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Differentially Expressed Genes under Cold Acclimation in *Physcomitrella patens*

Ming-Ming Sun[1,2,#], Lin-Hui Li[3,#], Hua Xie[4], Rong-Cai Ma[4,*] and Yi-Kun He[1,*]

[1]College of Life Science, Capital Normal University, Beijing 100037, China
[2]College of Elementary Education, Capital Normal University, Beijing 100080, China
[3]Department of Biology, China West Normal University, Nanchong 637002, China
[4]Beijing AgroBiotechnology Research Center, Beijing 100089, China

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Cold acclimation improves freezing tolerance in plants. In higher plants, many advances have been made toward identifying the signaling and regulatory pathways that direct the low-temperature stress response; however, similar insights have not yet been gained for simple non-vascular plants, such as bryophytes. To elucidate the pathways that regulate cold acclimation in bryophytes, we used two PCR-based differential screening techniques, cDNA amplified fragment length polymorphism (cDNA-AFLP) and suppression subtractive hybridization (SSH), to isolate 510 ESTs that are differentially expressed during cold acclimation in *Physcomitrella patens*. We used real-time RT-PCR to further analyze expression of 29 of these transcripts during cold acclimation. Our results show that cold acclimation in the bryophyte *Physcomitrella patens* is not only largely similar to higher plants but also displays distinct differences, suggests significant alteration during the evolution of land plants.

Keywords: cDNA-amplified fragment length polymorphism (cDNA-AFLP), Cold acclimation, Gene expression profile, *Physcomitrella patens* (Hedw.), Real-time RT-PCR, Suppression subtractive hybridization (SSH)

Introduction

Many plants increase in freezing tolerance upon exposure to low temperature, a phenomenon known as cold acclimation (Thomashow, 1999). In recent years, significant insights have been made into the low-temperature signaling and regulatory pathways underlying cold acclimation. For instance, it is now known that once primary sensors perceive a low-temperature signal, plants generate second messengers, such as inositol phosphates and reactive oxygen species, that, by modulating intracellular Ca^{2+} levels, can initiate protein phosphorylation cascades, ultimately activating proteins that provide cellular protection or transcription factors that control the expression of specific sets of cold-regulated genes (COR), such as the *LEA* (late embryogenesis abundant) family genes (Thomashow, 2001), which are induced in many plant species in response to the dehydration caused by cold stress. The regulation of *COR* genes' expression involves multiple mechanisms, including ABA-dependent, and ABA-independent pathways (Thomashow, 2001; Xiong *et al.*, 2002).

Studies on *COR* genes expression in *Arabidopsis* have shown that members of the CBF/DREB1 transcriptional factor family, which are induced early and transiently by cold stress, play a key role in cold acclimation (Liu *et al.*, 1998; Gilmour *et al.*, 2000; Fowler and Thomashow, 2002). In addition, evidence has accumulated indicates that plant hormones, such as ABA, play important roles in stress signal transduction. In higher plants, cold stress can cause an increasing accumulation of ABA (Taylor *et al.*, 2000). Recent genetic evidence suggests that the ABA-inducible bZIP transcription factors, ABF/AREB, regulate the stress-signaling pathways that activate *LEA*-like genes in higher plants (Yamaguchi-Shinozaki and Shinozaki, 2006). Brassinosteroids (BRs), which are steroidal compounds that appear to play a role in protecting plants from a variety of environmental stresses, could promote growth recovery of maize seedlings and tomato fruit setting after cold acclimation. The growth-promoting effects of BRs have also been observed in rice during cold acclimation (Krishna, 2003).

Various studies have indicated that cell membranes are the primary site of freezing injury in plants (Thomashow, 1999).

[#]co-first authors: These authors have equal contribution to the study.

*To whom correspondence should be addressed.
Tel: 86-10-51503831; Fax: 86-10-51503980
E-mail: marongcai@baafs.net.cn (Rong-Cai Ma)
Tel: 86-10-68902345; Fax: 86-10-68902328
E-mail: yhe@mail.cnu.edu.cn (Yi-Kun He)

Therefore, it is not surprising that the key function of cold acclimation is stabilizing membranes against freezing injury. For instance, in rye and other plants, cold acclimation can prevent the serious freezing-induced membrane damage caused by the formation of hexagonal phase lipids. Cold acclimation can also induce changes in lipid composition of plant plasma membranes, especially increasing the amount of unsaturated fatty acid in the membranes; increased levels of unsaturated fatty acid contribute to membrane stability (Wada *et al.*, 1990; Hitz *et al.*, 1994). Additionally, several other factors, including the accumulation of soluble sugars, increased levels of antioxidants, and modulation of activities of various metabolic enzymes, also appear to play important roles in alleviating freezing-induced cellular damage (Livingston and Henson, 1998; Thomashow, 1999).

Other environmental stresses, including drought and pathogens, can also affect cold acclimation. The transcriptional profiles of stress-responsive genes indicate that cross-talk among signal transduction pathways is responsible for various stress responses (Xiong and Zhu, 2001).

