An apoplastic chitinase CpCHT1 isolated from the corolla of wintersweet exhibits both antifreeze and antifungal activities

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Abstract

The shrub *Chimonanthus praecox* L. (wintersweet) which is native to Chinese montane forests produces its flowers in the midst of winter. This indicates that the floral organs of this species are adapted to growth and development under freezing temperatures. Here, we report the isolation and preliminary characterisation of a 33 kDa apoplastic antifreeze chitinase (CpCHT1) from the petals and its corresponding cDNA. The chitinase activity of CpCHT1 was confirmed by activity staining. Antifreeze activity was validated in terms of the formation of bipyramidal ice crystals and high thermal-hysteresis values. CpCHT1 was also found to affect the germination of fungal spores of four major plant pathogens. In addition, the gene and protein are expressed constitutively not only in flowers, but also in leaves, bark and root tissues. From these data we hypothesize that this protein is multifunctional and may protect wintersweet from freezing injury and provide nonspecific disease resistance.

Additional key word: *Chimonanthus praecox.*

Introduction

Freezing temperatures experienced in many temperate countries during the winter months pose a severe threat to the survival of both flora and fauna. With regards to plant species, these harsh environmental conditions represent a major constraint on growth and development and the acquisition and retention of water. In recent years the phenomenon of temperature stress and adaptation to such stress in plants grown in cold environments has been widely investigated both at a physiological and molecular level (Xin and Browse 2000, Yeh *et al.* 2000, Kreps *et al*. 2002, Xiong *et al*. 2002, Atici and Nalbantoglu 2003, Nakamura *et al.* 2008, Zhang *et al*. 2010). These studies and corresponding investigations in cold tolerant animals and microoganisms, have demonstrated that freezing tolerance is a complex trait with multigenic inheritance (Thomashow 1999), and that many organisms produce a range of antifreeze proteins (AFPs; Atici and Nalbantoglu 2003, Nakamura *et al*. 2008).

Under most circumstances, freezing induces the formation of ice in the intercellular spaces and cell walls of plant tissues. Thus, freezing injury is mainly caused by cellular dehydration and for this reason, freezing stress, drought stress and salt stress share many physiological and genetic features in common. Antifreeze proteins protect cells against fatal intracellular and extracellular ice formation *via* inhibition of ice crystal formation (Atici and Nalbantoglu 2003). They are thought to act by binding to the faces of ice crystals, inhibiting recrystallization or *via* thermal hysteresis (Duman and Olsen 1993, Ewart *et al.* 1999, Griffith and Yaish 2004, Bravo and Griffith 2005). To date, more than thirty AFPs have been isolated from a variety of plant species (Atici and Nalbantoglu 2003). According to Antikainen and Griffith (1997) all freezing tolerant monocotyledons studied to date accumulate AFPs in the apoplast during cold acclimation. However, in dicotyledonous species AFP activity was not detected in the apoplast of spring canola and spinach and was only recorded at very low levels in the apoplast of winter

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Abbreviations: AFPs - antifreeze proteins; PDA - potato dextrose agar; PR - pathogenesis-related proteins; CLPs - chitinase-like proteins.
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canola and kale (Moffat *et al.* 2006). Therefore, the mechanism of action of AFPs in dicotyledonous species is yet to be fully resolved.

Recent studies have indicated that a sub-set of AFPs, such as those expressed in shoot tissues of winter rye (*Secale cereale*), are of dual function. It has been shown that some of these proteins bear molecular similarity to pathogenesis-related (PR) proteins. The majority of these and related proteins are induced through the action of the signaling compounds salicylic acid, jasmonic acid, or ethylene, and possess antimicrobial activities *in vitro* through hydrolytic activities on cell walls, contact toxicity, and perhaps an involvement in defense signaling. Isolated AFPs have been identified as glucanase-like (GLPs), chitinase-like (CLPs) and thaumatin-like (TLPs) (Hon *et al.* 1994, Moffat *et al.* 2006, Nakamura *et al.* 2008). Recently, chitinase-like AFPs have also been indentified in cold-acclimated and nonacclimated leaves of winter rye (Pihakakski-Maunsbach *et al.* 2001) and in brome grass (Nakamura *et al.* 2008).

Wintersweet is famous for its winter flowering (Fig. 1). It is logical to hypothesize that such cold tolerant structures must express substances that enable these usually delicate and fragile organs to survive such an extreme abiotic stress. Therefore, the objective of the current study was to determine if such AFPs were expressed in the corolla of wintersweet and if so, whether such proteins also exhibited features/activities associated with PR proteins.

