Functional Characterization of Three Recombinant Chitinase-Like Proteins of *Picea glauca*

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ABSTRACT

North temperate tree species such as white spruce (*Picea glauca* [Moench] Voss) have evolved strategies to protect themselves against abiotic and biotic stresses that trees encounter during the inclement winter months. Chitinases not only play well-documented roles in plant defense but also function during physiological and developmental preparations for overwintering, including growth cessation, cold and desiccation acclimation, and dormancy acquisition. Transcript abundance profiling suggested roles for three white spruce chitinases during the growth-to-dormancy transition. In silico analyses of these three sequences suggested that two of the CHITINASES under study belonged to Cluster I(Pg_GQ0183_A06 and Pg_GQ03904_P01) which was associated with both cold tolerance and antifreeze activity. Pg_GQ0046_J03.1 belonged to cluster VI which was associated with cell wall modification. Chitinase activity assays showed that two of three tested total soluble protein fractions were exhibiting endochitinase activity. Taken together, our results imply that dormancy-associated chitinases act in concert to(1) confer protection against freezing injury, pests, and pathogens, (2) store nitrogen, and(3) promote cell maturation that precedes growth cessation.

Key words: Defense, Antifreeze proteins, Cell wall, Quantitative RT-PCR, White spruce, Norway spruce.

INTRODUCTION

Woody perennials, such as the forest tree *Picea glauca (Moench, Voss),* are challenged on a seasonal basis by exposure to growth-limiting conditions. During this time, they must halt growth and most physiological processes while maintaining the ability to resume activity once favourable conditions return. Perennials must also establish defences against the harsh abiotic conditions of winter. These objectives are accomplished through co-occurring adaptations including bud formation, dormancy acquisition, nitrogen accumulation and cold tolerance, which altogether result in the establishment of an over-wintering state (Cooke and Weigh 2005; Olsen 2010). During the transition from active growth to the over-wintering state, many changes occur at the cellular and biochemical level. Apical and lateral meristems, the major sites of active growth, suspend cell division and are reduced in overall size (Druart *et al.* 2007). Storage vacuoles found in phloem parenchyma and ray cells fill with proteins or lipids (Stepien *et al.* 1994; Cooke & Weigh 2005). Soluble carbohydrates, which are thought to contribute to osmotic regulation, increase in concentration (Morin *et al.* 2007). These changes, brought about by environmental signals such as reduced day length and lower temperature, are sensed and mediated in large part by molecular events (Olsen 2010).

Gene expression profiling previously conducted has shown that there is substantial transcriptome reprogramming in *Picea glauca* during the transition from active growth to a dormant state (El Kayal *et al.* 2011). Integrated transcriptomic and proteomic studies further showed that in some cases differential expression is maintained at the protein level (Gonzalez *et al.* 2012). Of all the genes identified by the integrated –omics work mentioned above, members of the CHITINASE gene family were identified as having particularly significant differential expression profiles.

Plant chitinases are well known to act in defence against pathogens by cleaving the β-1, 4-glycosidic bond found in the chitin of arthropods and fungi. Some plant chitinases are produced constitutively, though expression is further regulated by hormones and stress factors including infection, wounding, drought, salinity, cold and excess light (Xu *et al.* 2007). Plant chitinases are also known to function in growth and development by generating or degrading signalling molecules, and by contributing to programmed cell death (PCD). Furthermore, a chitinase-like protein in Arabidopsis was associated with growth and development through cell wall modification (Johnston *et al.* 2006; Sanchez-Rodriguez *et al.* 2012). In addition, certain apoplatic chitinases have been shown to have antifreeze activity (Yeh *et al.* 2000).

Plant chitinases typically contain a chitin-binding domain, hinge domain, catalytic domain and C-terminal extension; however individual chitinases may omit the chitin-binding domain or C-terminal extension (Islam *et al.* 2011). All vacuolar

chitinases contain C-terminal extensions, while apoplast-secreted chitinases do not (Xu *et al.* 2007).