The potential of mosses as model systems to study plant biological processes was already recognized in the forties, especially *Physcomitrella. patens* (Schaefer and Zryd, 2001). Owing to several advantageous features including simple morphology for observation, easy cultivation under axenic medium, predominant haploid phase in its life cycle and high homologous recombination rate, *P. patens* is now considered as an excellent model plant for functional genomics (Cove, 2005; Cove *et al.*, 2006; Quatrano *et al.*, 2007). Transcriptomic analyses illustrate commonalities among plant lineages in gene content, structure, and regulation (Nishiyama *et al.*, 2003; Rensing *et al.*, 2005). However, to date, it remains unclear if it utilizes similar or special mechanisms with higher plants for stress tolerance (Oldenhof *et al.*, 2006). In fact, *P. patens* can survive throughout the world and are the dominant vegetation in some ecological regions such as Antarctica (Robinson *et al.*, 2003). The widespread viability of *P. patens* is due to their ability to successfully adapt physiologically to various extremely stressful environmental conditions, including low temperature. Since bryophytes have a key position in the evolution of plants, understanding cold acclimation in *P. patens* would lend insight into the evolution of stress response mechanisms in higher plants.

Although several cold-induced genes, including *PpDBF1*, *PPARs*, *PpSHP1* and *PpSHP2,* have been identified in cold-acclimated bryophytes (Minami *et al.*, 2003; Kroemer *et al.*, 2004; Liu *et al.*, 2007), little is known about cold-induced cell signaling pathways and freezing tolerance mechanisms in bryophytes, and what is known requires clarification. For instance, recent studies have shown that exogenously applied ABA can induce freezing tolerance in *P. patens*; however, endogenous ABA levels are not altered in cold-acclimated *P. patens* (Minami *et al.*, 2003; Minami *et al.*, 2005). In order to further illuminate the mechanisms associated with cold acclimation, we used gametophore cells of the moss *P. patens* for functional genomics studies.

We have used two powerful, reproducible PCR-based differential screening techniques, cDNA amplified fragment length polymorphism (cDNA-AFLP) and suppression subtractive hybridization (SSH), to isolate differentially expressed ESTs during cold acclimation in *P. patens*. These methods are used routinely to detect differentially expressed transcripts and have been successfully employed to isolate *COR* genes in other studies (Bahn *et al.*, 2001; Barret *et al.*, 2004). Furthermore, we have confirmed the increased expression of several of differentially expressed genes during cold acclimation using real-time RT-PCR. Transcriptional profiles obtained for the moss *P. patens* during cold acclimation may provide important insights into the regulatory pathways that have been evolved during the early stage of land plants.

Materials and Methods

Plant material and growth conditions. *P. patens* (kindly provided by Dr. Ralf Reski of the University of Freiburg, Germany) was cultured as previously described (Ashton *et al.*, 1979). Gametophores were grown axenically in solid medium containing 250 mg L^{-1} KH_2PO_4, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 250 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 12.5 mg L^{-1} $FeSO_4 \cdot 7H_2O$, supplemented with 0.055 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.055 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.614 mg L^{-1} H_3BO_3, 0.389 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.055 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.028 mg L^{-1} KI, 0.025 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 0.7% (w/v) agar, pH 7.0. Plants were cultured under standard conditions in a phytochamber under a 16 h light/8 h dark cycle with 50 μmol m^{-2} s^{-1} quantum irradiance at 25 ± 1°C. 12-day-old gametophores were harvested at 0, 3, 6, 12, 24, 48, and 72 h after cold treatment at 0°C on ice.

Freezing tests. For freezing tests, gametophores cold-acclimated at 0 for 0 h, 3 d and 7 d were frozen for 90 min at −4, −7, −9, −10 and −11°C, respectively. Following freezing, gametophores were placed in a fresh medium and incubated at 25°C under a 16 h light/ 8 h dark cycle for 10 days, and then the efficiency of survival was calculated.

RNA extraction and cDNA synthesis. Total RNA was obtained from gametophores using a phenol-based extraction method (Robaglia *et al.*, 1993). Poly $(A)^+$ RNA was isolated from total RNA using the Qiagen Oligotex mRNA Kit (Qiagen). For differential and real-time RT-PCR analyses, cDNA was synthesized using an RNA PCR Kit (Takara).

cDNA-AFLP analysis. cDNA-AFLPs were generated using cDNAs from control and cold-acclimated gametophores (0, 3, 6, 12, 24, 48, and 72 h) using the restriction enzyme combination *Eco*RI/*Mse*I as described previously (Bachem *et al.*, 1998). The primers used for pre-amplification were GACTGCGTACCAATTC for *Eco*RI, and GATGAGTCCTGAGTAA for *Mse*I. Pre-amplification PCR was carried out using the following profile: 94°C 30 s; 56°C 30 s; 72°C 1 min for 15 cycles. The primers used for mean amplification for *Eco*RI and *Mse*I were GACTGCGTACCAATTC+AN/+TN and GATGAGTCCTGAGTAA+CAN/+CTN (N = A, T, C, G). Mean

amplification PCR was as follows: 7 cycles: 94°C 30 s; 65°C –0.7°C/ cycle 30 s; 72°C 1 min, and 25 cycles: 94°C 30 s; 56°C 30 s; 72°C 1 min. After electrophoresis on vertical polyacrylamide gels, silver stained cDNA-AFLP fragments were excised from the gels and eluted with 20 µl of sterile water. Aliquots (5 µl) of the resuspended DNA were re-amplified using the specific cDNA-AFLP primers. PCR products were verified on 1.5% agarose gels, and subsequently cloned into the pGEM-T Easy vector (Promega, America) for sequencing (ABI 3100, Applied Biosystems, America).

