. Anthocyanins are water-soluble red-blue plant pigments that are produced in leaves in response to intense light, and in some fruit, during ripening (Taiz & Zeiger, 2006). In leaves, this pigment protects plants from photo-damage and photoinhibition by absorbing UV and blue-green light. The decreased level of anthocyanin production in ethephon-treated leaves suggests that the plants were sprayed with the hormone after the lag phase of anthocyanin production. Treatment with ethylene and ethylene-producing compounds at this time is known to decrease the levels of anthocyanin within plants. If the plants had been sprayed with ethephon during the early lag phase of anthocyanin synthesis the plants would have had a higher pigment content (Craker *et al.,* 1971).

Application of MeJa to plants causes dramatic physiological changes in plants not seen in any of the other treatments. The normally green leaves of winter rye developed a marked purple-red colour after only two days of MeJa treatment. In addition, their leaf tips began to curl and senesce. These dramatic phenotypic changes were not expected (Cheong & Choi, 2003). MeJa is involved in the upregulation of plant pigments including anthocyanins and carotinoids, and is a known inducer of senescence. However, the degree of damage to the tips of the treated winter rye leaves indicates that the concentration of MeJa required to produce the minimal amount of antifreeze activity observed in these samples is approaching toxic concentrations. For this reason it is unlikely that MeJa is the main signaling molecule involved in accumulation of antifreeze activity. MeJa signaling likely plays a larger role in the upregulation of PR-proteins for pathogen defense.

Another possibility is that winter rye uses another jasmonate, such as jasmonic acid to induce antifreeze activity. Although MeJa and jasmonic acid play similar roles in plants, each has distinct contributions to fine tune gene regulation. Thus, the high concentration of MeJa used in this research may have been sufficent to induce a pathway that this molecule does not normally affect. In addition, because a high dose of MeJa was sprayed on the plants, it is possible that cellular processes demethylated a small amount of the MeJa causing a normal response to jasmonic acid resulting in the upregulation of PR-like antifreeze proteins within the apoplast. Further studies must be conducted with regards to the induction of apoplastic antifreeze proteins by MeJa and other jasmonates in order to clarify their roles.

Treatment with 200mM hydrogen peroxide causes winter rye to excrete AFPs into its apoplast. Hydrogen peroxide is known to be produced by some plants in response to cold temperatures (Okuda *et al.,* 1991; Hung *et al.,* 2005) and improves survival of tomatoes after chilling injury (Chih-Wen *et al.,* 2006). However, this is the first report of hydrogen peroxide inducing antifreeze activity in plants. Increased growth rate and decreased anthocyanin levels were also associated with hydrogen peroxide treatment. These unexpected side effects should be studied in detail, as they have not been reported previously. It is possible that these effects were seen in winter rye because of the young age of the tissue. It is also possible that these effects are monocot or even cereal specific as most of the work on hydrogen peroxide signaling has been performed on older plants and on dicots such as tomato and tobacco. It is also possible that hydrogen peroxide acts indirectly to produce antifreeze activity as there are a number of proteins specifically involved in hydrogen peroxide perception (Hancock *et al.,* 2006). These proteins have additional roles, such as ethylene perception, or involvement in metabolic pathways such as glycolysis (Hancock *et al.,*, 2006). Hydrogen peroxide is

potentially involved in a large signaling cascade involving other plant hormones, such as ethylene that may lead to the accumulation of AFPs in the winter rye apoplast. Additional research will be necessary to determine the role of hydrogen peroxide on winter rye cold acclimation and growth. Understanding all of the signaling molecules needed to produce antifreeze activity in winter rye is a major step toward understanding complex expression pathways involved in cold acclimation.

4.2 Survey of β-1,3-endoglucanses of winter rye

Previous studies have relied on 1D SDS-PAGE to profile proteins within the winter rye apoplast. Although it appears that analysis of proteins within the NA apoplast were accurate, the analysis of cold-acclimated apoplastic proteins was incomplete.