Over time, many classification schemes have been proposed for plant chitinases. All chitinases are categorized as either endo- or exochitinases, dependent on whether they act at the middle or end of a chitin chain, respectively. Plant chitinases fall into the pathogenesis-related (PR) protein classes 3, 5 and 11. Plant chitinases are also grouped into seven biochemical groups (traditional classes I – VII) according to their primary structure, substrate specificity, catalytic mechanism and sensitivity to inhibitors. More recently, analysis of chitinase genes in the Arabidopsis and rice genomes yielded a phylogenetic classification composed of six clusters (Xu *et al.*2007). Analysis of the draft *P. glauca* genome revealed that it contains CHITINASE genes belonging to most of these clusters (Gonzalez *et al.* 2015).

A long-term objective of this research program is to functionally characterize the roles of individual *P. glauca* CHITINASES. Fine-scale expression profiles of CHITINASE genes during the transition to an over-wintering state were previously generated. The objective of this study was to predict and test the functions of CHITINASES differentially expressed during the transition to the overwintering state. Having already established a fine-scale temporal expression pattern, and coarse-scale spatial pattern (El Kayal *et al.* 2011), we investigated the sub-cellular localization of three CHITINASES in a model system. In addition, we produced recombinant CHITINASES for use in chitinase's functional assays.

MATERIALS AND METHODS

Plant material and experimental design

Two-year-old white spruce derived from Quebec (Canada) were used for all experiments. Seedlings were repotted and grown using a complete randomized block design in controlled-environment chambers at 20 °C under long days (LD; 16 h days/8 h nights) for 6 to 8 weeks. Plants were transferred to short days (SD), (8 h days/16 h nights) near the end of the active elongation phase – prior to initiation of apical buds as confirmed by inspection of shoot apices of a random sample of trees under a dissecting microscope – and harvested at 3, 7, 14, 28 and 70 d SD. Remaining plants were kept in SD for an additional 8–15 weeks, then transferred to low temperature (LT), (2–4 °C) for 3 to 4 weeks with continuing SD prior to harvest. This final time point is referred to as LT. At each time point, shoot tips (buds), current season foliage and current season stems with well-formed secondary growth were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C. Four independently replicated experiments, each with multiple plants per time point, were conducted in 2015 and 2016.

Construction of expression plasmids

cDNA sequences(GQ0046_J03, GQ0183_A06 and GQ03703_P01) obtained from GCAT3 were used to search the NCBI protein database with BLASTX in order to identify the protein coding sequence(CDS). Each CDS, excepting the final stop codon, was amplified using Platinum Taq DNA polymersase(Life Technologies Inc.) from cDNA templates reverse-transcribed from *P. glauca* RNA. Primers were designed to introduce BamHI and EcoRI sites or EcoRI and HindIII sites. The PCR fragments were subcloned into pGEMT-easy plasmids by TA cloning. pGEMT-easy plasmids containing the fragments described above were digested with FastDigest BamHI and EcoRI, or EcoRI and HindIII(Thermo Fisher Scientific Inc), after which the CDS fragments were subcloned into pET-32b(+) to construct pET-32b(+)-J03, pET-32b(+)-A06, and pET-32b(+)-P01. Clones were selected after transformation of *E. coli* DH5α and the insert integrity and orientation were verified by DNA sequencing.

Expression of recombinant chitinase protein

Individual colonies of *E. coli* BL21(DE3)pLysS transformed with the pET-32(b)-based plasmids were selected and stored as glycerol stocks. The bacterial cells were grown in LB medium containing 100 ug/L ampicillin per ml at 37° Covernight.

Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 – 2.0 mM and further propagation was carried out for 1-16 hours at 15 – 37 °C. Cells were harvested by centrifugation and resuspended in 20mM Tris-HCl pH 7.5. Solublization was allowed to proceed for 45 minutes at 4 C with occasional vortexing, after which the samples were centrifuged for 10 minutes at 7000 x g.

Each supernatant was loaded onto a 1 ml His60 Ni superflow resin column (Clontech Laboratories Inc.) and proteins were allowed to bind for 1 hour at 4 °C with continuous inversion. Purification under non-denaturing conditions was conducted according to the manufacturer's protocol. One ml elution fractions were monitored for protein concentration by Bradford assay (Bio-Rad Laboratories Ltd.). Protein content of the various fractions was visualized by SDS-PAGE and classical Coomassie stain, according to the Mini-PROTEAN II instruction manual (M1652940 Rev B, Biorad Laboratories Ltd.).