SSH analysis. SSH analysis was performed using the Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, America). For the library construction, non-acclimated (0 h) gametophores mRNA was used as the tester and cold-acclimated (3, 6, 12, 24, 48, and 72 h) gametophores mRNA was used as the driver. To prepare reverse subtracted cDNA for differential screening, we also performed SSH subtraction using mRNA from cold-acclimated (3, 6, 12, 24, 48, and 72 h) gametophores as the tester and mRNA from non-acclimated (0 h) gametophores as the driver. We cloned cDNA fragments into the vector pGEM-T (Promega, USA).

We used both cDNAs and forward and reverse subtracted PCR products probes to screen 768 clones, arrayed in duplicate at low density on 8 nylon membranes, by a dot-blot analysis. Differential screening was applied to identify up- and down-regulated transcripts. Driver cDNAs- and subtracted cDNA-probes were labeled with [α-^{32}P] dATP as described (Feinberg and Vogelstein, 1983), and hybridization was carried out on two identical membranes as described (Sambrook and Russell, 2001). Storage phosphor screens (Kodak) were visualized after exposure to a phospho-imager (Cyclone, Packard). The sum of signals was used to normalize the uniform background; the coefficient of normalization was 1.19 ($D_{nor} = D_{back} \times 1.19$).

Sequence analysis and functional annotation. Clusters of edited sequences were assembled using the SeqMan program (part of the DNASTAR suite from DNASTAR Inc, Madison WI). Assembly of clusters was performed using a match size of 50, minimum match percentage of 90, and a minimum sequence length of 100. To identify potential homologues to *P. patens* genes, we used the BLASTx and BLASTn algorithms (Altschul *et al.*, 1997) to compare the predicted protein sequences to those in the NCBI non-redundant and *P. patens* EST project databases (http://moss.nibb.ac.jp/blast/blast.html). Functional annotations were assigned to cluster sequences using GoFigure from Gene Ontology (GO, http://www.geneontology.org) (Khan *et al.*, 2003).

Real-time RT-PCR. Real-time RT-PCR was performed using a Rotor Gene 3000 Real-Time Thermal Cycler (Corbet Research, Australia). The SYBR Premix Ex Taq (Perfect Real Time) kit and RT-PCR reagents (Takara, Japan) were used for quantification of differentially expressed gene sequences. The specific primers used for selected transcripts are listed in Table 2. *Ppactin3* was used as the endogenous reference gene. Real-time RT-PCR amplification was performed as follows: 95°C 15 s; 60°C 20 s; 72°C 15 s for 45 cycles. These experiments were repeated three times. PCR efficiencies for the target and reference genes were determined by generating standard curves. Melting curve analysis was performed to exclude the occurrence of primer dimers and unspecific PCR products using Dissociation Curves Software Rotor-Gene 6.0. The relative expression rates of target gene transcripts were calculated according to Pfaffl (Pfaffl, 2001) using the following formula:

$$\text{Relative expression} = (1 + E_{target})^{\Delta Ct\ target} / (1 + E_{ref})^{\Delta Ct\ ref}$$

Here, E_{target} is the PCR efficiency for the target gene transcript; E_{ref} is the PCR efficiency for the endogenous reference gene transcript; ΔCt_{target} is determined by subtracting the control Ct value from the sample Ct value of the target gene transcript; and ΔCt_{ref} is determined by subtracting the control Ct value from the sample Ct value of the endogenous reference gene transcript.

Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (sQRT-PCR) analysis. *P. patens* were grown in petri dishes for 12 days. Gametophores were collected after cold treatment at 0°C for 0, 3, 6, 12, 24, 48, and 72 h for analyzing single-time-point mRNA abundance. The mRNA abundance of *Pp-CPD1*, *Pp-DET2*, *Pp-BRI1*, *Pp-BIN*, *Pp-BES1* and *Pp-BZR1* was analyzed by reverse transcription polymerase chain reaction (RT-PCR). Total mRNA was prepared and reverse transcription was transcribed using a cDNA synthesis kit (Takara, Japan). Primers for PCR reactions were: *Pp-CPD1* (5'-CATGGGCTGGGTTGCTCA; 5'-CCGCCGAATGGGACGAA), *Pp-DET2* (5'-TGCAAACCCGG AACATTACCTG; 5'-CCCGTGCGAAGACCCATCA), *Pp-BRI1* (5'-ATCACGATTGCATACCCCACATTA; CGTCAGGAGCTCG AGCAGAACTAT), *Pp-BIN* (5'-ATCAGCGAATGCCCCTTGTTT ACG; 5'-CCACGGGTGCGCCTTGATTTGT), *Pp-BES1* (5'-CTCC GGGGCAGAAGGGTGGTC; 5'-TCAGCTGCGCCGGGGTAGG AA), *Pp-BZR1* (5'-CGGGCTGGACGGTGGAAGAGGAC; AGC CGCGGCCAGGAAAGGGTGAT), and *Ppactin3* (5'-CAAAATG CAAGCCTGAAAAATGT; 5'-CGTACGTGGCGCTGGACTTC). For PCR amplifications, 18 cycles were used for *Pp-CPD1*, *Pp-DET2*, *Pp-BRI1*, *Pp-BIN*, *Pp-BES1* and *Pp-BZR1*, and 20 cycles for *Ppactin3*. RT-PCR products were analyzed by agarose gel electrophoresis. Sufficient amounts of cDNA in each sample were estimated by video densitometry analysis using LabWorks™ analysis software Version 3.0 (UVP) and compared against *Ppactin3* gene.