Materials and methods

Wintersweet (*Chimonanthus praecox* L.) flowers produced during the winter of 2007 were harvested at anthesis and used directly for extraction of apoplastic proteins, total proteins and RNA. Other tissues including young leaves, current-year-roots and twig bark as well as corolla were used for extracting total proteins and RNA.

Apoplastic proteins were extracted as described by Hon *et al* (1994). For total protein preparations, the tissues were homogenized in an ice-cold solution comprising 100 mM Tris-HCl, pH 7.5, and 0.9 % (m/v) NaCl. The homogenate was then centrifuged at 4 °C for 30 min at 10 000 *g* and the supernatant collected and stored at -20 °C for future use.

The apoplastic protein solution was made to 50 % saturation with ammonium sulphate, and loaded onto a phenyl-sepharose column (1 × 20 cm) equilibrated with 20 mM ascorbic acid solution. The column was successively eluted using 20 mM ascorbic acid solution saturated with 50 - 0 % ammonium sulphate. Single fractions with chitinase activity were collected and dialyzed overnight against a phosphate buffer (20 mM, pH 7.5).

Dialyzed fractions were loaded onto a DEAE-cellulose column (1 × 20 cm). The column was washed with 30 cm^3 of 0.1 - 0.5 M NaCl. Proteins with chitinase activity were then dialyzed against HAc-NaAc buffer, pH 6.0, prior to ion-exchange chromatography. Samples were loaded onto a CM-sepharose fast flow column (3 cm^3 bed volume) adjusted with HAc-NaAc buffer (pH 6.0). Fractions with chitinase activity were eluted with 0.1 - 0.5 M NaCl pooled and stored at -20 °C.

Chitinase activity was evaluated using 4-methyl-umbelliferyl-D-N,N',N''-triacetylchitotriose [4-MU-$(GlcNAc)_3$] (*Sigma*, St. Louis, USA). A reaction mix (0.3 cm^3) was prepared (0.250 cm^3 of reaction buffer, 0.03 cm^3 of sample, and 0.02 cm^3 of substrate at concentration 0.1 mg cm^{-3}) and incubated at 40 °C for 4 h; reactions were terminated by the addition of one volume of 0.2 M Na_2CO_3. Chitinase activity was measured by the mean fluorescence estimated in a fluorometer model *450* (*Turner Biosystems*, Sunnyvale, USA; 340-nm interference filter and 415-nm cut filter). One unit of chitinase activity was defined as the quantity of enzyme required to release 1 μM of 4-methylumbelliferone in 1 min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12 % SDS-PAGE) was performed according to Laemmli (1970). In order to identify chitinase activity two gels were run in parallel, one stained in Coomassie blue, whilst the second was transferred into washing buffer (1 % casein, 2 mM EDTA, 40 mM Tris-HCl, pH 8.0) for the removal of SDS from the gel and the renaturation of the chitinase. After several washes in double distilled water and sodium acetate buffer (pH 4), the gel was covered with 1 % (m/v) low melting-point agarose supplemented with 0.2 mM 4-MU-$(GlcNAc)_3$ in 100 mM potassium phosphate buffer (pH 6.0). The gel was then inspected for the presence of fluorescent bands when viewed under UV radiation following incubation at 40 °C for 120 min; which indicated the presence of chitinase (Tronsmo and Harman 1993).

Two methods were used to test for antifreeze activity: *1*) AFPs have been shown previously to alter the morphology of ice crystals in solution. In the current study, the shape of ice crystals in the purified protein solution (0.1 or 1 mg cm^{-3}) and BSA solution (5 mg cm^{-3}, as a negative control) were observed using a microscope (*BHS313*, *Olympus*, Tokyo, Japan) equipped with a *LTS120* temperature controller. In order to make these observations, a droplet of the sample solution was frozen and subsequently heated until a single ice crystal was observed, as described previously (Kobashigawa *et al.* 2005). *2*) Thermal hysteresis values were measured by differential scanning calorimetry (DSC, *Dupont 910*, Ventura, USA) following the protocol of Hansen and Baust (1988). Each sample (0.1, 0.5, 1, 1.5 mg cm^{-3}) was quickly frozen to -25 °C, held at that temperature for 5 min, then warmed at 0.5 °C per 60 s to an annealing temperature near the sample melting temperature. When the sample was in the state of partial melting, it was slowly cooled to -10 °C at 1 °C per 60 s. BSA solution (5 mg cm^{-3}) was used as the negative control which was subjected to the same

treatment. The maximum difference between the annealing temperature and the onset of freezing during the slow cooling steps was used as an approximation to the samples freezing point depression activity. All measurements were performed in triplicate.

