Chitinase Genes Responsive to Cold Encode Antifreeze Proteins in Winter Cereals[1]

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Antifreeze proteins similar to two different chitinases accumulate during cold acclimation in winter rye (*Secale cereale*). To determine whether these cold-responsive chitinases require post-translational modification to bind to ice, cDNAs coding for two different full-length chitinases were isolated from a cDNA library produced from cold-acclimated winter rye leaves. *CHT9* is a 1,193-bp clone that encodes a 31.7-kD class I chitinase and *CHT46* is a 998-bp clone that codes for a 24.8-kD class II chitinase. Chitinase-antifreeze proteins purified from the plant were similar in mass to the predicted mature products of *CHT9* and *CHT46*, thus indicating that there was little chemical modification of the amino acid sequences in planta. To confirm these results, the mature sequences of *CHT9* and *CHT46* were expressed in *Escherichia coli* and the products of both cDNAs modified the growth of ice. Transcripts of both genes accumulated late in cold acclimation in winter rye. Southern analysis of winter rye genomic DNA indicated the presence of a small gene family homologous to *CHT46*. In hexaploid wheat, CHT46 homologs mapped to the homeologous group 1 chromosomes and were expressed in response to cold and drought. We conclude that two novel cold-responsive genes encoding chitinases with ice-binding activity may have arisen in winter rye and other cereals through gene duplication.

Many overwintering plants survive the formation of extracellular ice in their tissues. For example, when winter rye (*Secale cereale*) plants are exposed to subzero temperatures, ice initially forms in intercellular spaces and xylem vessels of the leaves at temperatures ranging from −2°C to −5°C (Pearce, 1988; Pearce and Ashworth, 1992). It is the ability of a plant to withstand this initial freezing process that distinguishes a freezing-tolerant from a freezing-sensitive plant. As the temperature is lowered further, the intercellular ice creates a vapor pressure differential that results in the progressive loss of water from the cell (Burke et al., 1983). The lowest survival temperature of the plant is thought to be related to its capacity to survive this freeze-induced dehydration (Sakai and Larcher, 1987).

During cold acclimation, many freezing-tolerant plants accumulate antifreeze proteins (AFPs; Griffith et al., 1992; Urrutia et al., 1992; Duman and Olsen, 1993; Griffith and Ewart, 1995). AFPs inhibit the growth and recrystallization of intercellular ice by adsorbing onto the surface of ice crystals via van der Waals interactions and/or hydrogen bonds (DeVries, 1986; Knight and Duman, 1986; Ewart et al., 1999). In cold-hardened winter rye leaves, AFPs are present in epidermal cell walls, in cell walls surrounding intercellular spaces, and in the secondary cell walls of xylem vessels of the leaves (Antikainen et al., 1996; Pihakaski-Maunsbach et al., 1996). These are all sites where ice can propagate into and throughout the plant, and so the presence of AFPs may influence the growth of ice in the apoplast.

Upon exposure to cold temperatures, winter rye secretes six AFPs ranging in size from 15 to 35 kD into the apoplast (Griffith et al., 1992; Hon et al., 1994, 1995). These AFPs are not unique proteins (Hon et al., 1995), rather they are similar to pathogenesis-related (PR) proteins that are normally secreted in response to infection by pathogens as part of the mechanism for disease resistance (Stinzi et al., 1993). Two of the rye AFPs are glucanases, two are chitinases, and two are thaumatin-like proteins (Hon et al., 1995). One 35-kD protein that accumulates at cold temperature in the apoplast of winter rye leaves was purified to homogeneity and shown to exhibit both endochitinase and

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antifreeze activities, thus proving that it is a dual function enzyme (Hon et al., 1995). Cold-induced disease resistance and accumulation of PR proteins such as chitinases, osmotin, and polygalacturonase inhibitor protein have been observed in other plants, including barley, potatoes, bermudagrass, and carrots (Tronsmo et al., 1993; Zhu et al., 1993; Gatschet et al., 1996; Ergon et al., 1998; Worrall et al., 1998; Meyer et al., 1999), but with the exception of carrot polygalacturonase inhibitor protein, it is not known whether these PR proteins can also bind to ice. This distinction is an important one because Hiilovaara-Teijo and coworkers (1999) recently showed that winter rye plants infected at warm temperature by snow mold, a pathogen of winter cereals, accumulate PR proteins, including glucanases, chitinases, and thaumatin-like proteins, in the apoplast of winter rye leaves. These PR proteins induced by pathogens have no antifreeze activity, whereas PR proteins that accumulate in the apoplast of rye leaves in response to cold exhibit antifreeze activity and antifungal enzymatic activities.

The goal of this project was to determine the origin of antifreeze activity in the PR proteins that accumulate during cold acclimation in winter rye. We focused our study upon the two chitinase-AFPs (CHT-AFPs) with molecular masses of 28 and 35 kD (Hon et al., 1995). One way that these two winter rye CHT-AFPs may have acquired antifreeze activity is through the evolution of gene sequences encoding variants of chitinases that have both ice-binding domains and catalytic domains. Another possibility is that the mature amino acid sequences of the chitinases are chemically modified at cold temperatures to allow the proteins to bind to ice. The ice-binding domain alternatively may arise from a combination of events in which the products of variant chitinase genes are also chemically modified. Our specific objectives were to clone and characterize the genes encoding CHT-AFPs from winter rye. After the isolated cDNAs were sequenced to confirm that they encoded two different cold-responsive chitinases, they were expressed in heterologous hosts and assayed for antifreeze activity to prove that the translation products bind to ice. To determine whether further chemical modifications of the mature proteins were involved in binding to ice, the masses of the 35- and 28-kD CHT-AFPs purified from winter rye were determined by mass spectrometry and compared with the predicted translation products of the cDNAs. Our results show that the cDNAs *CHT9* and *CHT46* encode dual function ice-binding chitinases that do not require further chemical modification for their activities.

RESULTS

Strategy to Isolate Chitinase cDNAs from Winter Rye

Plant chitinases have been categorized into at least four classes (Collinge et al., 1993; Beintema, 1994; Graham and Sticklen, 1994; Meins et al., 1994). Class I chitinases are composed of three domains: a Cys-rich chitin-binding domain, a Pro-rich hinge region, and a highly conserved catalytic domain. Class II chitinases lack the hinge region and chitin-binding domain, but their catalytic domain is nearly identical to Class I chitinases. Class III lack the chitin-binding domain and have little sequence identity to the Class I catalytic domain. Class IV chitinases contain the Cys-rich chitin-binding domain with little homology to the Class I catalytic domain. All plant chitinases have an N-terminal or C-terminal targeting sequence that directs them first to the ER and then to either the vacuole or the apoplast. In earlier experiments, the 35-kD rye CHT-AFP was shown to have endochitinase activity and to bind to colloidal chitin (Hon et al., 1995), so we expected it to be a Class I chitinase. The 28-kD CHT-AFP was expected to be a Class II chitinase because it did not bind to the chitin-affinity column, yet it was immunodetected by the same antiserum as the 35-kD CHT-AFP (Hon et al., 1995; Antikainen et al., 1996). We reasoned that genes encoding both rye CHT-AFPs could be recovered using the conserved catalytic domain as a probe and used the cDNA *HvCHT2a* (GenBank accession no. X78671), which encodes a basic class II barley chitinase, to screen the cold-acclimated (CA) winter rye leaf cDNA library. On northern blots performed under high stringency conditions, p*HvCHT2a* hybridized with transcripts of two different sizes isolated from CA winter rye leaves (data not shown). These transcripts were 1.25 and 1.00 kb, which corresponded to the predicted transcript sizes for the 35- and 28-kD rye CHT-AFPs, respectively.

Approximately 92,700 colonies from the λ-Zap-cDNA library made from poly(A)$^+$ mRNA isolated from CA winter rye leaves were screened with p*HvCHT2a.* Forty-eight putative clones were identified, which were denoted recombinant plasmids p*CHT1* to p*CHT48*. Preliminary restriction mapping revealed insert sizes ranging from 305 bp to 2,655 bp. Analysis of the clones by G-tracking (Sanger et al., 1977), by rehybridizing with p*HvCHT2a* at high stringency, and by choosing clones with inserts greater than 950 bp revealed two distinct groups of cDNAs encoding chitinases that accounted for 23 of the positives. *CHT9* (GenBank accession no. AF280437) and *CHT46* (GenBank accession no. AF280438) were sequenced completely because they were the longest representative clones from the two groups.

Characterization of *CHT9*

The cDNA *CHT9* was isolated from a group of four positive clones and was 1,193 bp in length with a poly(A)$^+$ tail. The predicted open reading frame contained 318 amino acids that began 48 bp downstream from the 5′ terminus of the insert and ended with the stop codon 955 bp from the initiating Met codon (Fig.

21). PSORT and SignalP analyses indicated with a certainty of 82% that there was a signal sequence of 20 amino acids that targets the protein via a vesicular pathway to the outside of the cell (Nielsen et al., 1997). There were 190 bp of non-coding region at the 3′ terminus. The predicted mature gene product of *CHT9* consisted of 301 amino acids with a molecular mass of 31.7 kD and a pI of 6.96. The mature form of CHT9 was identical to the known sequence of 32 amino acids at the N terminus of the native 35-kD CHT-AFP (Hon et al., 1995).