Results

Freezing tolerance of *P. patens* gametophores. The increase of freezing tolerance in *P. patens* gametophores following different periods of cold acclimation is shown in Fig. 1 and Table 1. Non-acclimated plants did not survive at –7°C; about 70% of plants acclimated at 0 for 3 d survived at –7°C, but did not survive at –10°C. However, most plants cold acclimated for 7 d at 0°C did survive at –10°C.

cDNA-AFLP and SSH analysis of cold acclimation of *P. patens*. For cDNA-AFLP (cAFLP) analysis, we used the enzyme combination *Eco*RI/*Mse*I. From forty *Eco*RI/*Mse*I primer combinations, ten (E1M4, E2M4, E3M4, E7M4, E2M6, E4M1, E5M7, E7M3, E6M1, E8M1) were scored for differentially expressed genes in cold-acclimated *P. patens* (Fig. 2). The *Eco*RI primer was extended with two selective nucleotides, and *Mse*I primers were extended with three

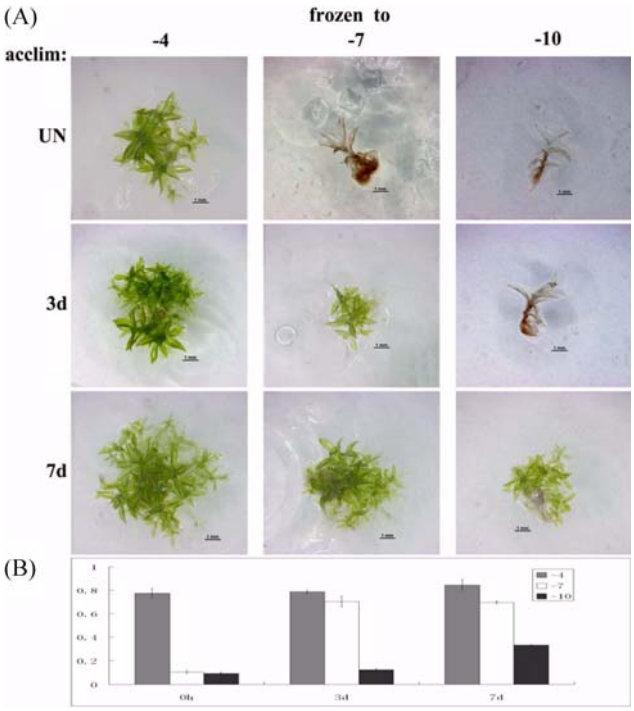

Fig. 1. Freezing survival of *P. patens* gametophores after different acclimation (acclim) treatments. 12-day-old plants were acclimated at 0°C with a 16-h photoperiod for 0 (UN), 3 d, or 7 d. A: The plants shown were frozen at −4°C (left), −7°C (middle), or −10°C (right). After thawing overnight at 0°C, tissues were grown on a fresh medium at 25°C for 10 d. Bars = 1 mm. B: The efficiency of survival after 10 d refreshing growth. The value was originated from Table 1. N: 57 plants.

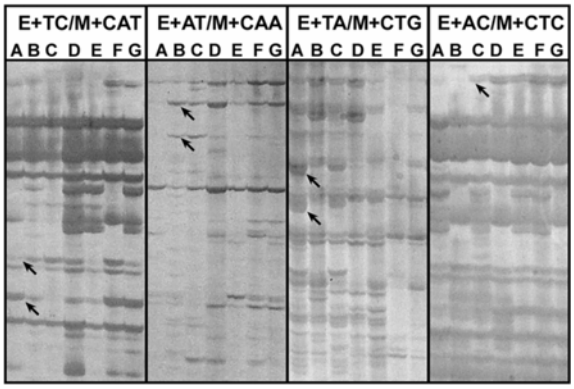

Fig. 2. Cold acclimation-associated changes in the gametophores of *P. patens* were determined by cDNA-AFLP. mRNAs were purified from control plants (0 h, lane A), and from cold-acclimated plants (3 h, lane B; 6 h, lane C; 12 h, lane D; 24 h, lane E; 48 h, lane F; 72 h, lane G). The arrows indicate differentially expressed genes after cold stress.

nucleotides. In reducing the occurrence of non-specific fragments generated in the cDNA-AFLP, the cycle number used in the pre-amplification and the amount of secondary template used were key parameters. By comparing the effects of 15, 20, and 25 cycles for pre-amplification, combined with dilutions of 10- or 20-fold, on the profiles of the fingerprint of the cold-treated *P. patens*, we found that a 15 cycle pre-amplification followed by a 20-fold dilution resulted in the best qualification (data not shown). Among approximately 1630 transcript-derived fragments (TDFs) in the size of 100 to 1000 bp, 119 (7.3%) were specific to cold-acclimated *P. patens*. These fragments were verified by Reverse-Northern blot analysis. Differentially amplified PCR fragments were excised from gels and reamplified using selective cDNA-AFLP primers. Finally these cDNA fragments were cloned for further analyses.

Of the 768 independent clones arrayed from the SSH cDNA library, approximately 50.9% were differentially regulated during cold acclimation (Fig. 3); we identified 201 up-regulated, and 190 down-regulated clones using the subtracted PCR product probes. These clones were sequenced for further analysis. In all, we sequenced 510 EST clones for further analysis from the two libraries. These sequences have been deposited in the NCBI dbEST database (GenBank accession number EE295950 to EE296459).