To ascertain whether CpCHT1 had antifungal activity, several agronomically important plant pathogens were selected: *Alternaria alternata, Fusarium oxysporum*, *Magnaporthe grisea* and *Verticillium dahliae.* In order to obtain sufficient spores, all four pathogens were isolated from newly infected organisms and grown on potato dextrose agar (PDA). After sporulation, spores were collected, washed three times in sterile distilled water, and resuspended in 0.1 % (m/v) glucose. To examine the effects of the chitinase on spore germination, spores were used directly. To examine the influence on elongation of germ tubes, spores were preincubated under normal conditions (potato dextrose liquid medium, 25 °C), until a germ tube had appeared. Conidial suspension (0.1 cm^3) was then mixed in the presence or absence of serially diluted quantities of purified CpCHT1 in the wells of sterile depression slides, and incubated at 25 °C for 10 h. The results were recorded by light microscopy (*Olympus BHS313*).

For Western blot analysis primary antibodies were raised against wintersweet chitinase in rabbit to detect CpCHT1 in crude protein extracts of wintersweet tissues run alongside the purified protein. A rabbit antisera specific to the purified chitinase (1:2000 dil.) was prepared according to Harlow and Lane (1988). Secondary alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:1000 dil.) were used to develop the immunoblots. All antibodies were diluted in 5 % (m/v) defatted dry milk powder in TBS (100 mM Tris-HCl pH 7.5 and 0.9 % NaCl). Western blotting was performed by transferring proteins from SDS-PAGE to nitrocellulose membranes. After transfer and membrane blocking with defatted milk, the presence of the chitinase was detected using primary and secondary antibodies as described above.

A full length wintersweet cDNA library was constructed using a *SMART*™ cDNA *Library Construction Kit* (*Clontech*, Tokyo, Japan) from winter- sweet corolla material. Partial clones from the library were randomly selected and sequenced. The partial sequence of each clone was then compared with sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/). A partial cDNA with chitinase characteristics was then used to make a radiolabeled probe. Positive clones were selected and sequenced in the normal manner in order to isolate and identify a full-length cDNA clone (Sambrook *et al.* 1989). The cDNA encoding the wintersweet AFP-chitinase was designated *CpCHT1*.

The different tissues of wintersweet (corolla, bark, leaf and root) were collected for total RNA extraction. Northern blot analysis was performed as described Sambrook *et al.* (1989). Hybridization was performed using ultrasensitive hybridization buffer (*ULTRAhyb*™, *Ambion*, Tokyo, Japan) at 42 °C (probe concentration 25 ng cm^{-3}). Anti-sense single stranded DNA probes derived from *CpCHT1* were labeled with *DIG* (*Roche*, China) according to the manufacturer's protocol. The forward and reverse primers used to make probes for these experiments were as shown below and were cloned and sequenced in *pGEMT* (5'-ATGAGAACTCAAGCC-TTAGCCTT-3' and 5'-AGCATTGAAAGGCCTCTG-3').

Genomic DNA was extracted and Southern blotting was performed in the normal manner (Sambrook *et al*. 1989). DNA samples were digested to completion with the following restriction enzymes: *Xba*I, *Sma*I, *Xho*I, and *Bam*HI, and electrophoresed on 0.8 % m/v agarose gels, blotted onto *Hybond*^{N} membrane (*Amersham*, Buckinghamshire**,** UK) using capillary transfer. The cloned, *CpCHT1* gene fragments (ORF) listed previously were used as probes in these experiments. Southern blot analyses were performed under high-stringency conditions.

Results

The chitinase specific activity of the crude protein extract was found to be 32.4 U mg^{-1}. In order to obtain a purified CpCHT1 sample, three methods of column chromatography were used in series, which resulted in a 41.3 fold enrichment in chitinase specific activity with 5.5 % recovery of purified CpCHT1 (Table 1). Both the purity and molecular mass of the enzyme were determined by SDS-PAGE (Fig. 2*A*), from which the molecular mass of chitinase was observed to be 33 kDa. Confirmation of the purified CpCHT1 as a chitinase was obtained by activity staining of the SDS-PAGE gels (Fig. 2*B*). These data indicated that the 33-kDa protein band (CpCHT1) possesses chitinase activity.