Characterization of *CHT46*

Although there were 19 clones that could encode the 28-kD CHT-AFP, we sequenced the longest cDNA of this group completely in both directions and found that *CHT46* encoded a full-length chitinase. *CHT46* had a 998 bp insert with 78 bp upstream from the first ATG that initiated the open reading frame of 252 amino acids, followed by 163 bp of 3′-untranslated region (Fig. 2). The predicted gene product had a molecular mass of 26.8 kD and a pI of 8.25. PSORT and SignalP predicted that this protein

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-40                                                  CACAATCAGCAGCTGAGCGGACTCTGCTTCATTGCCCAAG -1

  1 ATG AGA GGA GTT GTG GTG GTG GCC ATG CTG GCC GCG GCC TTC GCC GTG 48
    M  R  G  V  V  V  V  A  M  L  A  A  A  F  A  V
 49 TCT GCA CAC GCC GAG CAG TGC GGC TCG CAG GCC GGC GGG GCG ACG TGC 96
    S  A  H  A  E  Q  C  G  S  Q  A  G  G  A  T  C
 97 CCC AAC TGC CTC TGC TGC AGC AAG TTC GGC TTC TGC GGC TCC ACC TCC 144
    P  N  C  L  C  C  S  K  F  G  F  C  G  S  T  S
145 GAG TAC TGC GGC GAC GGC TGC CAG AGC CAG TGC AAC CGC TGC GGC GGC 192
    E  Y  C  G  D  G  C  Q  S  Q  C  N  R  C  G  G
193 ACA CCA GTA CCG GTA CCG ACC CCC ACC GGC GGC GGC GTG TCC TCC ATT 240
    T  P  V  P  V  P  T  P  T  G  G  G  V  S  S  I
241 ATC TCG CAG TCG CTC TTC GAC CAG ATG CTG CTG CAC CGC AAC GAT GCG 288
    I  S  Q  S  L  F  D  Q  M  L  L  H  R  N  D  A
289 GCG TGC CTG GCC AAG GGG TTC TAC AAC TAC GGC GCC TTC ATC GCC GCC 336
    A  C  L  A  K  G  F  Y  N  Y  G  A  F  I  A  A
337 GCC AAC TCG TTC TCG GCG TTC GCG ACC ACG GGT GGC ACC GAC GTC AGG 384
    A  N  S  F  S  A  F  A  T  T  G  G  T  D  V  R
385 AAG CGC GAG GTG GCC GCG TTC CTA GCT CAG ACC TCC CAC GAG ACC ACC 432
    K  R  E  V  A  A  F  L  A  Q  T  S  H  E  T  T
433 GGC GGG TGG CCC ACG GCG CCC GAC GGC CCC TAC TCG TGG GGC TAC TGC 480
    G  G  W  P  T  A  P  D  G  P  Y  S  W  G  Y  C
481 TTC AAC CAG GAG CGC GGC GCC CCC TCC GAC TAC TGC TCG CCG AGC TCG 528
    F  N  Q  E  R  G  A  P  S  D  Y  C  S  P  S  S
529 CAG TGG CCG TGC GCG CCG GGC AAG AAG TAC TTC GGG CGC GGG CCC ATC 576
    Q  W  P  C  A  P  G  K  K  Y  F  G  R  G  P  I
577 CAG ATC TCA TAC AAC TAC AAC TAC GGG CCG GCG GGG CGG GCC ATC GGC 624
    Q  I  S  Y  N  Y  N  Y  G  P  A  G  R  A  I  G
625 ACG GAC CTA CTC AAC AAC CCA GAC CTC GTG GCC ACG GAC GCC ACC GTG 672
    T  D  L  L  N  N  P  D  L  V  A  T  D  A  T  V
673 TCA TTT AAG ACG GCA CTG TGG TTC TGG ATG ACG CCG CAG TCA CCA AAA 720
    S  F  K  T  A  L  W  F  W  M  T  P  Q  S  P  K
721 CCT TCG AGC CAC GAC GTG ATC ACG GGC CGG TGG AGC CCC TCG GGC GCC 768
    P  S  S  H  D  V  I  T  G  R  W  S  P  S  G  A
769 GAC CAG GCG GCG GGG AGG GTG CCT GGG TAC GGC GTG ATC ACC AAC ATC 816
    D  Q  A  A  G  R  V  P  G  Y  G  V  I  T  N  I
817 ATC AAC GGT GGG CTC GAG TGC GGG CGC GGG CAG GAT GCT CGT GTC GCC 864
    I  N  G  G  L  E  C  G  R  G  Q  D  A  R  V  A
865 GAC CGA ATC GGG TTC TAC AAG CGC TAC TGT GAC CTC CTC GGC GTC ACG 912
    D  R  I  G  F  Y  K  R  Y  C  D  L  L  G  V  T
913 TAC GGC GAC AAT CTG GAC TGC TAC AAC CAG AGG CCG TTC GCA TAG TCA 960
    Y  G  D  N  L  D  C  Y  N  Q  R  P  F  A  *

961 ATCGGCTATATGATGCGAGAAGACATCCAATAATCGCTCTCTAAATATTGTAACAATGGCGTC 1023
1024 GCGATAGACCTCGCCTAGTACTCGTACAAGCGAATAATGGTGCAATCCATCGCAAGATGCGTG 1086
1087 CGTACTCAACTATAATGCAATAAATGGTGTGTTCCTATACAAAAAAAAAAAAAAAAAAA      1145
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Figure 1. The nucleotide and deduced amino acid sequences of *CHT9*. The nucleotide sequence (1,193 bp) is numbered relative to the first nucleotide in the translational start codon ATG and the stop codon is indicated by an asterisk. The predicted amino acid sequence is shown directly below the nucleotide sequence with the predicted first amino acid of the mature protein shown in bold and the signal sequence shown in italics. The region used for the gene-specific probe (30-mer oligonucleotide) is underlined.

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-78                                                                  CGGCACGAGCAAGCT -64

-63 CTCCGCTCTCCTTGAGCTATACTCCATTGCACGAGCTGATCAGCTGCAGGTGAGGTGGTGAGC -1

  1 ATG GCG CGG TTT GCT GCG CTC GCC GCG CTT CTT CTC GCC GTG GCG GTG 48
  1 M  A  R  F  A  A  L  A  A  L  L  L  A  V  A  V  16
 49 GGC GGC GCC GCG GCG CAG AGC GTG GGC TCC GTC ATC ACG CAG TCC ATG 96
 17 G  G  A  A  A  Q  S  V  G  S  V  I  T  Q  S  M  32
 97 TAC GCG AGC ATG CTG CCC AAC CGC GAC AAC TCG CTG TGC CCG GCC AGG 144
 33 Y  A  S  M  L  P  N  R  D  N  S  L  C  P  A  R  48
145 GGG TTC TAC ACG TAC GAC GCC TTC ATC GCC GCC GCC AAC ACC TTC CCG 192
 49 G  F  Y  T  Y  D  A  F  I  A  A  A  N  T  F  P  64
193 GGC TTC GGC ACC ACC GGC AGC ACG GAC GAC GTC AAG CGC GAG GTC GCC 240
 65 G  F  G  T  T  G  S  T  D  D  V  K  R  E  V  A  80
241 GCC TTC TTC GGC CAG ACC TCA CAC GAG ACC ACC GGA GGG ACT AGA GGC 288
 81 A  F  F  G  Q  T  S  H  E  T  T  G  G  T  R  G  96
289 GCC GCC GAC CAG TTC CAG TGG GGC TAC TGC TTC AAG GAG GAG ATA AAC 336
 97 A  A  D  Q  F  Q  W  G  Y  C  F  K  E  E  I  N  112
337 AAG GCC ACG TCT CCA CCC TAC TAC GGA CGG GGA CCC ATC CAA TTG ACA 384
113 K  A  T  S  P  P  Y  Y  G  R  G  P  I  Q  L  T  128
385 GGG CGG TCC AAC TAC GAT CTC GCC GGG AGA GCC ATC GGG AAG GAC CTG 432
129 G  R  S  N  Y  D  L  A  G  R  A  I  G  K  D  L  144
433 GTG AGC AAC CCG GAC CTG GTG TCC ACG GAC GCG GTG GTT TCC TTC AGG 480
145 V  S  N  P  D  L  V  S  T  D  A  V  V  S  F  R  160
481 ACG GCC ATG TGG TTC TGG ATG ACG GCG CAG GGC AAC AAG CCA TCG TCC 528
161 T  A  M  W  F  W  M  T  A  Q  G  N  K  P  S  S  176
529 CAC GAC GTC GCC CTC CGC CGC TGG ACG CCG ACG GCT GCC GAT AAC GCT 576
177 H  D  V  A  L  R  R  W  T  P  T  A  A  D  N  A  192
577 GCG GGT CGG GTC CCT GGG TAC GGC GTA ATC ACC AAT ATC ATC AAC GGC 624
193 A  G  R  V  P  G  Y  G  V  I  T  N  I  I  N  G  208
625 GGG CTC GAG TGC GGC ATG GGC CGG AAC GAC GCC AAC GTC GAC CGC ATC 672
209 G  L  E  C  G  M  G  R  N  D  A  N  V  D  R  I  224
673 GGC TAC TAC ACA CGC TAC TGC GGC ATG CTT GGC ACG GCC ACC GGG GGC 720
225 G  Y  Y  T  R  Y  C  G  M  L  G  T  A  T  G  G  240
721 AAC CTC GAC TGC TAC ACC CAG CGA AAC TTC GCT AGC TAG ACA GTG TAT 768
241 N  L  D  C  Y  T  Q  R  N  F  A  S  *                        256

769 GCACGTGTTATAAATAAATGGCAATGCATATGCCATCCCCGAATAAATAATTCAACATGTGAC 831

832 AGTTGATTTGTATGGTAATACGAGTAAGTTGTTGCAACAAATTATGAATATTGAATAAAATCA 894

895 AATTTTATCAAAAAAAAAAAAAAAA                                        920
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Figure 2. The nucleotide and deduced amino acid sequences of *CHT46*. The nucleotide sequence (998 bp) is numbered relative to the first nucleotide in the translational start codon ATG and the stop codon is indicated by an asterisk. The predicted amino acid sequence is shown directly below the nucleotide sequence with the predicted first amino acid of the mature protein shown in bold and the signal sequence shown in italics. The region used for the gene-specific probe (a fragment generated by *Xho*I digestion) is underlined.

had a 21-amino acid signal peptide that directed CHT46 to the outside of the cell through the vesicular pathway with a certainty of 82%. The mature gene product of CHT46 was predicted to consist of 231 amino acids with a mass of 24.9 kD and a pI of 7.78.