It is unknown how many glucanases in total are present within the leaf of CA and NA winter rye. Two-dimensional SDS-PAGE on these extracts was performed to address this question. The stained gels were nearly identical, although there were a few proteins that were noticeably absent within the CA extract when compared to the protein profile of NA extract (see circled peptides in Figure 3-6). Immunoblots of these gels with anti-glucanase antibodies reveal the presence of NA β-1,3-endoglucanases and 3 CA β-1,3-endoglucanases (Figure 3-8). Each blot appeared to have a few points of weak signal which may be an additional β-1,3-endoglucanases. Immunoblots using IEF strips with a narrower pH range would help elucidate the validity of these spots.

One-dimensional SDS-PAGE analysis of apoplastic proteins lead researchers to the conclusion that these extracts contained 13 apoplastic proteins, seven of which are AFPs, 2 are β-1,3-endoglucanses. These results were successfully replicated (Figure 3-2) and the presence of two β-1,3-endoglucanases within these protein samples (Figure 3-3) was

confirmed by immunoblot analysis. The conclusions drawn from 1D-SDS PAGE analysis remain identical to previous conclusions.

Two-dimensional SDS-PAGE analysis of CA apoplastic extracts were in contrast 1D results. The data suggest that there there are at least 25 apoplastic polypeptides in these extracts (Figure 3-7). Immunoblot analysis (Figure 3-9) of the same samples indicated the presence of at least 1 basic and 4 acidic β-1,3-endoglucanases. The immunoblot showed 1 area of weak signal that may be a less abundant glucanase. Further analysis of these spots is essential before definitive conclusions are reached.

The identities of the original cold-induced apoplastic proteins were originally determined through sequencing of the isolated polypeptide (Hon *et al.,* 1995). Normally this method reveals if a protein band contains more than one polypeptide as was the case for a 35kDa band which contained a glucanase and a 36kDa chitinase. However, if a gel band contained proteins that were completely identical at their N-terminal there would be no conflicting signal, and nothing to indicate to the researchers additional proteins were present within that sample. In the absence of this information, scientists could be lead to false conclusions about the number of proteins within a sample, and is undoubtedly, what has happened in this situation. Immunoblot analysis shows that there are at least three glucanases within the apoplast of cold-acclimated winter rye, each with a slightly different pI value. This unique pI value is what allows these proteins of similar sizes to separate into distinct dots on a 2D SDS-PAGE. In fact, there may be additional glucanases with the same pI value within each dot on this 2D-PAGE.

This representation of the protein content within the CA apoplast may also be true for the chitinases, thaumatin-like proteins and lipid transfer proteins known to be present within

the CA apoplast. The results presented in this thesis show that the process of apoplastic protein accumulation during cold acclimation is more complex than previously thought. Complete understanding of the changes that occur in this extracellular compartment must be realized in order to fully understand its functional capacity. This could be accomplished by re-focusing extracts using IEF strips with a narrower pH range, and by immunoblot analysis using antibodies raised against all major apoplastic proteins: β-1,3-endoglucanses, chitinases, thaumatin-like proteins and lipid transfer proteins.

4.3 Promoter analysis and expression patterns of 5 β-1,3-endoglucananses of winter rye

It is impossible to determine the function of a glucanase based on its predicted protein sequence, as the similarities between winter rye glucanases are far too great. Due to the fact that the non-coding regions of cereal genomes, especially their promoter regions are evolving at a far greater pace then the coding regions (Guo and Moose, 2003) one of the aims of this research was to link cis-elements found within the promoter region of each gene to gene expression and possibly function.

Promoters are critical non-coding regions located directly upstream of the transcriptional start site. These regions work collectively with regulatory machinery or with other regulatory regions, such as motifs found in the intron, to direct RNA polymerase transcription initiation of the downstream region. The critical motifs within the promoter are referred to as cis-acting elements, they are generally quite short (4-6 nt) and are often conserved between genes that are regulated by similar transcriptional triggers. For instance, genes which are upregulated by MeJa would be expected to contain motifs known to interact with transcriptional factors within the MeJa transcriptional pathway. These genes may have

nothing in common with each other, for instance one gene may code for a membrane-bound channel protein and the other may code for a pathogenesis-related protein, but both are upregulated through a transcriptional pathway involving MeJa and so both could contain identical MeJa cis-elements within their promoters.

