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Spring 2015

Identification of the ICE1 (inducer of CBF/DREB1 expression) gene in *Pinus nigra*.

Abstract

Low temperatures are an environmental stress that influence plant growth and development, limits a plant's natural range, and causes major crop loss world wide. Plants have evolved a cold acclimation mechanism that enhances their tolerance to freezing temperatures. The accumulation of these defenses against freezing trauma is dependent on the activation of Cold Regulated (*COR*) gene expression. This study aims to amplify and characterize the *ICE1* (inducer of *CBF/DREB1* expression) gene in *Pinus nigra* (black pine) making use of degenerate primers constructed by using *ICE1* sequences of 12 angiosperms. A region of the *ICE1* gene in black pine was PCR amplified and sequenced. This study presents the first and only sequence information on the *ICE1* gene in pine.

Introduction

Freezing temperatures cause the destabilization of plant cell membranes caused by cytoplasmic dehydration, limiting a plant's natural range and causing major crop loss worldwide. Membrane systems are susceptible to severe water loss from within the cell to the intercellular space due to the formation of extracellular ice as temperatures drop below 0^0C. In temperate regions where temperatures can vary greatly between summer and winter seasons, plants can acquire a tolerance for freezing temperatures with exposure to low nonfreezing temperatures (0-15^0C). The mechanism, plant cold acclimation, has been studied in a model organism, Arabidopsis thaliana, along with other angiosperm species that are

agriculturally significant, like Vitis vinifera and Solanum lycopersicum. The process of cold acclimation is dependent on the Cold Regulated (COR) proteins, induced by the cold signaling pathway, that stabilize membranes against injury resulting from freeze induced cellular dehydration. Understanding the nature of the genes and mechanisms regulating this pathway provides insight as to how temperature can affect the distribution of plant assemblages and the evolution of a plant population, and it can provide better strategies to improve the freezing tolerance of agronomically significant plants [1,2].

Our understanding of the cold signaling pathway which involves the *ICE* (inducer of *CBF/DREB1* expression)*, CBF/DREB1* (C-repeat binding factor/dehydration responsive element binding) and *COR* genes continues to grow but much work needs to be done, in diverse species of angiosperms and gymnosperms, to grasp the full array of effects freezing temperatures have on plants. As presently understood, signal transduction begins as the temperature drops below 15^0C. An unknown sensor triggers cytoplasmic calcium levels to increase which signals the activation of kinases in the cytosol. The inactive ICE1 binding protein, constitutively expressed in the cytosol, gets phosphorylated allowing this transcription factor to bind a cis-element in the promoter of *CBF3/DREB1A* activating its expression. Induction of the *CBF3/DREB1A* gene produces a transcription factor that binds to cis-elements in the promoter of the COR genes that encode: chloroplast membrane phospholipids able to withstand freeze induced membrane trauma (*COR15a*), dehydrins important for membrane stabilization and the prevention of protein aggregation at low temperatures (*COR47*), and polypeptides that reduce membrane permeability (*COR6.6*). ICE1 is negatively regulated by HOS1, an E3 ubiquitin-protein ligase, and positively regulated

by SIZ1, an E3 SUMO-protein ligase, via ubiquitylation and SUMOylation respectively [1,2,3,4].

My study aims to extend our knowledge of cold acclimation in seed plants by investigating a gene involved in the cold signaling pathway of gymnosperms. Pines are valued for their timber and wood pulp, and grow densely in temperate and tropical regions often outcompeting hardwood species [5]. The goal of this project is to amplify and characterize the *ICE1* gene in *Pinus nigra* (black pine) making use of degenerate primers constructed from 12 angiosperm *ICE1* sequences.

Methods

Homology Analysis-degenerate primer design

An alignment was performed on 12 *ICE1* sequences (Tab.1) belonging to diverse angiosperms using CLUSTALW [6]. Regions of conservation were located to design degenerate primers (Tab.2) spanning 20-22 bases and produce an expected PCR product of 531 bases.