Hexagonal shaped ice crystals were observed to form in the presence of a 0.1 mg cm^{-3} solution of the sample protein (Fig. 3*A*). However, when the concentration of the purified protein solution was increased to 1 mg cm^{-3}, bipyramidal shaped ice crystals were observed to form (Fig. 3*B*). In contrast, only flat shaped ice crystals were observed in the negative control (BSA 5 mg cm^{-3}; Fig. 3*C*). The thermal hysteresis value is another important index

Table 1. Purification of apoplastic chitinase from wintersweet flowers.

Purification step	Total activity [U]	Specific activity [U mg^{-1}(prot.)]	Purification [fold]	Yield [%]
Crude enzyme	9503.0	32.4	1.0	100.0
Phenyl-sepharose	5189.0	673.9	20.8	54.6
DEAE-sepharose	1140.0	1150.2	355.0	12.0
CM-sepharose	522.7	1338.1	41.3	5.5

routinely used in the studies of antifreeze proteins. Previous studies have shown that AFPs at a range of concentrations produce varying degrees of thermal hysteresis. In the current study, 0.1, 0.5, 1 and 1.5 mg cm^{-3} solutions of the sample were tested for their affect on thermal hysteresis and the highest reading of 0.52 was obtained at a concentration of 1.5 mg cm^{-3} (Table 2).

Fig. 1. Flowers of *Chinmonanthus praecox* (wintersweet) in bloom in Shandong Province P.R. of China mid-winter 2006.

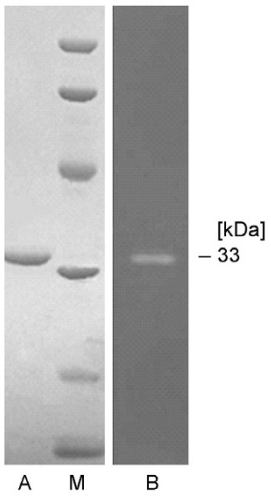

Fig. 2. SDS-PAGE of purified CpCHT1. *A* - Coomassie brilliant blue R-250 staining. *B* - Activity staining. *M* - marker proteins (94, 67, 43, 30, 20.1, 4.4 kDa).

Table 2. Thermal hysteresis activity of the purified AFP-chitinase (CpCHT1) solutions and bovine serum albumin as control. All measurements were performed in triplicate and the maximum difference between annealing temperature and onset of the freezing exotherm during the slow cooling steps was used as an approximation to the samples' freezing point depression activity. T_{hd} - hold temperature, T_0 - temperature at the onset of the freezing exotherm during the slow cooling, T_H - thermal hysteresis temperature, equal to (T_{hd} - T_0).

Sample	Concentration [mg cm^{-3}]	T_{hd} [°C]	T_0 [°C]	T_H [°C]
BSA	5.0	-0.80	-0.80	0.00
CpCHT1	0.1	-0.65	-0.85	0.20
CpCHT1	0.5	-1.18	-1.49	0.31
CpCHT1	1.0	-0.87	-1.31	0.44
CpCHT1	1.5	-1.83	-2.35	0.52

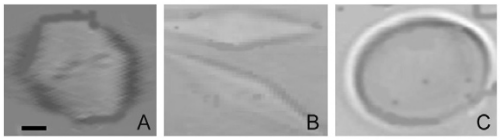

Fig. 3. Antifreeze activity of purified AFP-chitinase. *A* - Hexagonal ice crystals, which mean a low antifreeze activity, were observed in 0.1 mg cm^{-3} solution of CpCHT1. *B* - Bipyramidal crystals, which mean a high antifreeze activity, were observed in 1 mg cm^{-3} CpCHT1 solution. *C* - BSA solution (5 mg cm^{-3}) was used as a negative control that lacks antifreeze activity. *Scale bar* represents 10 µm.

To detect *in vitro* antifungal activity of CpCHT1, 0.1 cm^3 of each fungal spore stock was cultured in the presence or absence of a serial dilution of the purified protein (0.1, 1, 10, 100, 150 µg cm^{-3}) at 25 °C for 10 h. In these experiments it was observed that spore germination and germ-tube elongation of the tested fungi were all inhibited to different degrees by the sample as compared with the phosphate buffer control (Table 3). For example, at a sample concentration of 100 µg cm^{-3}, the *Fusarium oxysporum* and *Alternaria alternata* germination was inhibited by 95 % and germ tube elongation by 85 - 90 % (Table 3, Fig. 4).