Sequence analysis and functional annotation of differentially expressed transcripts. Using the assembly algorithms in the SeqMan software (McCarter *et al.*, 2003), the 510 differentially expressed ESTs could be assembled into 365 EST clusters. Table 3 lists the 14 most abundant EST clusters. These abundant transcripts only accounted for 17.8% of the total ESTs generated. Six of the most abundantly represented transcripts encode proteins of unknown function; of these, one

Table 1. Freezing tolerance of *P. patens* gametophores after cold acclimation

	−4°C		−7°C		−10°C	
	Efficiency of survival (%)[#]	SD	Efficiency of survival (%)	SD	Efficiency of survival (%)	SD
UN*	77.30	0.041	78.68	0.015	84.43	0.044
3d	10.32	0.013	70.27	0.045	69.56	0.012
7d	9.26	0.011	12.31	0.013	33.46	0.003

*UN: non-acclimated plants; SD: standard deviation.
[#]: this test was repeated three times. for each test, the number of plants used in each line was higher than 57.

Table 2. Sequence (genes analyzed using real-time RT-PCR) similarity of sequenced cDNA-AFLP and SSH clones

Clone	GeneBank Accession No.	EST No.	Length	Maximum similarity[#]				Real-time RT-PCR primers
				Gene product	E-value	Accession No.	Organism	
*Pp-SSH7**	EE296090, EE296095, EE296131, EE296167, EE296205, EE296225, EE296273, EE296278, EE296287	9	328	LEA1 protein	6e-07	BAC80266	*T.æstivum*	[a]f-CCACTCAGGACAAGGCTTCG, r-AATACGTTGGCCACAGCATC
*Pp-cAFLP254**	EE296067	1	539	LIM domain-containing protein	9e-31	CAB16816	*A.thaliana*	f-GATGCCGCTAAGAGCGCTAC, r-GTAGCTTCGCCTGCCTTGTG
Pp-SSH251	EE296111	1	332	TspO/MBR-related protein	5e-11	ZP00471521	*C.salexigens*	f-CCACTCTTCGGCTTGATCTG, r-CAAGAGTGACGCTGTGGAAC
*Pp-SSH-cAFLP2**	EE296078, EE296138, EE296144, EE296151, EE296157, EE296158, EE296185, EE296210, EE296288, EE296290, EE296291	11	486	putative 3-β hydroxysteroid dehydrogenase	6e-38	AAM61751	*A.thaliana*	f-GCCGATACCTTGTAGCGCTG, r-TGATTCTTCTGTCCAATCCA
*Pp-SSH18**	EE296122, EE296140, EE296213, EE296264, EE296276	5	440	similar to calmodulin 1	1e-05	XP544274	*C.intestinalis*	f-TGTGTTGTGTTCGGATGATT, r-CAGCTGGCAGAAGTCTTCAA
Pp-SSH125	EE296281	1	250	putative calmodulin	5e-19	AAY78631	*A.thaliana*	f-TTATTACGGCAGCAGAGCTT, r-AACAGGAATGTCCAGGCTCG
Pp-SSH108	EE296214	1	587	Unknown				f-CGAGCTGTGCCGATGATGAG, r-AATCAATGTCGTCCAGTCTC
*Pp-SSH213**	EE296103	1	672	violaxanthin de-epoxidase precursor	2e-31	AAC49373	*L.sativa*	f-CTATTACCGAGGCAAGAATG, r-CGTGTGTTGTCAGTTGTGGT
*Pp-cAFLP62**	EE296012, EE296014	2	395	Cytochrome P450 78A11	4e-29	Q7Y1V5	*O.sativa*	f-CTCTGACCTTGACGTACTGG, r-GATTGCACAACCATTAGCTT
*Pp-SSH20**	EE296318, EE296332, EE296345, EE296350	4	686	DREB subfamily of ERF/AP2 transcription factor	5e-22	AT1G77200	*A.thaliana*	f-ACAAGAGGTCACGTATCTGC, r-GGAGATCTGATGGAGGAGAC
Pp-SSH89	EE296392	1	605	putative transcription factor BHLH5	2e-33	AAL55712	*A.thaliana*	f-TTAAGCCATGTGCAAGCGGA, r-CCGATTGCAGCTTGCTCGTA
*Pp-SSH215**	EE296437	1	407	MYB binding protein	2e-10	XP493841	*O.sativa*	f-CTCTGTGCATCTTGACGTTG, r-GATCCTTCGCTCTAACATCA
Pp-SSH216	EE296430	1	458	putative protein phosphatase 2C	2e-29	AAM14262	*A.thaliana*	f-GGTCATGATGGTGAAGCTGC, r-TTCACAGCATCTTCCACACA
Pp-SSH94	EE296311	1	540	putative phosphatidylinositol 4-kinase	8e-42	CAB37928	*A.thaliana*	f-CTGCATCTGAATCCATTACC, r-TGGTCACATCATTCACATCC
*Pp-cAFLP72**	EE295953, EE295954	2	473	serine/threonine specific protein kinase-like	2e-28	BAD23327	*O.sativa*	f-AATAGACCAAGTGGTGAGCA, r-TGAGAATTGACCTTCCAAGC
Pp-SSH129	EE296356	1	448	similar to mitogen-activated protein kinases	3e-06	AAS07241	*O.sativa*	f-TTACTCATCACGGCCACCTC, r-GGCTCGTTCGGTTCCGTCTA
*Pp-SSH294**	EE296147	1	499	Omega-6 fatty acid desaturase	2e-27	AAV41001	*O.europæa*	f-ATTCCTATGTGTGGTGTCAC, r-TGTGCCTTGAGTGTAGAATA
*Pp-SSH321**	EE296326	1	590	plasma membrane intrinsic protein 1a	1e-80	CAB56217	*S.oleracea*	f-TGGTGATGTAGAGGAAGAGA, r-TAGTAGAGTGCACGGTTAAG
*Pp-cAFLP361**	EE295968	1	282	putative stress-inducible membrane pore protein	3e-19	AAU44206	*O.sativa*	f-GTGTATACGCCGGAGTTGAA, r-AACATCTTGTTCTTCGTGGA
*Pp-SSH32**	EE296308, EE296407, EE296442	3	445	agglutinin isolectin 1precursor WGA1	2e-07	P10968	*T.æstivum*	f-AATGAGGTAGTGAGCGGCTT, r-TTCGTCAGTCGCTGAAGAGT
Pp-cAFLP354	EE296040	1	490	putative pyruvate orthophosphate dikinase	6e-74	AAT85082	*O.sativa*	f-TTACCTGCGTCTCCTGGAGC, r-TTGCGTACCAGGATAACCAC
*Pp-cAFLP299**	EE296023	1	460	hydroxyproline-rich glycoprotein family protein	1e-17	NP565899	*A.thaliana*	f-AATGTAATGACAGGCGATCC, r-GACATGCACCTGAGGACCTG
*Pp-SSH153**	EE296348	1	427	peroxiredoxin	3e-31	NP566268	*A.thaliana*	f-TCTGATGTCGCCTTGCAGGA, r-GAATGCACCAGGAAGACCAA
*Pp-SSH318**	EE296420	1	694	secretory peroxidase	2e -17	AAD33072	*N.tabacum*	f-GCAGCAAGCCTTGTATTAGC, r-AGAGCCGGTGCACTTCACAT
Pp-SSH152	EE296071	1	476	putative hypersensitive-induced protein	1e-49	CAA10289	*C.arietinum*	f-AAGCAGAAGGAGAAGCGGAA, r-TTCCTGGAACGTTGTTAGAA
Pp-SSH208	EE296232	1	291	drought-induced protein 1	4e-09	AAO91809	*G.latifolia*	f-GAGGAATGGTCCAGGTGGTG, r-CCTTCCTCCGATCATCGGTT
Pp-cAFLP291	EE296062	1	286	cell division cycle protein 48	7e-11	Q96372	*C.annuum*	f-TAGTCACCACTCTGCTAACC, r-AGGTACAAGTTGGATGGGTT
Pp-SSH238	EE296454	1	428	CDC48-interacting UBX-domain protein	8e-07	AAS78923	*A.thaliana*	f-CAAGTGTATCTCTTCCTTCT, r-ATTGGACACCTCTCATACTG
Pp-SSH119	EE296304	1	474	Unknown				f-TCAGAGCAGGACTCGAATAA, r-TGCTTGGATTCCTCATACTC
Pp-actin3								f-CGGAATGGTGAAGGTATGAT, r-CACGATGTGAAGAAGACGAT