Table 1. *ICE1* sequences used in the design of degenerate primers to amplify black pine DNA.

	description	accession number
1	*Arabidopsis thaliana*	NC_003074.8
2	*Vitis vinifera*	JQ707298.1
3	*Musa acuminata*	XM_009422758.1
4	*Camellia sinensis*	JX029153.1
5	*Malus domestica*	EF495202.1
6	*Glycine max*	NM_001251631.1
7	*Brassica napus*	JF268687.1
8	*Chrysanthemum dichroum*	JN129387.1
9	*Sorghum bicolor*	XM_002458977.1
10	*Lactuca sativa*	HQ848932.1
11	*Oryza sativa*	NM_001050547.1
12	*Hordeum vulgare*	EU887261.1

Table 2. Degenerate primers used to amplify *ICE1* gene in *Pinus nigra.*

primer orientation	oligo sequence	degenerate bases	oligos coded
forward	CAGCCHACTYTKTTYCAGAA	HYKY	24
reverse	GCATCNCCAAGNATDGADGCYC	NNDDY	288

DNA Extraction

Black pine needles were harvested and placed in a 1.5 mL tube. Needles were homogenized by adding coarse granite to the tube and using a bead mill to physically shear the cell wall. Genomic DNA was isolated by using the FastDNA™ SPIN Kit for Soil. *Pinus nigra* DNA was quantified and diluted to a 1ng/uL stock for PCR amplification.

PCR Amplification, Sequencing, and BLAST Analysis

PCR reactions were performed on 1 ng of Pinus nigra DNA using 45 pmoles concentration of degenerate primer in a 20 uL reaction, cycled 50 times with an annealing temperature of 57^0C. PCR products were isolated (Fig.1) with electrophoresis on a 2%(w/v) agarose gel and visualized with ethidium bromide under a UV lamp. The band near the expected size of 531 bases was excised and gel purified using the QIAquick Extraction Kit and sequenced. Sequence data was analyzed (Tab.3) on the NCBI website's BLAST [7].

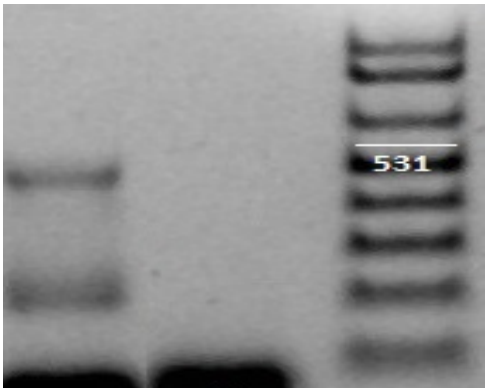
Results

PCR

Degenerate primers successfully amplified a ~500 base PCR product (Fig.1) using a 45 pmole concentration on 1 ng of Pinus nigra DNA. The primer concentration was increased, from 5 pmoles that is commonly used in 20 uL PCR reaction, to account for the decrease in potency of the degenerate primers (effective primer concentration decreases as the number

of oligonucleotides that are coded by the degenerate primer increases). The band was excised, purified by gel extraction, sequenced and analyzed with BLAST.

Figure 1. 2%(v/w) agarose gel visualized with ethidium bromide under a UV lamp. The first lane shows an ~500 base PCR product (top band), amplified from *Pinus nigra* DNA using degenerate primers, separated by electrophoresis.



BLAST

The BLAST analysis on the sequence produced a top match to white spruce (Fig.2), a relative of black pine in the Pinaceae family, along with subsequent close matches to *ICE1* sequences (Tab.3), belonging to angiosperms.

Figure 2. Closest BLAST result for the *Pinus nigra* PCR product that was sequenced. The sequence matched Picea glauca. commonly known as white spruce, a relative of black pine in the Pinaceae family.