The results of the Western blot analysis demonstrated the presence of CpCHT1 in many tissues other than the

Table 3. *In vitro* antifungal activity [%] of the purified AFP-chitinase (CpCHT1) in concentration from 0.1 to 150 µg cm^{-3}. Approximately 1000 fungal spores were cultured in the agar plate with or without a different mount of the chitinase at 25 °C for 10 h. Inhibition of spore germination was examined under a light microscope.

Pathogens	Inhibition of germination					Inhibition of germ tube elongation				
	0.1	1	10	100	150	0.1	1	10	100	150
Alternaria alternata	6	35	60	95	100	9	43	50	85	96
Fusarium oxysporum	8	30	65	90	100	7	55	76	90	100
Magnaporthe grisea	4	28	44	62	65	7	47	67	70	78
Verticillium dahliae	5	26	40	85	85	5	30	65	75	80

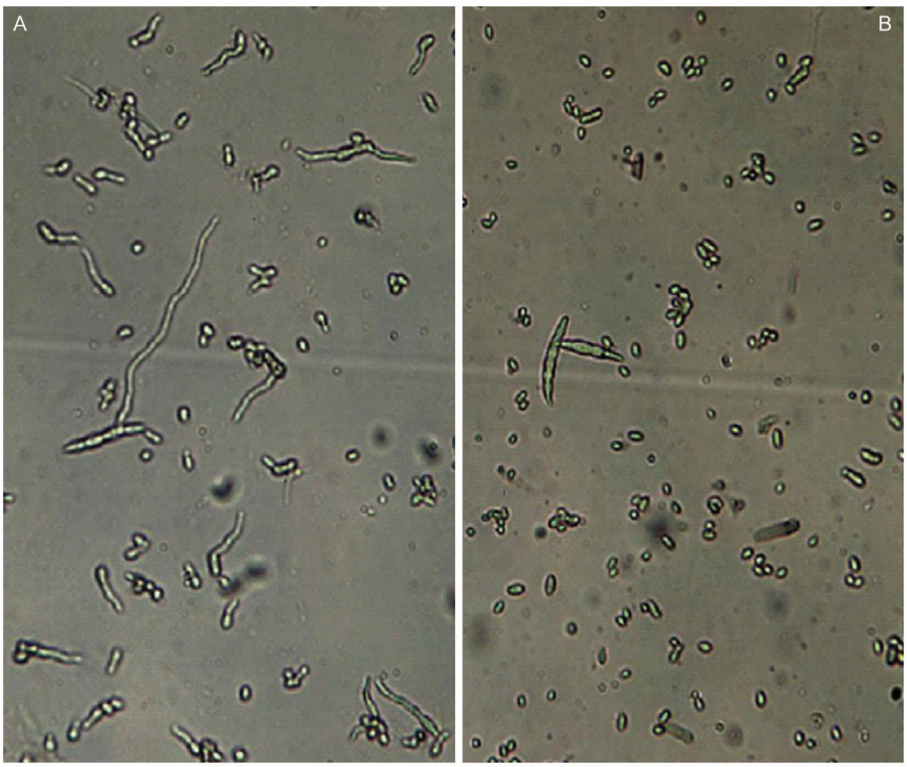

Fig. 4. Antifungal activity of the wintersweet AFP-chitinase. Inhibition of spore germination of *Fusarium oxysporum* was observed under a light microscope at 100x magnification after the spores were cultured at 25 °C for 10 h in the absence (A) or presence (B) of the purified chitinase (100 μg cm^{-3}).

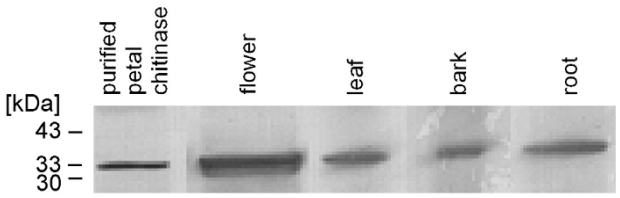

Fig. 5. Immunoblot of purified AFP-chitinase, protein extracts from flower, leaf, bark and root probed with antibody to apoplast chitinase from wintersweet petals. The samples were run on a single concentration (12 %, m/v) SDS-PAGE. Lane of purified CpCHT1 (10 μg) was loaded with purified petal apoplast chitinase, other lanes (flower, leaf, bark and root) were loaded with total proteins (10 μg) isolated from flower, leaf, bark and root, respectively.

corolla namely; the bark, root and leaf. No qualitative differences were observed in the chitinase profiles of the extracts from leaf, bark and root. However, the expression of the chitinase was greatest in corolla tissue (Fig. 5).