[a]f: forward primer; r: reverse primer

*: Included in contigs of *P. patens* gene library (http://moss.nibb.ac.jp)

[#]: BLASTX was used to search the GenBank databases. The best hits are listed, together with the corresponding values (probability that the match is due to chance). E-values for best matches with sequences of unknown function alone were greater than 1e-05.

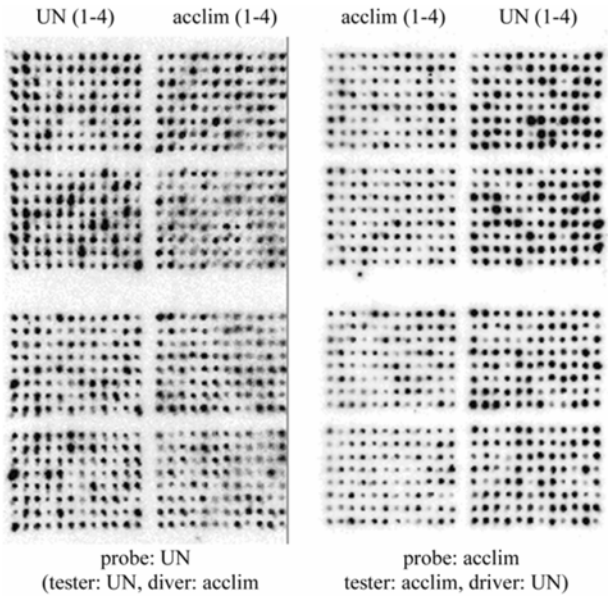

Fig. 3. Dot-blot analysis of 768 clones, up-regulated or down-regulated by cold acclimation. UN: non-acclimated plants (0 h); acclim: cold-acclimated plants (3, 6, 12, 24, 48, 72 h). Clones showing strong hybridization signals for both probes were classified as false-positives.

sequence is novel; the other five sequences are in the *P. patens* EST project databases. One of the abundant transcripts has homology to a known gene that encodes a LEA protein. We also found highly abundant, differentially expressed transcripts that encoded proteins involved in signaling transduction and transcription regulatory pathways (calmodulin and DREB subfamily of ERF/AP2 transcription factor), as well as components associated with carbohydrate and lipid metabolisms, including hydroxysteroid dehydrogenase, fructose-bisphosphate aldolase, chloroplast protein CP12, and ribulose bisphosphate carboxylase. In particular, we found only two of the 365 clusters belonging to ESTs using both cDNA-AFLP and SSH. One of these consisted of 11 ESTs that encode a 3-β hydroxysteroid dehydrogenase, a protein that may be involved in the biosynthesis of brassinosteroids. The other consisted of 4 ESTs that encoded ribulose bisphosphate carboxylase (Table 3).