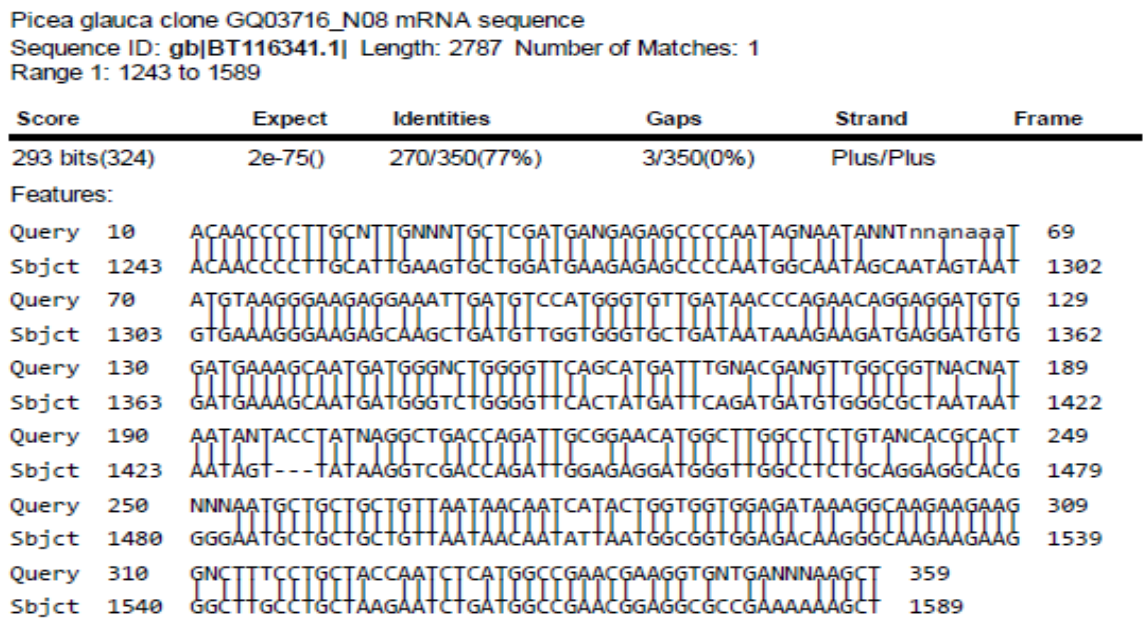


Table 3. List of closest matching BLAST results to amplified sequence.

Description	Query cover	Ident	Accession
Picea glauca clone GQ03716_N08 mRNA sequence	**96%**	**86%**	**BT116341.1**
Amborella trichopoda transcription factor ***ICE1***, mRNA	**19%**	**76%**	**XM_006844479.2**
Daucus carota inducer of CBF expression 1-b (***ICE1***-b) mRNA,	**18%**	**77%**	**KM487594.1**
Daucus carota inducer of CBF expression 1-a (***ICE1***-a) mRNA	**18%**	**77%**	**KM487593.1**
Tarenaya hassleriana transcription factor ***ICE1***-like , mRNA	**16%**	**78%**	**XM_010530677.1**
Glycine max ***ICE***d mRNA, complete cds	**16%**	**79%**	**HM989928.1**
Glycine max inducer of CBF expression 4 (***ICE***4), mRNA	**16%**	**79%**	**NM_001251778.1**

Discussion

The utility of degenerate primers is apparent when attempting to characterize homologous genes in novel organisms. The mixture of oligonucleotides encoded by the a degenerate primer can reduce the specificity of the PCR amplification but it increases the likelihood of hybridization to the novel gene. This study demonstrated that the *ICE1* gene is encoded in the genome of Pinus nigra, a cold hardy gymnosperm. This sequence information is a prelude to

cold regulated gene expression studies in *Pinus* and can be used to explore contiguous regions of the black pine *ICE1* gene. Logically, research into *Pinus nigra HOS1* and *SIZ1* genes should be done to understand the mechanism that regulates the ICE1 transcription factor in response to seasonal changes. Determining the nature of the genes and processes responsible for the activation of the cold-acclimation response will help our understanding of the natural world [1].

References

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