A full-length AFP-chitinase cDNA (GenBank accession number, FJ560717) was cloned *via* screening a wintersweet cDNA library. The cDNA (*CpCHT1*) is 1140 nucleotides in length, of which 952 form a single open reading frame, predicting a 317 amino acid polypeptide (Fig. 6). The polypeptide has a sequence of 20 amino acids at the N-terminus with several features common to eukaryotic signal sequences (Von Heijne 1986). The amino acids at the C-terminal end include alanine at a putative cleavage site preceded by an aromatic phenylalanine residue; these are both features characteristic of the C-terminal region of eukaryotic signal sequences (Von Heijne 1986).

Comparison of the 317 amino acid sequence with known proteins demonstrated a strong homology with class 1 chitinases from rye, barley, wheat, rice and bromegrass (Fig. 6). The CpCHT1 amino acid sequence contains two consensus motifs (a chitin-binding domain and a catalytic domain) normally found in members of this family. Of most significance was the homology to the winter rye sequence CHT9 which is also a putative dual function AFP-chitinase. The deduced amino acid sequence of CpCHT1 showed 70.22 % similarity/homology to CHT9 (31.7 kDa, AF280437) and 45.60 % similarity to CHT46 (24.8 kDa, AF280438). A simple tree to show the relatedness of CpCHT1 to other angiosperm class 1 chitinases is shown in Fig. 7.

The expression of the *CpCHT1* gene in different tissues was studied using Northern blot analysis. It would appear that the *CpCHT1* gene is constitutively expressed, and that expression levels are highest in the flower (corolla), followed by leaf, root and bark tissue (Fig. 8).