Sequence Comparison of *CHT9* and *CHT46* Does Not Identify the Ice-Binding Domain

BLAST searches confirmed that we had isolated cDNAs encoding a class I and a class II chitinase from the CA winter rye library (Fig. 3), as we had predicted from the results of earlier experiments. The *CHT46* sequence was 85% identical at the nucleotide level to p*HvCHT2a,* which encodes a barley class II chitinase, and 96% identical at the amino acid level. The predicted amino acid sequence of *CHT9* exactly matched the known N-terminal amino acid sequence of the native 35-kD rye CHT-AFP (Hon et al., 1995) and was most similar to a class I chitinase from Chinese Spring wheat (*Triticum aestivum;* Liao et al., 1994; GenBank accession no. X76041) with 96% and 92% identity at the nucleotide and amino acid levels, respectively. Sequence comparisons between the mature forms of *CHT9* and *CHT46* revealed they shared

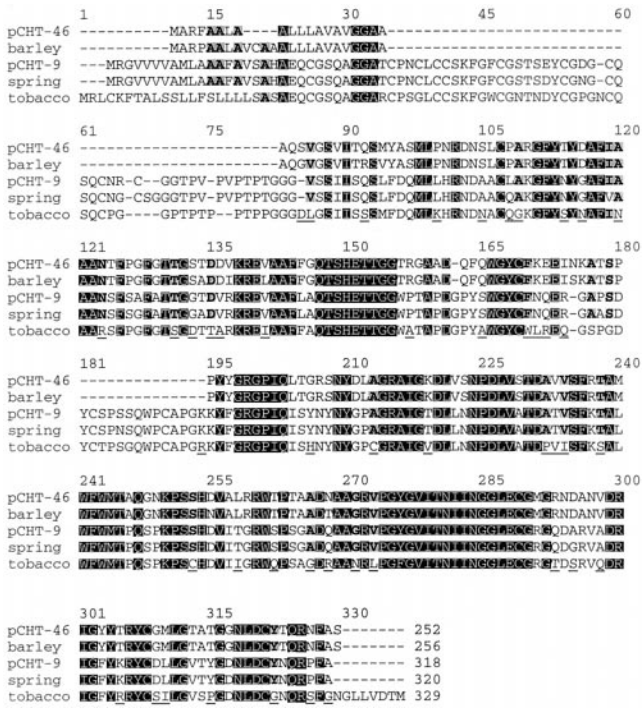

Figure 3. Multiple sequence alignment of amino acid sequences for chitinases from rye and other species. The predicted amino acid sequences for CHT9 and CHT46 were aligned with the barley chitinase HvCHT2a (T. Bryngelsson, personal communication, GenBank accession no. X78671), a tobacco chitinase (Shinshi et al., 1990; GenBank accession no. X16939), and a Chinese spring wheat chitinase (Liao et al., 1994, GenBank accession no. X76041). Identical amino acids are highlighted on a black background, similar amino acids are shown on a gray background, and dashes indicate gaps. Within the mature proteins, amino acids that differ between tobacco, which lacks antifreeze activity, and both CHT9 and CHT46, which exhibit antifreeze activity, are underlined.

57% identity at the nucleotide level and 49% identity at the amino acid level.

If the antifreeze activity were associated with a novel domain introduced into the rye CHT-AFPs, we would expect to see an amino acid sequence that was similar between CHT9 and CHT46, but not found in other chitinases lacking antifreeze activity. Therefore, we compared the rye chitinase sequences with a class I tobacco chitinase sequence because we had previously demonstrated that this tobacco chitinase lacks antifreeze activity (Hon et al., 1995). As shown in Figure 3, there does not appear to be a specific domain that is the same in CHT9 and CHT46 and different for tobacco that could account for antifreeze activity. The ice-binding domains of AFPs from fish have only been identified by solving the three-dimensional structures of the proteins and determining the surface features that interact with ice. In native AFPs, the ice-binding domain has been characterized as a flat area on the surface of the proteins with polar residues spaced at regular intervals to allow the formation of hydrogen bonds with the ice crystal lattice (Sicheri and Yang, 1995; Sönnichsen et al., 1998). This three-dimensional structure can be achieved by folding many different primary amino acid sequences, so the lack of an ice-binding domain in the primary sequences of the chitinases was expected.

Post-Translational Modification of CHT-AFPs Does Not Account for Antifreeze Activity

The 35-kD chitinase purified from apoplastic extracts of CA rye plants had a molecular mass of 31,693 ± 7 D when examined by mass spectrometry (Fig. 4). It was unfortunate that we obtained various adducts of the protein that diminished the accuracy

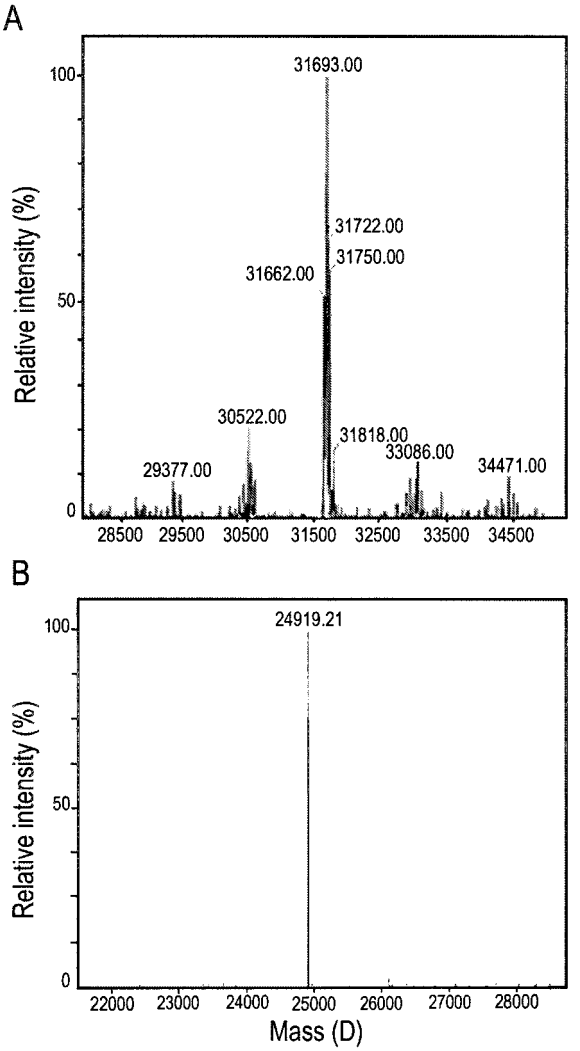

Figure 4. Molecular masses of apoplastic CHT-AFPs purified from CA winter rye leaves determined by mass spectrometry. The two rye CHT-AFPs were purified by column chromatography and their identities were confirmed by SDS-PAGE, immunoblotting, and the presence of antifreeze activity. Samples containing 0.8 μg of protein were examined by mass spectrometry. Raw data (*m/z*) were processed with the MaxEnt algorithm of MassLynx 2.0 to yield spectra on a true molecular mass scale. A, The molecular mass of the 35-kD CHT-AFP was 31,693 ± 7 D. B, The molecular mass of the 28-kD CHT-AFP was 24,919 ± 3 D.

of the mass determination (Fig. 4A). The mass of the purified protein was only 34 D larger than the translated sequence of the mature form of *CHT9*, which had a predicted size of 31,659 D, assuming that all the cysteines were oxidized. Class I chitinases were previously reported to be post-translationally modified by hydroxylation of the prolines present in the hinge region of the protein (Sticher et al., 1992, 1993). The increased mass of the CHT-AFP purified from rye leaves over the predicted translated sequence was consistent with hydroxylation of two of the four Pro residues found in the hinge region. The presence of hydroxy-Pro in chitinases also causes the proteins to migrate in SDS-PAGE as though they were several kilodaltons larger than the corresponding unmodified proteins (Sticher et al., 1993), which would explain why this CHT-AFP migrated as a larger polypeptide in SDS-PAGE.

The molecular mass of the 28-kD chitinase purified from CA winter rye leaves was determined by mass spectrometry to be 24,919 ± 3 D (Fig. 4B). The translation product of *CHT46* had a predicted molecular mass of 24,920 D, thus demonstrating that the CHT46-AFP was not post-translationally modified after removal of the signal sequence.