Chitinase activity assays

Total soluble protein and purified protein fractions as described above were assayed using the colorimetric chitinase assay kit, according to the manufacturer's instructions(CS0980, Sigma-Aldrich Inc.). Absorbance was measured using a Nanoquant Infinite M200 (Tecan Inc.).

Construction of Green Fluorescent Protein (GFP)fusion plasmids

Each CDS was amplified using Platinum Taq DNA polymersase (Life Technologies Inc.) from cDNA templates reverse-transcribed from *P. glauca* RNA. Primers for GQ0046_J03, GQ0183_A06 and GQ03703_P01were designed to introduce XbaI and Kpn2I sites. PCR fragments were digested using FastDigest XbaI and Kpn2I (Thermo Fisher Scientific Inc), after which they were subcloned into pAN580. Individual clones of *E. coli* DH5α transformed with the pAN580-based plasmids were selected and stored as glycerol stocks. The insert integrity and orientation were verified by DNA sequencing.

Biolistic transformation of onion epidermal cells

For each individual transformation or "shot", 20 ul of the tungsten particles (50 mg/ml) was combined with 2 ul plasmid DNA(~2mg/ml), 20 ul $CaCl_2$(2.5 M) and 8 ul spermidine (0.1M) and left on ice with occasional vortexing for 20 minutes. 80 ul 100% ethanol was added to precipitate the DNA onto the tungsten particles, after which they were washed 3 times in 100% ethanol and resuspended in 8 ul 100% ethanol. Six ul of the particle suspension was pipetted into the center of a macrocarrier disk and placed in a petri dish with Drierite. DNA-coated particles as above were used within 2 hours of preparation. Onion epidermal cells were bombarded with plasmid DNA-coated tungsten particles using a PDS-1000/He particle delivery system(Bio-Rad Laboratories Ltd.) according to Armin(2007). Cells were left at room temperature for 16 hours and imaged using the LSM 510 Axio imager and Plan Apochromat 20X objective(Carl Zeiss AG).

Statistical analysis

All test and manipulations, as well as subsequent statistical analyses were performed with Statistical Analysis Software 9.2 (SAS 9.2). Normality tests were performed and, where necessary to normalize distribution, data sets were transformed using a Log_{10} function. Significant differences were determined by Tukey's HSD test with overall α= 0.05.

RESULTS AND DISCUSSION

In *silico* characterization

A comparison was conducted based on the literature review to elucidate any overall relationship between phylogenetic classification and functional roles in plant chitinases, with an emphasis on functions other than pathogen response (Table 1).

Our previous expression data, predicted domains, and the literature review information summarized in Table 1 were analyzed to assign predicted subcellular localizations and functions to each CHITINASE. Phylogenetic cluster I was associated with both cold tolerance and antifreeze activity. Two of the CHITINASES under study belonged to this cluster (Pg_GQ0183_A06.2and Pg_GQ03904_P01.1).

Phylogenetic cluster VI was associated with cell wall modification(Hermans *et al.* 2010), through chitinase-like proteins from *Arabidopsis* which were proposed to bind nascent glucan chains in the apoplast(Sanchez-Rodriguez *et al.* 2012). One differentially expressed *CHITINASE* belonged to this cluster(Pg_GQ0046_J03.1). Interestingly, this was the sole *CHITINASE* which was found to be down-regulated during the transition to an over-wintering state in our previous studies (El Kayal *et al.* 2011; Gonzalez *et al.* 2012).

Protein overexpression and chitinase activity assays

Multiple different induction conditions were tested and though all 3 recombinant *CHIITNASES* were successfully overexpressed, the induced protein accumulated in the insoluble fraction (Figure 1). The *CHITINASES*, presumably present in inclusion bodies, were denatured and then purified under non-denaturing conditions using Ni-affinity chromatography.

Table1: Correspondence between classification schemes and the characterized functions of previously classified proteins. References to the literature supporting this analysis are given in the footnote below

Biochemical class	Characterized functions	References
I, II	Pathogen-response	Dong and Dunstan 1997; Ohnuma *et al.* 2011
	Cold-response	Antikainen *et al.* 1996; Olsen 2010
	Antifreeze	Druart *et al.* 2007; El Kayal *et al.*2011
II	Pathogen-response	Ohnuma *et al.* 2011
III	Vegetative Storage	Hossain *et al.* 2010; Islam *et al.* 2011
II, IV	Pathogen-response	Johnk *et al.*2005 ; Ohnuma *et al.*2011
	PCD/Embryogenesis	Islam *et al.* 2010
V	Pathogen-response	Melchers *et al.* 1994
	Chitinolytic	Meuriot *et al.* 2004
	Salt & Osmotic	Johnston *et al.* 2006; Meuriot *et al.*2004
II, VII	Pathogen-response	Arnim 2007; Ohnuma *et al.* 2011
	Cell wall	Morin *et al.* 2007 ; Nelson *et al.* 2007