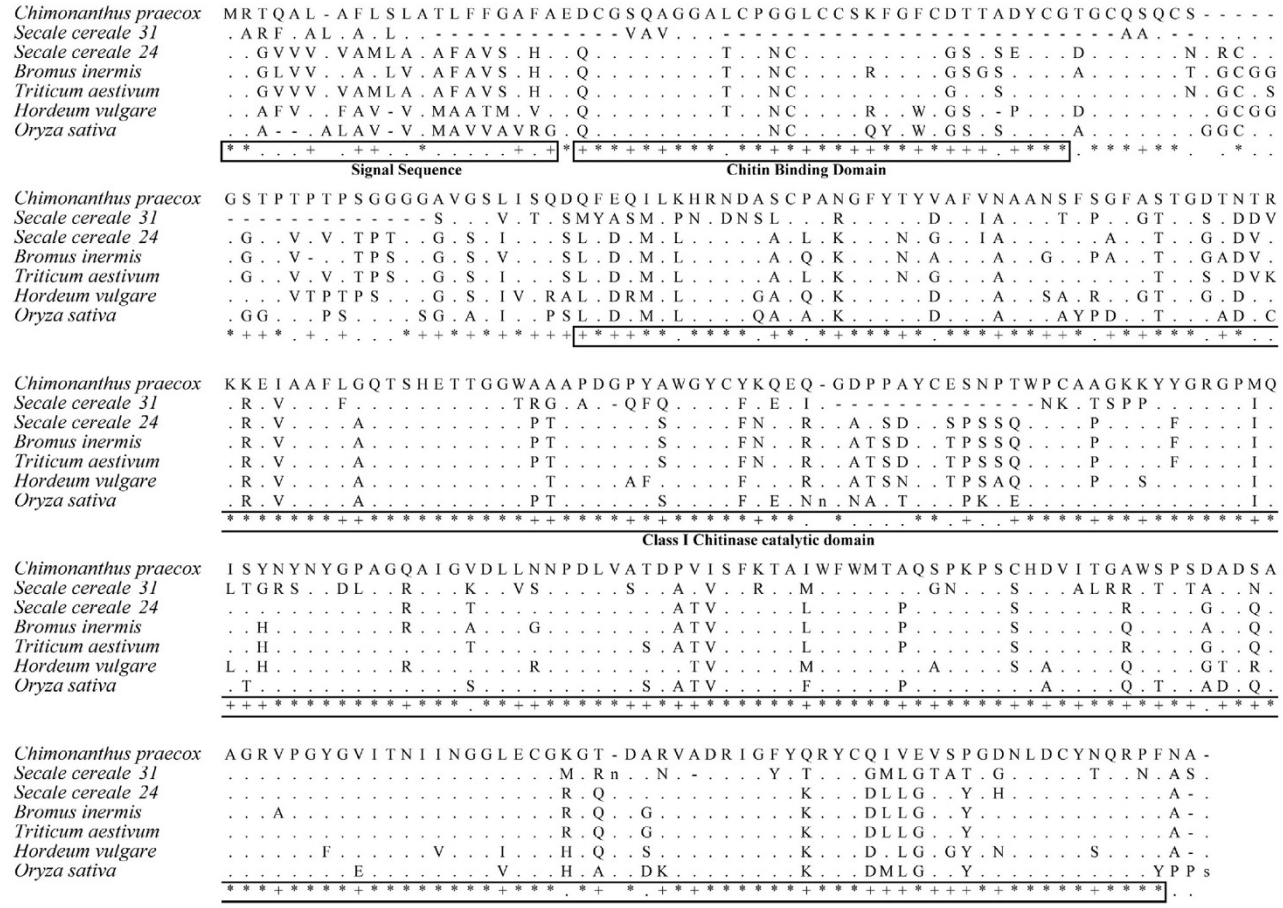

Fig. 6. Alignments of chitinase amino acid sequences deduced from nucleotide sequences of cDNA from wintersweet *CpCHTI1* (accession No. FJ560717), rye *CHT9* (No. AF280437), rye *CHT46* (No. AF280438), bromegrass *BiCHT1* (No. AB428423), wheat *Chi3* (No. AB029936), barley *CN9* (No. U02287) and rice *Cht-3* (No. D16223). *Asterisks* indicate conserved amino acid residues.

Discussion

The wintersweet is of particular interest in the context of cold tolerant angiosperms, since it is not only able to successfully overwinter in the extreme harsh winter conditions experienced in Northern China, but also blooms during the winter months. Furthermore, there is no record in the literature of studies of AFPs being performed on a dicotyledonous perennial such as wintersweet. Most previous investigations of this topic have focused on monocotyledonous annual crop species (Griffith and Yaish 2004, Nakamura *et al.* 2008). Hence, the objective of the current study was to ascertain if petal/corolla tissues of wintersweet express such AFPs and in particular to determine the presence or absence of AFP-chitinases with dual antifreeze and antifungal activity.

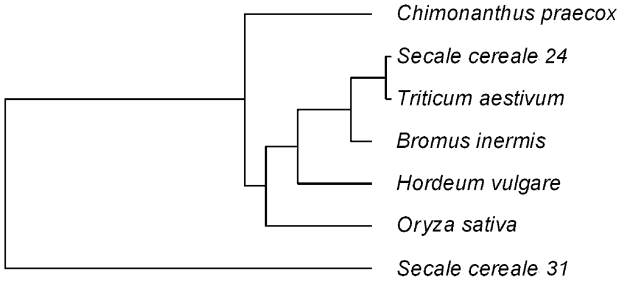

Fig. 7. Cladogram of *C. praecox* CpCHT1 and the most closely related members of the family generated by the *DISTANCES* program of *GCG*.

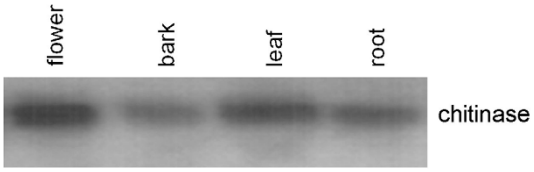

Fig. 8. RNA-blot analyses of the gene for chitinase. The cDNA hybridizes to a major 1.1 kb mRNA. Each lane was loaded with 15 μg of total RNA, and equal loading was verified by ethidium-bromide staining before blotting.