Expression of *CHT9* and *CHT46* Is Cold Responsive in Winter Rye Leaves

Gene-specific probes for *CHT9* and *CHT46* were designed from their respective 3′-untranslated regions (Figs. 1 and 2). The *CHT9* probe hybridized to a 1.25-kb transcript, whereas the probe for *CHT46* hybridized to a 1.0-kb transcript (data not shown). As shown in Figure 5A, *CHT9* transcripts were not detectable by the gene-specific probe in nonacclimated (NA) leaf tissue and accumulated to a high level only after 5 weeks of cold acclimation. *CHT46* transcripts were present at a low level in NA tissues and increased dramatically in response to low temperature, but only after 5 to 7 weeks of cold acclimation. When rye plants were returned to 20°C to de-acclimate, the transcripts for *CHT9* and *CHT46* were undetectable after 30 h (Fig. 5B), thus indicating that transcription of both genes is responsive to cold temperature.

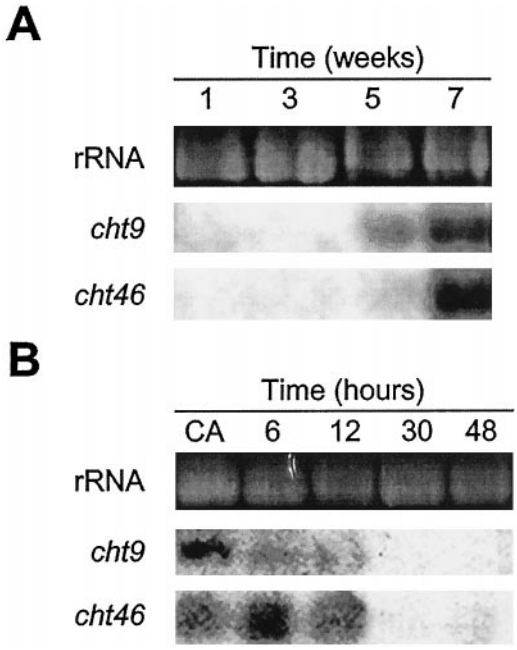

Figure 5. Expression of *CHT9* and *CHT46* in winter rye during cold acclimation and deacclimation. Total RNA was isolated from the youngest leaf tissue of plants at each time point. The RNA (10 μg) was denatured at 65°C for 15 min, separated in a formaldehyde 1.4% (w/v) agarose gel, and stained with ethidium bromide. The 25S rRNA of the ethidium-stained gel is shown above. The RNA was transferred to a nylon membrane and hybridized with radiolabeled gene-specific probes for *CHT9* and *CHT46*. The sizes of the transcripts were estimated using the RNA standard markers as indicated on the left. A, Winter rye plants were grown under cold-acclimating conditions for 1, 3, 5, and 7 weeks. Neither chitinase gene was expressed immediately in plants transferred to cold temperature. Instead, *CHT9* was detected as a 1.25-kb transcript and *CHT46* was detected as a 1.00-kb transcript in leaves only after the plants had been cold acclimated for at least 5 weeks. B, Plants that had been cold acclimated for 7 weeks were transferred back to 20°C for 6, 12, 30, and 48 h to deacclimate. The transcripts for *CHT9* and *CHT46* were not detectable within 30 h of transferring the plants to 20°C, thus indicating that expression of these genes is responsive to cold temperature.

CHT9 and *CHT46* Expressed in *Escherichia coli* Exhibit Antifreeze Activity

CHT9 was cloned into the bacterial expression vector pET12a, which contains the leader sequence of outer membrane protein T (OmpT) to target the CHT-AFPs to the periplasmic space. The leader sequence is necessary, but it is not sufficient for export into the periplasm because translocation also depends on the mature domain of the target protein. When polypeptides from BL21(DE3) cells transformed with pET12a/CHT9 were examined by SDS-PAGE, a 35-kD polypeptide accumulated in the periplasmic fraction after induction by isopropylthio-β-D-galactoside (IPTG) (Fig. 6A). Although the periplasmic fraction contained some of the CHT-AFPs, the majority of the protein remained intracellular (data not shown). Figure 6B shows that the periplasmic fraction exhibited hexagonally shaped ice crystals, thus indicating a low level of antifreeze activity. After the extract was concentrated 1,000-fold, a high level of antifreeze activity was observed as the ice crystals grew into hexagonal bipyramids with straight faces (data not shown). Parallel experiments conducted with the vector-control culture induced by IPTG showed no evidence of antifreeze activity (data not shown).

CHT46 was cloned into an expression vector designed to secrete the AFP with a His-tag from *E. coli* cells. However, the protein was not secreted into the

medium, rather a polypeptide approximately 29 kD in molecular mass accumulated in the soluble fraction of whole cell lysates after induction by IPTG. The purified protein had no antifreeze activity. After removal of the OmpA targeting sequence and the His tag by digestion with thrombin (Fig. 7A), the protein (0.06 mg protein mL^{-1}) exhibited antifreeze activity as shown by the formation of hexagonal ice crystals (Fig. 7B). Antifreeze assays of the protein after further concentration of the solution by ultrafiltration (1.35 mg protein mL^{-1}) yielded hexagonal ice crystals with clear evidence of *c*-axis growth (Fig. 7B). As observed with CHT9, no antifreeze activity was observed using the vector-control culture induced by IPTG in parallel experiments (data not shown).

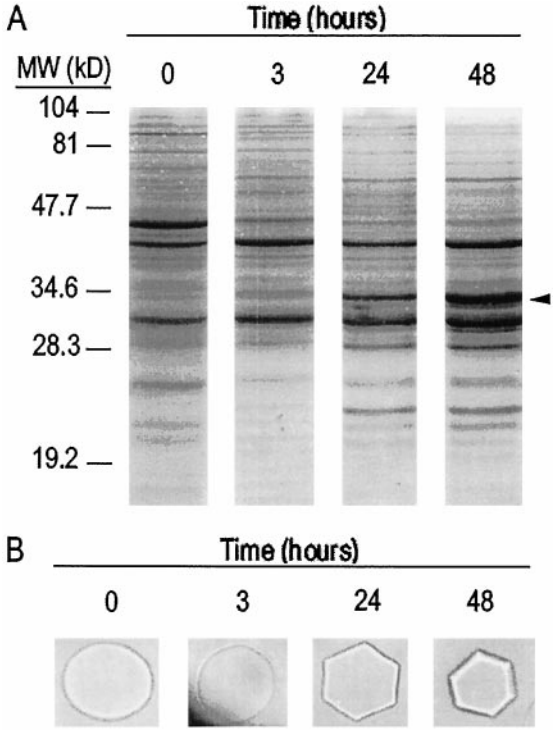

Figure 6. Expression of pET12α/*CHT9*-12 in *E. coli* BL21 cells and antifreeze activity of CHT9. A, Lanes 1 through 4 show the accumulation of the 35-kD CHT9 (arrowhead) in the periplasmic fraction of cells where expression of pET12α/*CHT9*-12 was induced by IPTG and the culture was incubated at 25°C for 0, 3, 24, and 48 h. Periplasmic proteins (20 μg $lane^{-1}$) isolated from the cells at each time point were solubilized and separated by SDS-PAGE on a 12% (w/v) polyacrylamide gel stained with Coomassie Blue. The molecular masses of Bio-Rad protein standards are shown on the left. B, Antifreeze activity was assayed by observing the growth of ice crystals in solutions of the periplasmic fraction isolated at each time point. The crystals are shown with the basal plane (*a*-axes) parallel to the plane of the page and the *c*-axis perpendicular to the page. The total protein concentration in each solution was adjusted to 1.2 mg mL^{-1}. The formation of hexagonally shaped ice crystals was observed 24 h after induction of pET12α/*CHT9*-12 expression. This increase in antifreeze activity was correlated with the accumulation of a 35-kD protein in the periplasm.

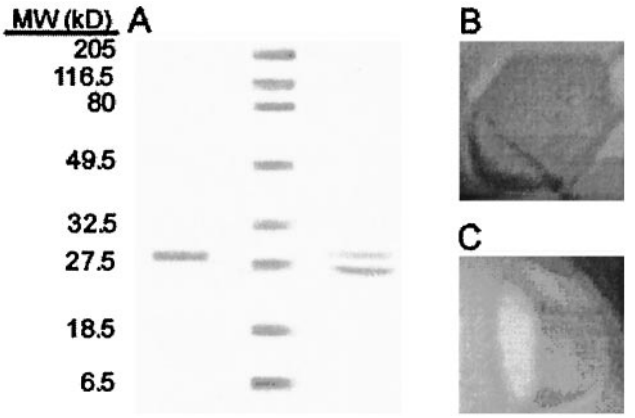

Figure 7. Purification and antifreeze activity of CHT46 produced by expressing OmpA/His6/*CHT46* in *E. coli* JM105 cells. A, As shown in the left lane, OmpA-His6-CHT46 (1.2 μg) was purified from cell lysates, solubilized, and separated on a SDS-polyacrylamide gel (12%, w/v) stained with Coomassie Blue. The right-hand lane shows the presence of CHT46 after thrombin cleavage of OmpA-His6-CHT46. The positions of Bio-Rad broad range prestained molecular mass protein standards (center lane) are shown on the left. B, After cleavage of the OmpA leader and His-tag, hexagonally shaped ice crystals grew during freezing of CHT46 solutions containing 0.06 mg of total soluble protein mL^{-1} (ice crystal shown with *c*-axis perpendicular to the plane of the page). C, After concentrating the solution containing CHT46 to 1.35 mg of total soluble protein mL^{-1}, the ice crystals grew to form hexagonally shaped columns (in the crystal shown, the *c*-axis is parallel to the plane of the page). The increased *c*-axis growth of the ice crystal indicates a greater amount of antifreeze activity.