Many plant cis-elements have been reported, and the number of known cis-elements grows everyday. The online plant cis-element database PLACE, allows researchers to search a putative promoter for known cis-elements. The resulting data provide a valuable starting point for promoter analysis but must be processed thoroughly to gain meaningful results; the sequences of cis-elements are short and thus may occur by chance. False conclusions can be made if database results are taken at face value. Promoters are mostly composed of non-functional sequence, that is each motif can be separated by hundreds of nucleotides that have nothing to do with the regulation of the gene. Because the length of each motif is so short there is a degree of probability that the regions in between active motifs could match the sequence of a known cis-element. Although there is a sequence match, this site may have nothing to do with the regulation of the gene; the sequence may have arisen in this area by chance.

To avoid mis-leading false positivies researchers must carefully filter the results to narrow down the number of putative cis-elements within a promoter region. This is done by analyzing each match to ensure that the specific conditions for the element to work as a regulatory region are in place. Many elements must be within a certain distance of the transcription start site in order to be active. Spacing is critical as elements often act by forming regulatory loops of certain sizes to enhance or silence transcription or interact with components of protein complexes. Some cis-elements are required to be present in multiple copies, and each copy may or may not require specific spacing between repeats in order to interact with

transcriptional machinery or other regulatory regions. Some cis-elements must work in concert with another distinct motif, or a repeat of several other motifs in order to form specific structures within the promoter.

The results presented in this thesis were evaluated to ensure that all cis-elements presented met all known functional criteria. The results were also screened to remove matches that were improbable based on their description in the literature. Although the presented cis-elements were rigourously screened, their inclusion in this analysis does not constitute proof that the elements are functional. There is still a high probability that at least some of the elements exist by chance. Promoter deletion analysis would be helpful in testing these, but this is next to impossible given that winter rye is not transformable.

In its place, semi-quantitative RT-PCR was used to test whether glucanase expression could be predicted by the placement of cis-elements within the promoter. RNA was extracted at specific time points from plants treated with cold, pathogens, wounding, dehydration, MeJa or ethephon. RT-PCR analysis of each sample was performed using primers specific to each of the five glucanases genes.

The promoter of scGlu6 contained motifs involved in disease resistance, seed development, carbon metabolism, light and growth regulator response. There were no cold-related elements within this promoter. Based on the trends and number of cis-elements within this promoter scGlu6 may be involved in seed production, germination, and carbon metabolism within the seed.

No scGlu6 transcripts were detected in any of the samples tested. The primers used in these reactions were known to be gene specific as they were also used for amplification of genomic analyses. The lack of scGlu6 amplification from these cDNA pools suggests two

possibilities – it is either a pseudogene or it is expressed under conditions not tested in these experiments. Although pollinated floral organs were used as an RT-PCR sample to evaluate floral/pollen tube expression, the flower may have been in the wrong stage of seed production for glucanase expression. scGlu6 may be involved in germination, and cDNA pools from young seedlings were not created for this study. In addition, there are a number of other treatments and stresses and different sampling time points that may have resulted in transcript production from this glucanase gene. Another possibility is that scGlu6 is a pseudogene, the regulatory regions of the gene may have mutated to the point where they no longer control transcription initiation. The coding region may encode for a glucanse, but the cellular machinery will not let the gene be realized as a protein. Although it is possible that scGlu6 is a pseudogene, this is the less likely scenario as the published scGlu6 sequence was obtained from a cold-treated cDNA library. A sequence cannot be obtained from a cDNA library unless the transcript was present within the at the time of RNA extraction.