Purified fractions containing a single major protein component corresponding to the induced recombinant *CHITINASE* were recovered in three of five cases (Figure 1). These fractions failed to show any activity when assayed for chitobiosidase, b-N-acetylglucosaminidase and endochitinase activity (Figure 2). In parallel with the activity assays conducted on the purified fractions, were also tested. Surprisingly, two of these fractions showed endochitinase activity. This suggests that although not evident in SDS-PAGE analysis, some of the induced recombinant *CHITINASE* is present in a soluble form.

The total soluble protein fractions which showed endochitinase activity corresponded to two *CHITINASES* upregulated during the transition to the overwintering state, to which were assigned hypothetical functions of antifreeze proteins (Pg_GQ03703_P01.1, Pg_GQ0183_A06.2). Since this activity was present, it cannot be excluded that these proteins are simply upregulated in preparation for defence against psychrophilic pathogens; though, if such a function was proven, it would not necessarily preclude additional functions. On the other hand, the total soluble protein fraction corresponding to Pg_Q0046_J03.1 failed to show endochitinase activity. Though preliminary, this result lends support to the notion that this protein has a function distinct from pathogen response such as, perhaps, cell wall modification.

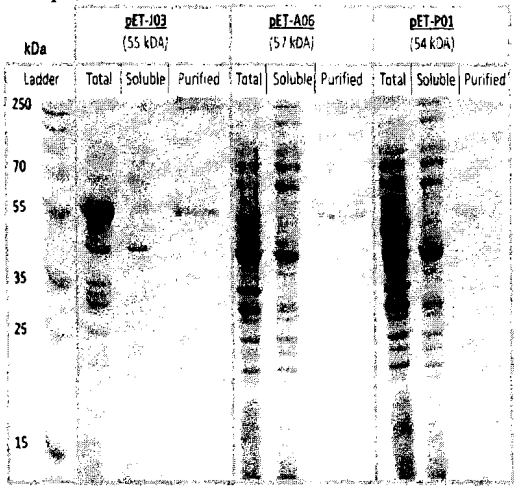

Figure 1: Total, soluble and purified recombinant protein extracted from E. coli BL21(DE3) transformed with expression constructs pET-J03, pET-A06 and pET-P01.

Numbers in brackets represent the expected size of the recombinant protein for each expression construct. Electrophoresis was conducted at 200 V for 45 minutes in a 12% discontinuous Laemmeli SDS-PAGE gel, followed by staining with classical Coomasie blue stain (Bio-Rad, M1652940).

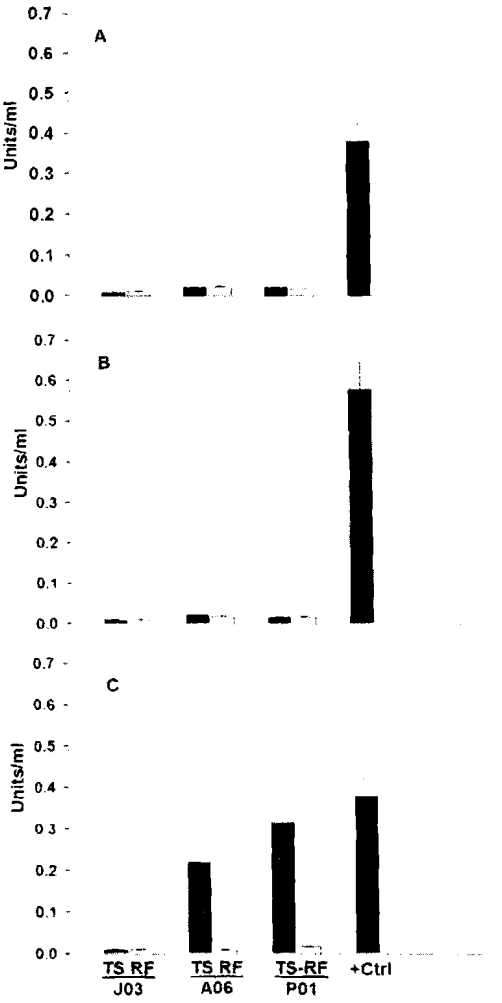

Figure 2: Calculated(A) chitobiosidase activity, (B) β-N-acetylglucosaminidase activity and(C) endochitinase activity of protein samples.