CHT46 Has Homologs in the Rye and Wheat Genomes

In Figure 8, the rye and wheat genomes were examined using the full-length *CHT46* cDNA as a probe of a Southern blot under very stringent hybridization and washing conditions. Both cultivars of winter rye, Musketeer and Puma, exhibited three strongly hybridizing fragments, indicating that there is a small family of *CHT46*-related genes in the rye genome. The Southern blot also revealed that the Chinese Spring wheat genome contained at least six fragments that strongly hybridized with the *CHT46* probe (Fig. 8). Chinese Spring wheat is a hexaploid wheat containing three genomes designated A, B, and D, with seven chromosome pairs per genome (AABBDD, 42 chromosomes; Kimber and Sears, 1987). Therefore, the strongly hybridizing fragments may represent multiple homologs of *CHT46* per genome.

One advantage of examining the wheat genome is that diteliocentric lines of Chinese Spring wheat are available to map the CHT46 homologs to specific arm(s) of each of the 21 chromosomes. In these wheat lines, the presence of the long or short arm of the chromosome is indicated by L or S, respectively. With the use of the ditelocentric series, we mapped all six fragments (48, 18, 16, 15.5, 14, and 13.2 kb) to the long arms of the homeologous group 1 chromosomes of all three genomes (A, B, and D) of hexaploid

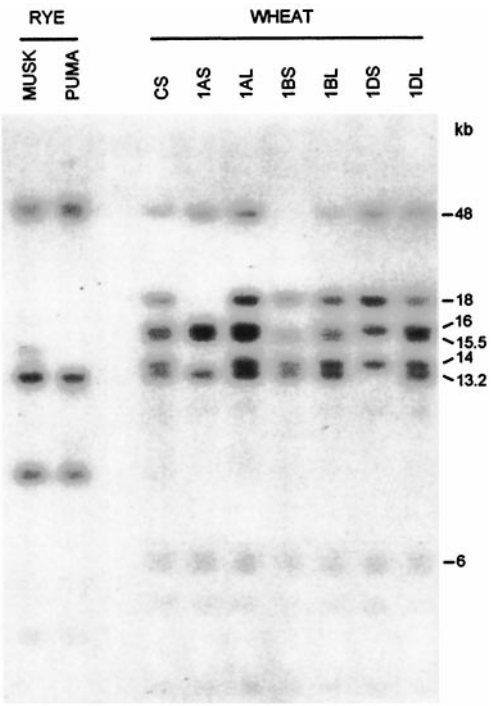

Figure 8. Estimation of gene copy number and mapping of *CHT46* homologs in winter cereals. DNA (1.8 μg) from winter rye cultivars Musketeer (MUSK) and Puma (PUMA), from the wheat cultivar Chinese Spring (CS), and from the group 1 chromosome ditelocentric series of Chinese Spring (1AS, 1AL, 1BS, 1BL, 1DS, and 1DL) were digested with *Xba*I. The last wash after hybridization of the membranes was in 0.2× SSC containing 0.1% (w/v) SDS at 68°C for 1 h. Numbers at right indicate estimated lengths of the fragments in kilobases.

wheat (Fig. 8). Fragments of 18 and 14 kb were missing from the chromosome 1AS line, fragments of 48 and 16 kb were missing from the chromosome 1BS line, and fragments of 15.5 and 13.2 kb were missing from the chromosome 1DS line. Because the S lines lack the long arms of the chromosomes, the missing restriction fragments indicated that the CHT-AFP homologs were located on the long arms of group 1 chromosomes.

CHT46 Homologs in Winter Wheat Are Responsive to Cold and Drought

We have previously shown by immunoblotting that winter wheat accumulates at least six CHT-AFPs during cold acclimation (Antikainen and Griffith, 1997; Chun et al., 1998). These may correspond to homologs of both CHT9 and CHT46 in hexaploid wheat. However, after probing northern blots of wheat RNA with the *CHT46* cDNA under high stringency, we obtained an intense, broad signal that likely represents the presence of several chitinase transcripts corresponding only to *CHT46* homologs (Fig. 9).

Transcripts that hybridized with the cDNA for *CHT46* were detected at high levels in the leaves (13-fold greater than NA leaves) and to a lesser extent in crowns (6-fold greater than NA leaves), but not in the roots of winter wheat plants that were cold acclimated for 36 d (Fig. 9). Expression was also examined in the leaves of winter wheat plants subjected to a variety of environmental stresses (Fig. 9). A low level of constitutive expression of genes encoding chitinases was apparent in the leaf RNA of NA, CA for 1 d, root, heat shock, salt, abscisic acid (ABA), low-light, and high-light plants. When compared with expression in NA plants, transcript levels in leaves increased 5-fold 6 d after plants were transferred to 5°C and 13-fold 36 d after the transfer, thus indicating that transcripts accumulated as long as the wheat plants were exposed to cold temperature. The signal decreased dramatically after deacclimation at 20°C for just 1 d. Transcripts that hybridized to *CHT46* were also detected at a level 5-fold greater in the leaves of winter wheat plants exposed to drought than observed in well-watered NA plants (Fig. 9).

DISCUSSION

CHT9 and *CHT46* Encode Cold-Responsive Chitinase-AFPs in Winter Rye

A molecular approach was used to determine if the rye CHT-AFPs are encoded by variants of chitinase genes. Two chitinase clones *CHT9* and *CHT46* were isolated from a cDNA library generated from

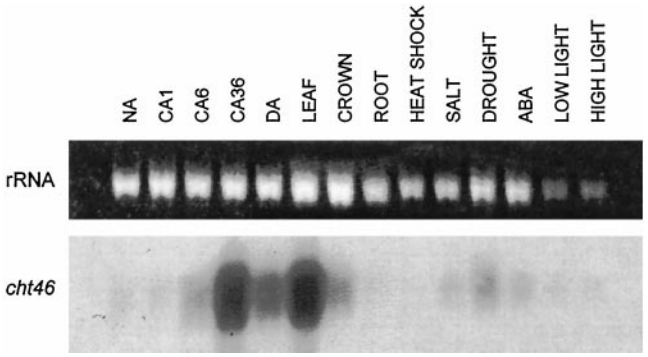

Figure 9. Developmental, environmental, and hormonal regulation of expression of *CHT46* homologs in winter wheat. Total RNA was isolated from the winter wheat cv Frederick grown at 20°C for 7 d (NA) at 5°C for 1, 6 and 36 d (CA1, CA6, and CA36, respectively), and deacclimated at 20°C for 1 d (DA). To examine tissue specificity, total RNA was isolated from CA36 plants divided into leaves, crowns, and roots. In addition, RNA was isolated from plants subjected to heat shock, salt stress, drought stress, low light (250 μmol photons m^{-2} s^{-1}), and high light (800 μmol photons m^{-2} s^{-1}), as well as plants treated with ABA. Northern blots were probed with p*CHT46* at high stringency. There was a low level of chitinase gene expression in NA, CA1, root, heat shock, salt, ABA, low light, and high light plants. Genes that hybridized to *CHT46* were up-regulated by cold (CA) and drought in leaves and crowns. The hybridization signal produced by CA plants was significantly reduced after transferring the plants back to 20°C to deacclimate (DA).

poly(A)$^+$-mRNA purified from CA rye leaves (Figs. 1 and 2). Our experiments show that these two cDNAs encode the two rye CHT-AFPs for the following reasons. First of all, the cDNAs encode chitinases because they exhibit a high level of identity with genes coding for chitinases from other plants (Fig. 3). Second, the products of the two clones correspond in molecular mass and/or N-terminal amino acid sequence to the two chitinases with antifreeze activity that accumulate during cold acclimation in winter rye leaves (Hon et al., 1995). Third, by using gene-specific probes, we showed that transcripts of *CHT9* and *CHT46* are both present at higher levels in plants grown at low temperature (Figs. 5 and 6). Fourth, the products of the two cDNAs exhibit antifreeze activity when they accumulate in *E. coli*. And last, the two cDNAs encode proteins with signal sequences that are predicted to target them for secretion via the vesicular pathway. This result agrees with earlier observations obtained by immunogold localization and electron microscopy that glucanase-AFPs (Pihakaski-Maunsbach et al., 1996) and CHT-AFPs (K. Pihakaski-Maunsbach, personal communication) are secreted from cells through a pathway that leads from the endoplasmic reticulum to Golgi bodies and then to the plasmalemma via vesicles. All these results lead us to the conclusion that the CHT-AFPs are encoded by variants of chitinase genes whose expression is responsive to cold temperature and whose products are targeted to the apoplast.