The putative promoters of scGlu9 and scGlu10 contained a diverse array of elements involved in carbon storage, biotic and abiotic stresses, light regulation and development as well as regulation by hormones such as ethylene and MeJa. These promoters contained the greatest number of individual elements related to cold-induced regulation. RT-PCR analysis showed that transcripts of scGlu9 were present after treatment with ethephon and low temperature, and were low after wounding and pathogen infection. Wounding and pathogen infection also regulate scGlu9 mRNA transcript levels, but to a lesser extent. A similar story was found for scGlu10. Promoter analysis shows that scGlu10 contains at least 4 cold-related cis-acting elements along with elements involved in other biotic and abiotic streses, light regulation,

carbon metabolism, and regulation by hormones such as MeJa. This gene is likely transcribed after treatment with cold, dehydration and infection with a pathogen.

Both cold and dehydration conditions induce antifreeze activity (Yu & Griffith, 2001) where as no antifreeze activity is seen in the apoplast of pathogen infected winter rye (Hiilovaara-Teijo *et al.,* 1999). Although both scGlu9 and scGlu10 are expressed under cold and dehydration conditions, neither may be responsible for the acquired antifreeze activity in the apoplast of CA winter rye. Instead, they may be involved in protection against pathogens such as the snow mould *M.nivale* which thrives at cold temperatures. If these proteins act as AFPs, their presence alone may not be enough to contribute antifreeze activity to the plant. Complexes of AFPs involving glucanases, chitinases and thaumatin-like proteins (Yu & Griffith, 1999) or changes to salt concentrations (Stressmann *et al.,* 2003) that are present after cold acclimation may be a requirement for antifreeze activity *in vivo*.

The promoter of scGlu11 contained similar cis-elements to the promoters of the glucanases already discussed. However, the expression pattern of scGlu11 differed from scGlu9 and scGlu10. Only low levels of scGlu11 transcripts were detected in cold-treated plants. This is in contrast to the abundant scGlu11 transcript level present after wounding. Treatment with MeJa and ethephon also resulted in the accumulation of scGlu11 mRNA. The transcript levels after wounding strongly suggest that scGlu11 has a large role in healing damaged plant tissues. Both MeJa and ethylene are involved in wound responses (Ryan & Pearce, 2001) which would agree with scGlu11 RT-PCR transcript abundance estimates. It would be interesting to know if the apoplast of wounded plants possesses antifreeze activity. If it does, then the correlation between the cold response and wound response of scGlu11 would be easy to explain. If the apoplast of wounded plants does not possess antifreeze activity this

may be due to a lack of expression of other proteins, compounds or salts that are essential for antifreeze activity. Another reasonable explanation for the presence of scGlu11 in both cold and wounded tissues is that the process of chilling a plant slightly damages the plant tissues. This damage is far from life threatening, but may be enough to induce proteins that are strongly responsive to wounding.

The promoter of the last gene studied, scGlu12, does not contain many known cis-elements, but those present are involved in biotic and abiotic stresses, light regulation, and carbon metabolism; all are similar to the cis-elements of the other genes. Remarkably, this promoter does not contain any elements specifically related to regulation by MeJa, which is the only treatment that resulted in detectable scGlu12 transcript levels. It is possible that other treatments affects scGlu12 mRNA levels, but this change is triggered at a time outside those tested in this study. Alternatively, treatment with a different pathogen, or changing light levels may lead to mRNA. At the very least, the fact that transcription of scGlu12 was induced by MeJa proves that this is not a pseudogene, as a transcript can be produced and there is nothing within the deduced amino acid sequence to suggest that a functioning protein would not be possible if the transcript were translated.

In summary, these promoters provide an interesting set of cis-elements that suggest glucanases are expressed in response to a variety of signals consistent with their known function.