Total soluble (TS) and column refolded (RF) protein samples were assayed using the Chitinase Assay Kit, according to the manufacturer's instructions (Sigma-Aldrich, #CS0980). Chitinase from *Trichoderma viride* was used as a positive control (Ctrl). One unit is defined as the release of 1 µmole of p-nitrophenol per minute at pH 4.8 and 37 °C. Error bars represent the standard deviation of technical replicates (N=3).

Subcellular localization

Onion epidermal peels were transformed with the constructs described above by biolistic bombardment. After biolistic bombardment, onion epidermal cells were observed using brightfield microscopy to confirm viability, and were found to have active cytoplasmic streaming up to 120 hours after peeling.

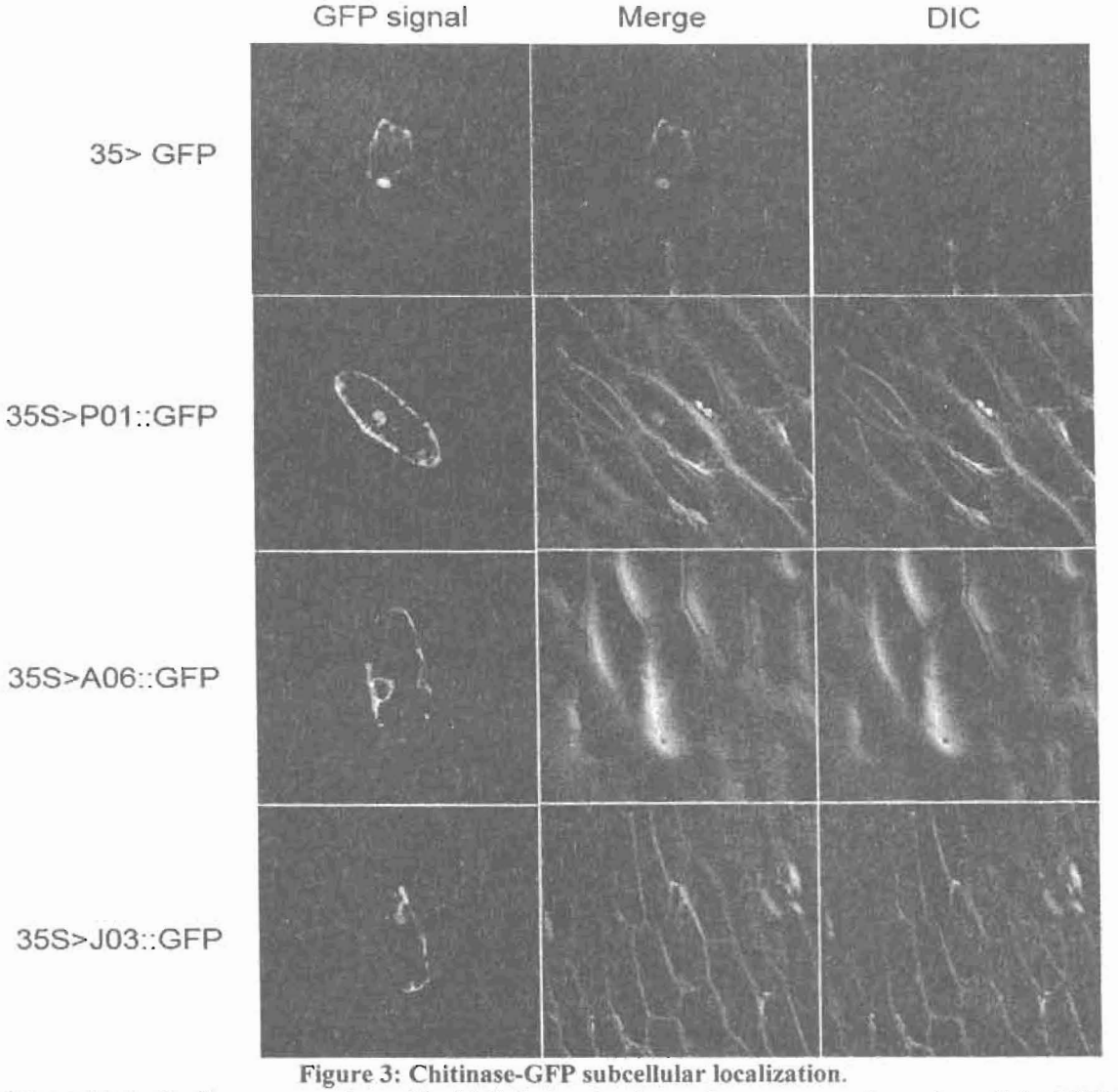

Figure 3: Chitinase-GFP subcellular localization.

Onion epidermal cells were transformed by biolistic bombardment. Images were taken using a Zeiss LSM 510 Axio imager and Plan Apochromat 20X objective. Image manipulations were carried out in imageJ using "channel merge" tool.

Transformed cells were identified using an epifluorescence dissecting microscope between 16 to 48 hours following biolistic bombardment, however, GFP fluorescence typically peaked around 24 hours after bombardment .The constructs described above were transformed into onion epidermal cells and successfully imaged using confocal microscopy(Figure 3). When expressed without a fusion partner, GFP appeared to fluoresce throughout the cytoplasm and nucleus, but was absent from the vacuole. When fused to either of the three chitinases, GFP fluorescence was seen in a similar pattern, except that it was no longer found in the nucleus except P01. The chitinases under study are expected to be targeted to the apoplast or the vacuole, and therefore localization results thus are unexpected. One of the most likely explanations is that expression from the 35S promoter overloads protein-targeting pathways in the onion cell, resulting in mislocalization of the chitinase::GFP fusion protein. It is also possible that fusion of GFP to the chitinase interferes with the peptide localization signal, or that chitinase localization requires factors not present in onion epidermal cells.

CONCLUSION