Are the AFPs also post-translationally modified beyond processing of the signal sequence? AFPs from Antarctic fish (DeVries, 1986), plants (Duman, 1994), and bacteria (Xu et al., 1998) are known to be glycosylated and the sugar residues are thought to interact with ice. However, the difference of 34 D between the molecular mass of the 35-kD class I CHT-AFP purified from CA rye leaves and the predicted size of the translated sequence of *CHT9* shows that this CHT-AFP is not glycosylated. The Pro residues may be hydroxylated, but we have previously shown that the tobacco class I chitinase, which is also hydroxylated (Sticher et al., 1992), does not have antifreeze activity (Hon et al., 1995). Therefore, we concluded that hydroxylation alone does not confer the ability of the protein to bind to ice. Moreover, we observed no chemical modification of the mature form of 28-kD CHT-AFP. At this point, our data indicate that ice-binding activity originates from the specific primary amino acid sequences of CHT46 and CHT9, but we have yet to identify the specific ice-binding domain.

Genes Encoding Chitinase-AFPs May Be Developmentally Expressed at Cold Temperature

Previous studies showed that the various AFPs accumulate at different rates in winter rye (Hon et al., 1995). Glucanase-AFPs accumulate first, followed by thaumatin-like AFPs, and finally CHT-AFPs. As shown in Figure 5, the steady-state transcript levels of *CHT9* and *CHT46* increase only after winter rye plants have been exposed to cold temperature for at least 5 weeks, which may be due to increased transcription and/or increased stability of the transcripts at low temperature. The transcripts then accumulate as long as the plants are held at cold temperature and degrade rapidly when the plants are shifted to warm temperature (Fig. 5). In contrast, the low temperature- and drought-responsive element found in cold-regulated genes isolated from Arabidopsis is activated within hours of a shift to cold temperature (Stockinger et al., 1997; Liu et al., 1998; Shinwari et al., 1998; Thomashow, 1999). At this time, we do not understand why *CHT46* transcript levels do not increase immediately after the rye plants are transferred to the cold in direct response to the shift in temperature. In our experiments, seeds are germinated at 20°C/16°C (day/night) and produce small plants within 7 d. After the plants are transferred to 5°C/2°C (day/night), their growth lags and new leaves completely developed at cold temperature first appear after 3 weeks (Krol et al., 1984; Griffith and McIntyre, 1993). Transcripts of *CHT46*, and the CHT-AFP itself (Hon et al., 1995), accumulate only in these leaves developed at low temperature, and not in leaves developed at warm temperature and shifted to cold, which indicates that a developmental process triggered by cold temperature may be involved in their transcriptional regulation. Chitinase genes are known to be expressed developmentally in healthy plants, possibly as a preemptive defense against pathogens or, in some cases, in a physiological role (Graham and Sticklen, 1994). We hypothesize that expression of *CHT9* and *CHT46* may be related to a developmental process that occurs at a later phase of cold acclimation, possibly at a time when plants in the field are more prone to be exposed to subzero temperatures and/or low temperature pathogens for long periods.

In winter wheat the expression of genes that hybridize to *CHT46* is induced by cold and to a lesser extent by drought, but not by ABA (Fig. 9). These results are consistent with the findings in Arabidopsis that expression of many cold-regulated genes is not responsive to ABA (Thomashow, 1999). In addition, transcripts of *CHT46* homologs accumulated more quickly in wheat than in rye, thus indicating that the regulatory mechanism may differ between cereals. In the future, isolation and analysis of the genes encoding *CHT9* and *CHT46* will be essential in elucidating their regulatory mechanism(s).

Chitinase-AFPs Belong to a Gene Family

Our Southern blots (Fig. 8) showed that the rye genome contains at least three genes closely related to *CHT46*. There is supporting evidence that at least three class II chitinases exist in rye: the CHT46-AFP, a seed-associated chitinase, and a pathogen-induced chitinase. The class II chitinase purified from rye seeds (Yamagami and Funatsu, 1993; PIR accession

no. JN0884) is not identical in amino acid sequence to the CHT46-AFP. A 28-kD chitinase accumulates in the apoplast of rye leaves infected by pathogenic snow molds, but this enzyme lacks antifreeze activity (Hiilovaara-Teijo et al., 1999) and is, therefore, distinct from the CHT46-AFP. Thus it is possible that the three rye chitinases are encoded by different genes. Future studies are planned to isolate the genes of the *CHT46* homologs and to determine whether the seed protein has antifreeze activity. The identification of closely related chitinases that differ in antifreeze activity would greatly aid in delineating regions important for binding to ice.

Chitinases homologous to *CHT46* mapped to the long arm of the homeologous group 1 chromosomes of all three genomes (A, B, and D) of hexaploid wheat (Fig. 8). These results substantiate a previous study of the Chinese Spring-Cheyenne wheat chromosome substitution lines that showed that Chinese Spring wheat plants carrying the homeologous group 1 chromosomes from Cheyenne winter wheat secrete greater amounts of apoplastic protein and exhibit higher antifreeze activity after cold acclimation than the parental line for Chinese Spring (Chun et al., 1998). Because increased accumulation of apoplastic proteins and greater antifreeze activity are highly correlated with increased winter survival (Chun et al., 1998), either the addition or enhanced expression of genes encoding CHT-AFPs may improve the survival of overwintering crops. Many traits such as the accumulation of AFPs and dehydrins are regulated by genes located on the homeologous group 5 chromosomes (Galiba et al., 1995; Limin et al., 1997; Chun et al., 1998, 1999), and so enhancement of the expression of cold-responsive genes in cereals may involve manipulation of transcription factor(s) located on wheat chromosome 5 (Sarhan et al., 1997).

Chitinase-AFPs May Have Evolved by Gene Duplication

Because of their dual activities, the winter rye AFPs provide a rare and exciting opportunity to study the adaptive interaction between an organism and its environment and the molecular events involved in the evolution of proteins as they acquire new functions. One way that the two rye CHT-AFP genes could have acquired antifreeze activity is through gene duplication in which a copy of each chitinase gene was modified to gain the additional function of binding to ice while still retaining the original function of hydrolyzing chitin. The diverse number of AFPs in nature, each with narrow distributions among related organisms, has led to conclusion that AFPs evolved recently and independently in organisms that survive exposure to freezing temperatures (Ewart et al., 1999). Some AFPs are truly novel proteins, whereas others retain homology to proteins with a different function. The AFPs isolated from winter cereals are the only AFPs shown to retain their original function (Hon et al., 1995; Hiilovaara-Teijo et al., 1999). Because antifreeze activity in rye originates with gene transcription without chemical modification of the mature proteins, there must be one set of cold-induced genes encoding PR proteins with antifreeze activity and a different set of pathogen-induced genes that code for PR proteins lacking antifreeze activity.

The modification of PR proteins for their roles as AFPs requires at least three changes in the gene sequence. One change would modify the surface of the protein to create an ice-binding domain, a second would target the proteins to the apoplast rather than the vacuole, and a third would induce expression at cold temperature. The modifications to the surface of cold-responsive chitinases that confer the ability to bind to ice may involve only minor changes in the sequence of rye chitinases, as indicated by the high level of sequence identity between cold-responsive rye chitinases and chitinases from other plants (Fig. 3). To date, no consensus sequence for an ice-binding domain has been identified. This will require further crystal structure analyses of multiple AFPs because the domain may only be evident in the native protein and may be as short as 15 amino acids in an α-helical configuration (Ewart et al., 1999). In the case of type II AFPs from fish, which are similar to C-type lectins, it was shown that conserved changes in only two amino acids within the carbohydrate binding domain were sufficient to eliminate ice-binding activity (Ewart et al., 1998). In the case of winter rye, the CHT-AFPs may have evolved from chitinases by gene duplication followed by minor, possibly conserved, changes in sequence to create the ice-binding domain.

MATERIALS AND METHODS

Cold Acclimation of Winter Rye

Winter rye (*Secale cereale* cv Musketeer) plants were grown at 20°C/16°Cday/night) in 15-cm pots of vermiculite (VIL Vermiculite Inc., Toronto) with a 16-h daylength and a photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}. NA and CA plants were grown for 1 week and watered as needed with modified Hoagland solution (Epstein, 1972) in which iron was supplied as $FeCl_3$. NA plants were grown under these conditions for an additional 2 weeks, whereas the CA plants were transferred to 5°C/2°C with an 8-h daylength for periods up to 8 weeks (Krol et al., 1984; Griffith and McIntyre, 1993).

Purification of Chitinases and Mass Spectrometry

The rye 35-kD CHT-AFP was purified from apoplastic extracts (25 mg of protein) obtained from CA rye leaves as described previously by Hon et al. (1994) by chitin-affinity column chromatography (Huynh et al., 1992; Hon et al., 1995). The CA chitinase was eluted by 20 mM acetic acid, pH 3.0, dialyzed against MilliQ water, concentrated using

a Centricon-10 microconcentrator (Amicon, Beverly, MA) and analyzed for purity by SDS-PAGE. Protein concentrations were assayed using the Bradford (1976) technique, as modified by Bio-Rad Laboratories (Mississauga, ON, Canada), with bovine serum albumin as the standard. The identity of the chitinase was confirmed by immunoblotting with antiserum produced against the rye native CHT-AFP (Antikainen et al., 1996). The purified chitinase was assayed for antifreeze activity and analyzed by mass spectrometry.