4.4 Evolution of antifreeze proteins

The ultimate goal of this thesis was to identify cold-responsive glucanse genes and compare their promoter sequences with glucanases not expressed in the cold. The presence of known cis-elements consistent with glucanase expression patterns supports the conclusion that

regulation of transcription initiation is involved in determining the abundance of mRNA transcripts. To appreciate the presented results and those of others studying this topic, it is necessary to have a consistent definition of an antifreeze protein. Plant antifreeze proteins are polypeptides that act *in vivo* to adsorb onto the surface of ice crystals and modify their growth (as reviewed by Griffith & Yaish, 2005). The key to this definition is the phrase '*in vivo'*. Studying a protein that only exhibits antifreeze activity *in vitro* under laboratory conditions and therefore provides no protection to a plant will not advance research in the cold-tolerance field. Yet, for over twenty year researchers have been using a nanoliter osmometer to test for antifreeze activity *in vitro*. It has been assumed that if a plant extracted ice modify in protein can structure ice *in vitro* it must also have this function *in vivo.* This assumption is flawed, but until a procedure to assay for ice-binding *in vivo* is developed, the assumption will stand.

The assumption that proteins that bind to ice *in vitro* must also bind ice *in vivo* is likely true for bacteria, invertebrates and vertebrates that specifically produce antifreeze proteins with the sole purpose of ice-modification. It would be evolutionary unfavorable for these organisms to produce a polypeptide without purpose. The antifreeze proteins in plants are very different from those produced by these non-photosynthetic organisms. With the exception of two species known to produce antifreeze proteins with no homology to any other known protein (Fei *et al.,* 2001; Wang & Wei, 2003, Pudney *et al.,* 2003), over-wintering plants excrete antifreeze proteins with dual activity into their apoplast (reviewed by Griffith & Yaish, 2005). At the same time, these plants will produce nearly identical proteins with similar functions elsewhere in the cell that apparently do not possess antifreeze activity. Or do they? Although there have been studies to show that plants infected with pathogens and those treated with hormones such as ABA and salicylic acid excrete pathogenesis-related proteins without

antifreeze activity into their apoplast, there have been no studies to determine if the PR-proteins located within the cell have antifreeze activity. This raises a number of questions. First, did plant antifreeze proteins evolve from pathogenesis-related proteins or did some pathogenesis-related proteins simply loose their ability to bind ice? Take the family of β-1,3-endoglucanses, for example, not all glucanases are excreted into the apoplast; some work inside the cell for maintenance and repair. Do only these excreted proteins have antifreeze activity?

A recent paper published by Yaish and his colleagues in 2006 provides evidence that glucanases extracted from non-acclimated tissue have a small degree of antifreeze activity if they are at concentrations of 2.5 $mg \cdot mL^{-1}$or higher. This result is contrary to all previous publications on winter rye AFPs. The difference between this study and those done earlier lies in the concentration at which the antifreeze proteins are kept during the antifreeze assay. Other studies routinely tested antifreeze activity at 1 $mg \cdot mL^{-1}$ or at native concentrations which are approximately 0.5 $mg \cdot mL^{-1}$ (Yu & Griffith, 1999. At these concentrations, NA glucanase does not have antifreeze activity (Yaish *et al.,* 2006). It is clear that although NA glucanases do not have the ability to modify ice *in vivo*, these proteins can modify ice *in vitro* if placed at concentrations higher then that found in nature. From this, we can conclude that all glucanases possess some degree of ice-modifying capability which varies between the proteins. This suggests that the glucanases have not gained the ability to modify ice, but some glucanases have gained mutations that enhance this ice modifying ability. It would be interesting to test this hypothesis on purified glucanase from a tropical plant, such as banana.

Glucanases with antifreeze activity are key proteins in winter rye cold-defense, yet winter rye antifreeze activity cannot be attributed to the presence of ice-binding glucanases

alone. Maximum antifreeze activity is only achieved if glucanases are expressed along side of other AFPs and cellular molecules. The evolution of glucanases with antifreeze activity is closely linked to the evolutionary changes that lead to their co-expression with other critical cellular components. The amount of information needed to fully understand the process of cold-acclimation is truly phenomenal. Still, the research presented this thesis provides a clearer understanding of antifreeze activity in winter rye, and the knowledge and suggestions presented offer numerous opportunities for future research on this complex subject.

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