White spruce *CHITINASES* differentially expressed during the transition to an over-wintering state were identified through qRT-PCR. A literature review, in conjunction with extensive *in silico* work conducted by previous lab members, allowed us to assign hypothetical functions and subcellular localizations to each of the *CHITINASES* under study. In general, there was agreement between the assigned functions the validity of these hypotheses is ongoing.

pET-32b(+)-based expression plasmids were successfully constructed for each CHITINASE under study. Two *E. coli* host strains were successfully transformed, and expression was tested in a factorial experiment. The 3 recombinant *CHITINASES* were successfully overexpressed as part of the insoluble protein fraction, denatured and purified using His-tag affinity chromatography. Activity assays showed that the purified fractions, presumably due to lack of correct folding, were not chitinolytic. In contrast, two of three tested total soluble protein fractions showed endochitinase activity, suggesting that a small amount of induced CHITINASE was soluble. pAN580-based fluorescent protein fusion plasmids were successfully constructed for each CHITINASE under study, and were expressed in onion epidermal cells. In the long term, it would be ideal to locate *CHITINASES* in white spruce cells using specific antibodies.

Altogether, a significant contribution to the functional characterization of white spruce *CHITINASES* was made in this study. Many additional studies flow naturally from the present work: the recombinant *CHITINASES* can be used in functional assays, the fluorescent protein fusion constructs can be used to assess localization in other systems, including whole *Arabidopsis* plants; N-terminal fusions constructs complementary to those described above could be generated to verify localization patterns; and antibodies can be generated in order to study subcellular localization in the endogenous system.

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REFERENCES

Antikainen M., Griffith M., Zhang J., Hon W., Yang D. and PihakaskiMaunsbach K.(**1996**). Immunolocalization of antifreeze proteins in winter rye leaves, crowns, and roots by tissue printing. *Plant Physiology* 1100.P, 845-857.

Arnim, A.(**2007**). Subcellular Localization of GUS- and GFP-Tagged Proteins in Onion Epidermal Cells. *Cold Spring Harb Protoc.* doi:10.1101/pdb.prot4689

Cooke J. and Weih M.(**2005**). Nitrogen storage and seasonal nitrogen cycling in populus: Bridging molecular physiology and ecophysiology. *New Phytologist* **167**, 19-30.