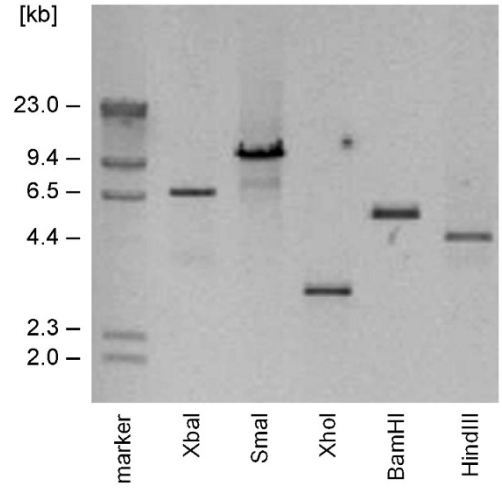

Fig. 9. Genomic DNA blot for *CpCHT1*. 15 - 20 µg genomic DNA extracted from young leaves was digested with *Hind*III, *Bam*HI, *Xho*I, *Sma*I or *Xba*I and fractionated on a 0.8 % (m/v) agarose gel. The blot was hybridized with the [^{32}P]dCTP-labelled *CpCHT1* ORF fragments. The marker lane is Lambda bacteriophage DNA digested with *Hind*III.

We have described the characterization of a 33 kD chitinase (CpCHT1) with high levels of antifreeze activity (Fig. 3) isolated from the apoplast of wintersweet petals. In addition, the corresponding cDNA was also identified and characterized, and an antisera raised against the purified protein. Northern blot analyses (Fig. 8) demonstrated that the *CpCHT1* gene is constitutively expressed throughout the plant (root, leaf, flower and bark). Highest expression levels were observed in the flowers followed by the leaves, roots and bark. The corresponding protein was detected by Western blot analysis. The higher content of this protein was observed in the flowers than in the other tissues tested.

Southern blot analysis of wintersweet genomic DNA probed under stringent conditions with radiolabelled CpCHT1 demonstrated that this gene most probably exists as a single copy in the wintersweet genome (Fig. 9). However, from the results of our database search, it is clear that this protein bears most similarity to the family of class 1 chitinases and to a previously isolated gene family of AFP-chitinases isolated from winter rye (*CHT9*) in particular (Yeh *et al.* 2000) (Figs. 6 and 7). The features common to the structure of this family of related mature proteins are that they all possess an N-terminal chitin binding domain and class I chitinase catalytic domain (Nakamura *et al.* 2008).

To investigate the antifreeze activity of the purified wintersweet protein we determined the complex degree of the ice crystal formation and the thermal hysteresis value for CpCHT1. The results demonstrated that incubation in the presence of CpCHT1 resulted in the formation of bipyrimidal ice crystals indicative of AFP activity at a protein concentration of 1 mg cm^{-3} (Fig. 3) and that a thermal hysteresis value of 0.52 was obtained at a concentration of 1.5 mg cm^{-3} (Table 2). These data are consistent with CpCHT1 having significant antifreeze activity *in planta* and are consistent with previously isolated AFP-chitinases (Antikainen and Griffith 1997, Yeh *et al.* 2000). The fact that at concentrations as low as 0.1 mg cm^{-3} the purified protein was able to produce hexagonal ice crystal formation progressing to bipyridimal ice crystals at higher concentrations (1 mg cm^{-3}) is indicative of a high antifreeze activity for CpCHT1 in the tissues of wintersweet consistent with its life cycle and winter blooming.

The putative chitinase activity of the protein was assayed. These data indicated both the presence of chitinase and a high specific activity (Table 1). This is consistent with data obtained from other putative AFP-chitinases reported in the literature (Nakamura *et al.* 2008, Wang *et al*. 2009) and strongly suggest that CpCHT1 has a high chitinase activity *in vivo*.

Previous studies have demonstrated that chitinases are produced very rapidly in response to attack by fungal pathogens and thereby confer plant disease resistance to certain important pathogenic species (Xu *et al*. 1996, Patil *et al*. 2000, Nishizawa *et al*. 2003, Guerra- Guimaraes *et al*. 2009). Wintersweet is well known to be resistant to many plant pathogens. The results of the current study have demonstrated a remarkable inhibitory effect of CpCHT1 on both spore germination and germ-tube elongation of both *Fusarium oxysporum* and *Alternaria alternate* (Table 3, Fig. 4) both of which are agronomically important pathogens.

In summary, here we present the isolation, cloning and preliminary characterization of the first apoplastic, putative AFP-chitinase (CpCHT1) from a dicotyledonous perennial, *Chimonanthus praecox*. Our data strongly indicate that CpCHT1 has both antifreeze and chitinase activities. We hypothesise that this dual functionality means that the plant is 'protected' all year round. In the warmer months the antifungal activity will be of evolutionary advantage as will the antifreeze activity during the winter months. Future studies will focus on the regulation of expression and properties of this protein *in planta*, and hopefully facilitate biotechnological applications of the *CpCHT1* gene and its encoded protein for cold tolerance in species of agronomic importance.

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