The 28-kD CHT-AFP was purified from apoplastic extracts of CA rye leaves by liquid chromatography. Apoplastic extracts were obtained by vacuum-infiltrating rye leaves with buffer A containing 10 mM ammonium acetate, pH 4.5, 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, and 1.25% (w/v) insoluble polyvinylpyrrolidone, followed by centrifugation at 1,200*g* for 30 min. The proteins were precipitated with 80% (w/v) ammonium sulfate, followed by centrifugation at 12,000*g* for 30 min. The pellet was resuspended in buffer A, dialyzed against three changes of 3 L of buffer A, and applied to a CM-Sepharose column. Proteins were eluted by a 0 to 0.25 M NaCl gradient in buffer A. Two distinct peaks were observed at A_{280}. Peak 2 corresponded to a purified 28-kD polypeptide by SDS-PAGE that exhibited antifreeze activity and was immunodetected by antiserum produced against the 35-kD CHT-AFP (Antikainen et al., 1996). To remove polyvinylpyrrolidone contamination for mass spectrometry, the 28-kD CHT-AFP was eluted from a Zorbax 300 SB-C18 reverse phase analytical column (4.6 mm × 25 cm) using a linear gradient of 5% to 95% acetonitrile (containing 0.1% [v/v] trifluoroacetic acid) with MilliQ water containing 0.1% (v/v) trifluoroacetic acid as the second solvent. The protein eluted at 49% acetonitrile.

Both CHT-AFPs (0.8 μg) were dialyzed against water, concentrated, and analyzed by mass spectrometry using a Quattro II triple-stage quadrupole mass spectrometer equipped with an electrospray ionization source (Micromass, UK). The raw data were deconvoluted using the Maximum Entropy algorithm (Ferrige et al., 1992) provided by the manufacturer (Masslyn, version 2.0).

Assay of Antifreeze Activity

Antifreeze activity was assayed qualitatively by observing the morphology of ice crystals growing in solution using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) and a phase-contrast photomicroscope (Olympus BHT, Carsen Medical and Scientific Co., Markham, ON, Canada). Ice crystals in water normally grow along the *a*-axes to form a flat round disc. AFPs at low concentration bind to the prism face of an ice crystal in solution, thus inhibiting growth along the *a*-axes and forming a hexagonally shaped crystal. Ice crystals grown at higher concentrations of AFPs form hexagonal columns and bipyramids as it becomes energetically favorable for the crystal to grow along the *c*-axis (DeVries, 1986).

Identification, Cloning, and Characterization of Chitinase cDNAs

The chitinase cDNAs *CHT9* and *CHT46* were isolated from a λ ZAP II library (Stratagene, La Jolla, CA) constructed from Poly(A)$^+$ mRNA purified from the leaves of 8-week-old CA winter rye plants (PolyATtract mRNA isolation system, Promega, Madison, WI). The library was screened with a barley chitinase cDNA probe (p*HvCHT2a*, 915 bp) kindly provided by Dr. Tomas Bryngelsson (Department of Plant Breeding Research, Swedish University of Agricultural Sciences, Svalöv, Sweden). All putative chitinase clones from the second screening were purified, subcloned in the Bluescript SK$^-$ plasmid (Stratagene), and sequenced using the Pharmacia ALFexpress DNA sequencer and Fragment Analysis System (Hospital for Sick Children Biotechnology Service Center, University of Toronto, Toronto). Sequence homologies were obtained using BLAST (Altschul et al., 1990) and amino acid sequences of the translated products were examined by ExPASy Proteomics Tools (Wilkins et al., 1999). All other molecular biology techniques were performed using the standard procedures of Sambrook et al. (1989).

For northern blotting, gene-specific probes designed from the 3′-untranslated region of each clone were synthesized by Bio/Can Custom oligonucleotides (Genosys, The Woodlands, TX) and were labeled using the terminal transferase labeling reaction (Boehringer Manheim Canada, Laval, Canada). The blots were washed for 10 min each at room temperature in 6× SSC, 1% (w/v) SDS and 2× SSC, 0.5% (w/v) SDS and then washed for 1 h at 55°C in 1× SSC, 0.5% (w/v) SDS.

Expression of *CHT9* in *Escherichia coli*

CHT9 was amplified by PCR with the synthetic oligonucleotides 5′-TTAAGGATCCGGAGCAGTGCGGCTCGCAGGC-3′and 5′-GGTTGGATCCTGCGAACGGCCTCTGGTTGTA-3′and fused in-frame with the signal peptide of pET12a. One clone named *CHT9*-12 was identified and confirmed by sequencing (T7 DNA sequencing kit, Pharmacia, Montreal, Canada). The expression of pET12a/*CHT9*-12 was examined in *E. coli* BL21(DE3) cells. Ten milliliters of an overnight culture harboring pET12a/*CHT9*-12 was used to inoculate 1.2 L of 2 × YT medium (8 g L^{-1} Bacto-tryptone, 5 g L^{-1} Bacto-yeast extract, and 5 g L^{-1} NaCl). The culture was grown to obtain an OD_{550} of 0.8 before the addition of IPTG and incubation continued at 25°C for another 5 h. The cells were harvested by centrifugation and the periplasmic fraction was prepared according to the Novagen pET system manual (Madison, WI). Periplasmic proteins were concentrated by ultrafiltration using a Centricon-10 (Amicon), analyzed by SDS-PAGE (Laemmli, 1970), and assayed for antifreeze activity.

Expression of *CHT46* in *E. coli*

CHT46 was amplified by PCR from the Bluescript construct SK*CHT46* with the synthetic oligonucleotides, 5′-TCAGATCAGCATATGAGTGTGGGCTCCGTCATCA-3′

and 5′-GGTTCTGCAGCAGTTAGCTAGCGAAGTTTC-G-3′ as primers to generate *Nde*I and *Pst*I sites at the ends of the cDNA. The PCR-amplified fragment was subcloned into the secretory expression vector OmpA/His6/*CHT46*/Par8, whichwas constructed by inserting the His6 sequence from pET16B and the gene encoding *CHT46* into the Tac cassette of Wong and Sutherland (1993). The OmpA/His6/*CHT46*/Par8 construct was transformed into *E. coli* JM105 cells and a single colony was used to inoculate 2 L of 2 × YT medium. The culture was incubated at 37°C for 5 h to attain an OD_{550} of 0.8, then induced by 1 mM IPTG, and incubated at 25°C to slow protein synthesis to promote transport to the periplasm or culture medium (Wong and Sutherland, 1993). Proteins present in the medium and cells were collected at 0, 3, 24, 48, and 72 h and examined by SDS-PAGE. His-tagged CHT46 was purified in the denatured form using a His-resin column as recommended by Invitrogen (Carlsbad, CA). His-tagged CHT46 was eluted at pH 4.0 in the presence of 8 M urea and 0.2 M β-mercaptoethanol (20 mL), allowed to refold slowly by dropwise addition of 500 mL of water overnight, then concentrated 4-fold by Centriprep10 ultrafiltration (Amicon). No antifreeze activity was observed, so the OmpA leader sequence as well as the His-tag sequence were removed by incubating 1 mL of the protein solution (0.06 mg mL^{-1}) with 5 units of thrombin at room temperature for 2 h, as confirmed by SDS-PAGE. The resulting solution was concentrated by ultrafiltration (Centricon10, Amicon) to 1.35 mg mL^{-1} and assayed for antifreeze activity.

Southern Blotting and Chromosome Mapping

Southern blots were performed on genomic DNA isolated from two cultivars of winter rye (cv Musketeer and cv Puma) and from spring wheat (*Triticum aestivum* cv Chinese Spring). The ditelocentric series of Chinese Spring wheat obtained from the U.S. Department of Agriculture E.R. Sears collection was used to map *CHT46* homologs to a specific chromosome. In the Chinese Spring ditelocentric series, all chromosomes are present in each line, except that one chromosome pair is represented by only the telocentric chromosomes of one arm. Genomic DNA was extracted from rye and wheat leaves as described by Rogers and Bendich (1988) and quantified using the diphenylamine colorimetric assay (Burton, 1968). DNA samples were digested using *Xba*I, separated by electrophoresis, blotted, and probed with p*CHT46* (Limin et al., 1997). The last wash for the filters after hybridization was in 0.2× SSC containing 0.1% (v/v) SDS at 68°C for 1 h.

Expression of *CHT46* Homologs in Winter Wheat

Winter wheat (cv Fredrick) seeds were germinated in water-saturated vermiculite for 7 d at 20°C with an irradiance of 250 μmol photons m^{-2} s^{-1} and a daylength of 15 h, then watered daily with a 20:20:20 (N:P:K) nutrient solution. These seedlings were the NA controls. For cold acclimation, seedlings were transferred to 5°C with a 12-h daylength. For heat shock, seedlings were transferred to 40°C for 3 h. Salt stress was imposed by incubating seedlings in a nutrient solution containing 500 mM NaCl for 24 h. Drought stress was created by lifting seedlings from the vermiculite and holding them at 20°C without water for 20 h. Plants were treated with ABA (mixed isomers, Sigma, St. Louis) by adding 10^{-4} M ABA to the nutrient solution and also by spraying the foliage with 10^{-4} M ABA and 0.02% (v/v) Tween 20. For low light, NA plants were grown for an additional 2 weeks under the control conditions described above. For high light, seedlings were grown under NA conditions for a total of 3 weeks, except that the light level was 800 μmol photons m^{-2} s^{-1}. mRNA was isolated from winter wheat leaves, crowns, and roots of seedlings as described in Limin et al. (1997). Northern blots were probed with *CHT46* and washed under high stringency (0.2× SSC containing 0.1% [w/v] SDS at 68°C for 1 h). Blots were quantified using ImageQuant version 5.0 (Molecular Dynamics, Sunnyvale, CA).