Of the 365 transcript clusters, 326 (89.3%) exhibited similarity to genes present in the public databases. 148 of the transcripts having known sequence homologies contain conserved protein domains that have known biochemical and physiological functions in other organisms. These clusters were classified based on the putative functions of the proteins they encode using the Gene Ontology (GO) hierarchy, which segregates protein domains into three classes: biological process (Table 4), cellular component (Table 5), and molecular function (Table 6). In the Biological Processes Classification, most clusters were associated with metabolisms, cell growth and maintenance, and ion transport (Table 4). Of the 94 clusters mapped into the Cellular Component Classification (Table 5), almost all were mapped to the intracellular components and membrane category. Within these categories, representation was the most significant in the plastid, ribosomal and integral membrane protein subcategories. 140 clusters mapped into the Molecular Function Classification (Table 6). Of the major categories, catalytic activity, binding activity, oxireductase activity, and transporter activity were best represented in the cluster collection.

Expression analysis of selected genes by real-time RT-PCR. Quantitative RT-PCR analysis was carried out for twenty-nine of the differentially expressed transcripts (Table 2). The 3, 6, and 12 h time points were chosen for early responsive genes, and the 24, 48, and 72 h time points for late responsive genes. For these analyses, seven transcripts identified by cDNA-AFLP, twenty-one transcripts identified by SSH, and one transcript identified by both SSH and cDNA-AFLP were studied for quantitative expression analysis under cold

Table 3. most abundant transcripts in the *P. patens* ESTs

Cluster	Length	Description	e-value	species
17	228	unknown		---
11	486	3-β hydroxysteroid dehydrogenase	6e-38	*A. thaliana**
9	434	unknown	0	*P. patens*
9	328	LEA1 protein	6e-07	*T. aestivum**
7	487	unknown	1e-62	*P. patens*
5	440	calmodulin	1e-05	*C. intestinalis**
5	350	fructose-bisphosphate aldolase	1e-50	*H. brasiliensis**
4	686	DREB subfamily of ERF/AP2 transcription factor	5e-22	*A. thaliana**
4	578	putative chloroplast protein CP12	3e-18	*A. thaliana**
4	608	unknown	3e-91	*P. patens*
4	529	unknown	0	*P. patens*
4	358	unknown	1e-118	*P. patens*
4	384	interferon inducible protein	1e-100	*M. musculus**
4	466	ribulose-1,5-bisphosphate carboxylase large subunit	1e-36	*I. malinverniana**

*: Included in contigs of *P.patens* library (http://moss.nibb.ac.jp/)

Table 4. Gene ontology (GO) mappings: Biological processes (135 clusters)

Categories and subcategories	Representation	% of Total
Cellular Processes	(5)	(3.7)
Cell communication(Signal transduction)	2	1.5
Cell motility	3	2.2
Cell Death	1	0.7
Cell Growth & Maintenance	(33)	(24.4)
Cell proliferation	6	4.4
Transport	27	20
Development	2	1.5
Physiological Processes	(194)	(143.7)
Behavior	1	0.7
Cell Growth & Maintenance	(33)	(24.4)
Cell proliferation	6	4.4
Transport	27	20
Death	2	1.5
Metabolism	20(136)	14.8(100.7)
Amine metabolism	1	0.7
Amino Acid metabolism	2	1.5
Biosynthesis	39	28.9
Carbohydrate metabolism	10	7.4
Carbon utilization	5	3.7
Catabolism	11	8.1
Electron transport	15	11.1
Lipid metabolism	2	1.5
Nitrogen metabolism	2	1.5
Nucleic acid metabolism	6	4.4
One carbon cpd metabolism	6	4.4
Phosphorous metabolism	1	0.7
Protein metabolism	12	8.9
Transcription	4	3.0
Photosynthesis	10	7.4
Response to External Stimuli	1(3)	0.7(2.2)
Response to abiotic	1	0.7
Response to biotic	1	0.7
Response to Stress	2(8)	1.5(5.9)
Response to DNA damage	2	1.5
Response to oxidative stress	2	1.5
Response to pest/pathogen	2	1.5
Secretion	1	0.7

acclimation. In case where several ESTs lead to one cluster sequence, the clone that contributed the longest sequence stretch to the cluster sequence were used for PCR amplification. Three repetitions were performed for each experiment. In these experiments, there was no significant change in the transcript level of the endogenous reference gene, *Ppactin3*, indicating that our experimental conditions and real-time RT-PCR analyses were valid. The correlation coefficient (R^2) for the *Ppactin3* reference sequence was 0.99776. The PCR efficiency (E) calculated from the slope of the standard curve was 105%. The R^2 and E values for the target gene transcripts varied from 0.99095 to 1 and from 94% to 133%, respectively (Supplementary Table). Taken together, we found that 18 transcripts had increased relative expression levels after cold treatment, 10 were repressed, and the expression of one was initially repressed, but was ultimately increased following cold acclimation.