Dong J. and Dunstan D.(**1997**). Endochitinase and beta-1,3-glucanase genes are developmentally regulated during somatic embryogenesis in picea glauca. *Planta* 201, 189-194.

Druart N., Johansson A., Baba K., Schrader J., Sjodin A., Bhalerao R.R., Resman L., Trygg J., Moritz T. and Bhalerao R.P.(**2007**). Environmental and hormonal regulation of the activity-dormancy cycle in the cambial meristem involves stage-specific modulation of transcriptional and metabolic networks. *Plant Journal* **50**, 557-573.

El Kayal W., Allen C.C.G., Ju C.J., Adams E., King-Jones S., Zaharia L.I., Abrams S.R. and Cooke J.E.K.(**2011**). Molecular events of apical bud formation in white spruce, picea glauca. *Plant Cell and Environment* **34**, 480-500.

Gonzalez L.M.G., El Kayal W., Ju C.J.., Allen C.C.G., King-Jones S. and Cooke J.E.K.(**2012**). Integrated transcriptomic and proteomic profiling of white spruce stems during the transition from active growth to dormancy. *Plant Cell and Environment* **35**, 682-701.

González, L.M.G., El Kayal, W., Morris, J.S. and Cooke J. E. K. (**2015**). Diverse chitinases are invoked during the activity-dormancy transition in spruce. *Tree Genetics & Genomes* **11**: 41. doi:10.1007/s11295-015-0871-0

Griffith M., Antikainen M., Hon W., PihakaskiMaunsbach K., Yu X., Chun J. & Yang D.(**1997**). Antifreeze proteins in winter rye. *Physiologia Plantarum* **100**, 327-332.

Hermans C., Porco S., Verbruggen N. and Bush D.R.(**2010**). Chitinase-like protein CTL1 plays a role in altering root system architecture in response to multiple environmental conditions. *Plant Physiology* **152**, 904-917.

Hietala A., Kvaalen H., Schmidt A., Johnk N., Solheim H. and Fossdal C.(**2004**). Temporal and spatial profiles of chitinase expression by norway spruce in response to bark colonization by heterobasidion annosum. *Applied and Environmental Microbiology* **70**, 3948-3953.

Hossain M.A., Noh H., Kim K., Koh E., Wi S., Bae H., Lee H. and Hong S.(**2010**). Mutation of the chitinase-like protein-encoding AtCTL2 gene enhances lignin accumulation in dark-grown arabidopsis seedlings. *Journal of Plant Physiology* **167**, 650-658.

Islam, M.A., Sturrock, R.N. and Ekramoddoullah, A.K.M.(**2011**). Conifer Chitinases. In: Al-Mughrabi K(Ed) Plant science and biotechnology in North America: Focus on Canada II. The Americas Journal of Plant Science and Biotechnology Volume 5 Special Issue **1**: 22-36.

Islam M.A., Sturrock R.N., Williams H.L. and Ekramoddoullah A.K.M.(**2010**). Identification, characterization, and expression analyses of class II and IV chitinase genes from douglas-fir seedlings infected by phellinus sulphurascens. *Phytopathology* **100**, 356-366.

Johnk N., Hietala A., Fossdal C., Collinge D. and Newman M. (**2005**) Defense-related genes expressed in norway spruce roots after infection with the root rot pathogen ceratobasidium bicorne (anamorph: Rhizoctonia sp.). *Tree Physiology* **25**, 1533-1543.

Johnston, M. D., Hamburger, B. R., Friedman, M., and Douglas, C. J.(**2006**). Functional genomics of plant chitinase-like genes: role in primary and secondary cell wall formation. ASPB abstract no. P18041

Melchers L., Apothekerdegroot M., Vanderknapp J., Ponstein A., Selabuurlage M., Bol J., Cornelissen B., Vandenelzen N P. and Linthorst H.(**1994**). A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *Plant Journal* **5**, 469-480.

Meuriot F., Noquet C., Avice J., Volenec J., Cunningham S., Sors T., Caillot S. and Ourry A.(**2004**). Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in medicago sativa taproots. *Physiologia Plantarum* **120**, 113-123.

Morin X., Ameglio T., Ahas R., Kurz-Besson C., Lanta V., Lebourgeois F., Miglietta F. and Chuine I.(**2007**). Variation in cold hardiness and carbohydrate concentration from dormancy induction to bud burst among provenances of three european oak species. *Tree Physiology* **27**, 817-825.