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LITERATURE CITED

Altschul SF, Gish W, Miller W, Meyers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Antikainen M, Griffith M (1997) Antifreeze protein accumulation in freezing-tolerant cereals. Physiol Plant **99:** 423–432

Antikainen M, Griffith M, Zhang J, Hon WC, Yang DSC, Pihakaski-Maunsbach K (1996) Immunolocalization of antifreeze proteins in winter rye leaves, crowns, and roots by tissue printing. Plant Physiol **110:** 84 5–857

Beintema JJ (1994) Structural feature of plant chitinases and chitin-binding proteins. FEBS Lett **350:** 159–163

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 341–374

Burke MJ, Anderson JA, Buchanan DW, Rajashekar CB, George MF (1983) Evidence for large negative pressure potentials in plant cells during freezing. Cryobiology **20:** 748–748

Burton A (1968) Determination of DNA concentration with diphenylamine. Methods Enzymol **12:** 163–166

Chun JU, Kim DC, Kwon BS, Griffith M, Nam JH (1999) Genetic analysis of vernalization response in wheat chromosome substitution lines. Korean J Breed **31:** 82–87

Chun JU, Yu X-M, Griffith M (1998) Genetic studies of antifreeze proteins and their correlation with winter survival in wheat. Euphytica **102:** 219–226

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases: minireview. Plant J **3:** 31–40

DeVries AL (1986) Antifreeze glycopeptides and peptides: interactions with ice and water. Methods Enzymol **127:** 293–303

Duman JG (1994) Purification and characterization of a thermal hysteresis protein from a plant, the bittersweet nightshade *Solanum dulcamara*. Biochim Biophys Acta **1206:** 129–135

Duman JG, Olsen TM (1993) Thermal hysteresis protein activity in bacteria, fungi, and phylogenetically diverse plants. Cryobiology **30:** 322–328

Epstein E (1972) Mineral Nutrition of Plants: Principles and Perspectives. John Wiley & Sons, New York

Ergon Å, Klemsdal SS, Tronsmo AM (1998) Interactions between cold hardening and *Microdochium nivale* infection on expression of pathogenesis-related genes in winter wheat. Physiol Mol Plant Pathol **53:** 301–310

Ewart KV, Li ZJ, Yang DSC, Fletcher GL, Hew CL (1998) The ice-binding site of Atlantic herring antifreeze protein corresponds to the carbohydrate-binding site of C-type lectins. Biochemistry **37:** 4080–4085

Ewart KV, Lin Q, Hew CL (1999) Structure, function and evolution of antifreeze proteins. Cell Mol Life Sci **55:** 271–283

Ferrige AG, Seddon MJ, Green BN, Jarvis SA, Skilling J (1992) Disentangling electrospray spectra with maximum entropy. Rapid Commun Mass Spectrom **6:** 707–711

Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW (1995) RFLP mapping of the vernalization (*vrn1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat. Theor Appl Genet **90:** 1174–1179

Gatschet MJ, Taliaferro CM, Porter DR, Anderson JA, Jackson KW (1996) A cold-regulated protein from bermuda grass crowns is a chitinase. Crop Sci **36:** 712–718

Graham LS, Sticklen MB (1994) Plant chitinases. Can J Bot **72:** 1057–1083

Griffith M, Ala P, Yang DS, Hon WC, Moffatt BA (1992) Antifreeze protein produced endogenously in winter rye leaves. Plant Physiol **100:** 593–596

Griffith M, Ewart KV (1995) Antifreeze proteins and their potential use in frozen foods. Biotechnol Adv **13:** 375–402

Griffith M, McIntyre HCH (1993) The interrelationship of growth and frost tolerance in winter rye. Physiol Plant **187:** 335–344

Hiilovaara-Teijo M, Hannukkala A, Griffith M, Yu X-M, Pihakaski-Maunsbach K (1999) Snow mold-induced apoplastic proteins in winter rye leaves lack antifreeze activity. Plant Physiol **121:** 665–674

Hon WC, Griffith M, Chong P, Yang DSC (1994) Extraction and isolation of antifreeze proteins from winter rye (*Secale cereale* L.) leaves. Plant Physiol **104:** 971–980

Hon WC, Griffith M, Mlynarz A, Kwok YC, Yang DSC (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiol **109:** 879–889

Huynh QK, Hironaka CM, Levine EB, Smith CE, Bvormeyer JRE, Shah DM (1992) Antifungal proteins from plants: purification, molecular-cloning, and antifungal properties of chitinases from maize seed. J Biol Chem **267:** 6635–6640

Kimber G, Sears ER (1987) Evolution in the genus *Triticum* and the origin of cultivated wheat. *In* EG Heyne, ed, Wheat and Wheat Improvement, Ed 2. American Society of Agronomy, Madison, WI, pp 154–164

Knight CA, Duman JG (1986) Inhibition of the recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. Cryobiology **23:** 256–262

Krol M, Griffith M, Huner NPA (1984) An appropriate physiological control for environmental temperature studies: comparative growth kinetics of winter rye. Can J Bot **62:** 1062–1068

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Liao YC, Kreuzaler F, Fischer R, Reisener HJ, Tiburzy R (1994) Characterization of a wheat class 1b chitinase gene differentially induced in isogenic lines by infection with *Puccinia graminis*. Plant Sci **103:** 177–187

Limin AE, Danyluk J, Chauvin L-P, Fowler DB, Sarhan F (1997) Chromosome mapping of low-temperature induced *Wcs120* family genes and regulation of cold-tolerance expression in wheat. Mol Gen Genet **253:** 720–727

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10:** 1391–1406

Meins F, Fritig B, Linthorst HJM, Mikkelsen JD, Neuhaus J-M, Ryals J (1994) Plant chitinase genes. Plant Mol Biol Rep **12:** S22–S28

Meyer K, Keil M, Naldrett MJ (1999) A leucine-rich repeat protein of carrot that exhibits antifreeze activity. FEBS Lett **447:** 171–178

Nielsen H, Engelbrecht J, Brunal S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng **10:** 1–6

Pearce RS (1988) Extracellular ice and cell shape in frost-stressed cereal leaves: a low-temperature scanning-electron-microscopy study. Planta **175:** 313–324

Pearce RS, Ashworth EN (1992) Cell shape and localization of ice in leaves of overwintering wheat during frost stress in the field. Planta **188:** 324–331

Pihakaski-Maunsbach K, Antikainen M, Griffith M, Maunsbach AM (1996) Immunogold localization of glucanase-like antifreeze protein in cold-acclimated winter rye. Protoplasma **191:** 115–125

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. *In* SBG Levin, RA Schilperoort, DPS Verma, eds, Plant Molecular Biology Manual. Kluwer Academic Press, Dordrecht, The Netherlands, pp 1–10

Sakai A, Larcher W (1987) Frost Survival of Plants: Responses and Adaptation to Freezing Stress. Springer-Verlag, New York

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nickleu S, Coulson AR (1977) DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Sarhan F, Ouellet F, Vazquez-Tello A (1997) The wheat *wcs120* gene family: a useful model to understand the molecular genetics of freezing tolerance in cereals. Physiol Plant **101:** 439–445

Shinshi H, Neuhaus J-M, Ryals J, Meins F (1990) Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol Biol **14:** 357–368

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An Arabidopsis gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun **250:** 161–170

Sicheri F, Yang DSC (1995) Ice-binding structure and mechanism of an antifreeze protein from winter flounder. Nature **375:** 427–431

Sönnichsen FD, Davies PL, Sykes BD (1998) NMR studies on antifreeze proteins. Biochem Cell Biol **76:** 284–293

Sticher L, Hofsteenge J, Milani A, Neuhaus J-M, Meins F Jr (1992) Vacuolar chitinases of tobacco: a new class of hydroxyproline-containing proteins. Science **257:** 655–657

Sticher L, Hofsteenge J, Neuhaus J-M, Boller T, Meins F Jr (1993) Post-translational processing of a new class of hydroxyproline-containing proteins. Plant Physiol **101:** 1239–1247

Stinzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant pathogenesis-related proteins and their role in defense against pathogens. Biochimie **75:** 687–706

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Nat Acad Sci USA **94:** 1035–1040

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol **50:** 571–599

Tronsmo AM, Gregersen P, Hjeljord L, Sandal T, Bryngelsson T, Collinge DB (1993) Cold-induced disease resistance. *In* B Fritig, M Legrand, eds, Mechanism of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 369

Urrutia M, Duman JG, Knight CA (1992) Plant thermal hysteresis proteins. Biochim Biophys Acta **1121:** 199–206

Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF (1999) Protein identification and analysis tools in the ExPASy server. Methods Mol Biol **112:** 531–552

Wong WKR, Sutherland ML, inventors. June 29, 1993. Excretion of heterologous proteins from *E. coli*. U.S. Patent 5,223,407

Worrall D, Elias E, Ashford D, Smallwood M, Sidebottom C, Lillford P, Telford J, Holt C, Bowles D (1998) A carrot leucine-rich-repeat protein that inhibits ice recrystallization. Science **282:** 115–117

Xu H, Griffith M, Patten CL, Glick BR (1998) Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12–2. Can J Microbiol **44:** 64–73

Yamagami T, Funatsu G (1993) The complete amino acid sequence of chitinase-c from the seeds of rye (*Secale cereale*). Biosci Biotech Biochem **57:** 1854–1861

Zhu B, Chen THH, Li PH (1993) Expression of an ABA-responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. Plant Mol Biol **21:** 729–735