Nelson B.K., Cai X., and Nebenführ A.(**2007**). A multi-color set of *in vivo* organelle markers for colocalization studies in *Arabidopsis* and other plants. *Plant Journal* **51**:1126-1136.

Ohnuma T., Numata T., Osawa T., Mizuhara M., Lampela O., Juffer A.H., Skriver K. and Fukamizo T.(**2011**). A class V chitinase from arabidopsis thaliana: Gene responses, enzymatic properties, and crystallographic analysis. *Planta* **234**, 123-137.

Ohnuma T., Numata T., Osawa T., Mizuhara M., Varum K.M. and Fukamizo T.(**2011**). Crystal structure and mode of action of a class V chitinase from nicotiana tabacum. *Plant Molecular Biology* **75**, 291-304.

Olsen J.E.(**2010**). Light and temperature sensing and signaling in induction of bud dormancy in woody plants. *Plant Molecular Biology* 73, 37-47.

Sanchez-Rodriguez C., Bauer S., Hematy K., Saxe F., Ibanez A.B., Vodermaier V., Konlechner C., Sampathkumar A., Rueggeberg M., Aichinger E., Neumetzler L., Burgert I., Somerville C., Hauser M. and Persson S.(**2012**). CHITINASE-LIKE1/POM-POM1 and its homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in arabidopsis. *Plant Cell* **24**, 589-607.

Stepien V., Sauter J. and Martin F.(**1994**). Vegetative storage proteins in woody-plants. *Plant Physiology and Biochemistry* **32**, 185-192.

Wiweger M., Farbos I., Ingouff M., Lagercrantz U. and von Arnold S.(**2003**). Expression of Chia4-pa chitinase genes during somatic and zygotic embryo development in norway spruce (picea abies): Similarities and differences between gymnosperm and angiosperm class IV chitinases. *Journal of Experimental Botany* **54**, 2691-2699.

Xu F, Fan C and He Y.(**2007**). Chitinases in Oryza sativa ssp. japonica and Arabidopsis thaliana. *J Genet Genomics* **34**: 138–150

Yeh S., Moffatt B., Griffith M., Xiong F., Yang D., Wiseman S., Sarhan F., Danyluk J., Xue Y., Hew C., Doherty-Kirby A. and Lajoie G.(**2000**). Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. *Plant Physiology* **124**, 1251-1263.

Yu X. and Griffith M.(**1999**). Antifreeze proteins in winter rye leaves form oligomeric complexes. *Plant Physiology* **119**, 1361-1369.

الملخص العربى

التوصيف الوظيفي لثلاث من بروتينات الـ chitinases في أشجار الاسبروس الأبيض

وليد السيد الكيال

قسم الغابات وتكنولوجيا الأخشاب- كلية الزراعة- جامعة الاسكندرية- مصر

أنواع الأشجار التابعة للمناطق الشمالية لها استراتيجيات عديدة لحماية انفسها من الاجهادات الحيوية وغير الحيوية التي قد تواجها في فترات الشتاء. وأثبتت العديد من الدراسات أن جينات الـ Chitinases تلعب دوراً هاماً في مقاومة الأشجار لتلك العوامل ولكن ايضاً في العمليات الفسيولوجية التي تساعد الأشجار على تحمل البرودة مثل توقف النمو ودخول الأشجار في طور السكون. التعبير لحيني لثلاث من تلك الجينات اظهر دورهم في التحول من نشاط النمو إلى السكون. اظهرت نتائج دراسات التتابع الجيني ان اثنين من تلك الجينات يمكن تصنيفها مع جينات مقاومة والصقيع اما الجين الثالث محل الدراسة فهو يتبع مجموعة الجينات التي تتحكم في تغيرات الجدار الخلوي. وكان تحليل النشاط الانزيمي كان موجباً لاثنين من تلك البروتينات بينما لم يظهر البروتين الثالث أي نشاط. نتائج هذه الدراسة تلخص ثلاث ادوار رئيسه تقوم بها هذه الجينات هي:

١- الحماية ضد اضرار الصقيع والاصابات الحشرية والطفيلية.

٢- تخزين النيتروجين.

٣- استحثاث نضج الخلايا وبالتالي توقف النمو.