Among the transcripts that had altered expression levels under cold acclimation, we found a number that were transcribed from genes having putative regulatory functions in cold acclimation signal transduction pathways or performing putative protective functions in plants (Table 2). Nine target transcripts showed dramatic increases in relative expression levels; at their peaks, expression levels of these transcripts were more than 10-fold greater in cold-acclimated plants than they were in non-acclimated plants (Fig. 4A). The genes that had dramatically increased expression under cold acclimation encode a LEA-like protein (*Pp-SSH7*, maximum value 75-fold), a LIM domain-containing protein (*Pp-cAFLP254*, maximum value 54-fold), a TspO/MBR-related protein (*Pp-SSH251*, maximum value 37-fold), a 3-β hydroxysteroid dehydrogenase (*Pp-SSH-cAFLP2*, maximum value 31-fold), two calmodulin-related proteins (*Pp-SSH18*, maximum value 28-fold; *Pp-SSH125*, maximum value 19-fold), and a protein of unknown function (*Pp-SSH108*, maximum value 11-fold). The genes that were repressed by cold acclimation encode

Table 5. Gene ontology (GO) mappings: Cellular component (94 clusters)

Categories and subcategories	Representation	% of Total
Cell	2(116)	2.1(123.4)
Cell wall	2	2.1
Cell fraction	2	2.1
Intracellular	3(76)	3.2(80.9)
Cytoplasm	4(63)	4.3(67)
Cytoskeleton	2	2.1
Cytosol	3	3.2
Endoplasmic reticulum	2	2.1
Golgi apparatus	3	3.2
Mitochondrion	6	6.4
Plastid (Chloroplast)	27	28.7
Ribosome	11	11.7
Vacuole (Lysosome)	2	2.1
Microbody (Peroxisome)	3	3.2
Nucleus(nucleoplasm)	10	10.6
Membrane	12(34)	12.8(36.2)
Endomembrane system	1	1.1
Organelle Inner membrane	3	3.2
Integral to membrane	14	14.9
Mitochondrial membrane	3	3.2
Plasma membrane	1	1.1
Extracellular	2	2.1

violaxanthin de-epoxidase (*Pp-SSH213*, maximum value 12-fold), and cytochrome P450 78A11 (*Pp-cAFLP62*, maximum value 10-fold).

Of the 29 differentially expressed genes that we investigated further, sixteen were early responsive genes, and eight were late responsive genes according to the time point at which expression levels peaked. The 16 early responsive genes were *Pp-SSH251*, *Pp-SSH18*, *Pp-SSH125*, *Pp-SSH213*, *Pp-SSH20*, *Pp-SSH89*, *Pp-SSH215*, *Pp-SSH216*, *Pp-SSH94*, *Pp-cAFLP72*, *Pp-SSH129*, *Pp-SSH294*, *Pp-SSH321*, *Pp-cAFLP299*, *Pp-SSH153*, and *Pp-SSH238*; 8 late responsive genes were *Pp-SSH7*, *Pp-cAFLP254*, *Pp-SSH-cAFLP2*, *Pp-SSH108*, *Pp-cAFLP62*, *Pp-SSH318*, *Pp-SSH208*, and *Pp-SSH119*. Expression of the other four genes exhibited no obvious changes (*Pp-cAFLP361*, *Pp-SSH32*, *Pp-cAFLP354*, *Pp-cAFLP291*). Expression of *Pp-SSH152* was initially repressed, but was later up-regulated by cold stress (Fig. 4 and Table 2). The largest group of early responsive genes is involved in signal transduction, and most late responsive genes are involved in metabolisms.

Effect of BRs on cold acclimation of *P. patens*. Using real-time RT-PCR, the *Pp-SSH-cAFLP2* expression level was found to be drastically increased under cold acclimation (Fig. 4A, Table 2). *Pp-SSH-cAFLP2* encodes a 3-β hydroxysteroid dehydrogenase that is involved in the biosynthesis of brassinosteroids (Fujioka and Sakurai, 1997). We compared the sequences of BR biosynthesis and signaling genes that have been identified in *Arabidopsis* with EST sequences of *P. patens* (http://www.cosmoss.org and http://moss.nibb.ac.jp) to identify potential orthologues. The mRNA abundance for 6 potential orthologs in these pathways, *Pp-CPD1*, *Pp-DET2*, *Pp-BRI1*, *Pp-BIN*, *Pp-BES1* and *Pp-BZR1*, were analyzed by semi-quantitative reverse transcription–polymerase chain reaction (sQRT-PCR). *CPD1* and *DET2* are key enzymes in the BR biosynthesis pathway (Bishop and Koncz, 2002); *BRI1*, *BIN*, *BES1* and *BZR1* are key regulators in the BR signaling pathway (Sablowski and Harberd, 2005). As shown in Fig. 5, the expression levels of all 6 transcripts changed under cold acclimation. The expression *Pp-CPD1* and *Pp-DET2* increased during early cold acclimation (3 h), but was later repressed. During late cold acclimation (24 h), expression of *Pp-CPD1* and *Pp-DET2* increased once again. In the signaling pathway, the *Pp-BIN* expression level was similar to that of *Pp-CPD1* and *Pp-DET2*, but the *Pp-BRI1*, *Pp-BES1* and *Pp-BZR1* expression levels increased continuously from 3 h to 48 h.

Discussion

Freezing test indicated that cold acclimation improves the freezing tolerance of *P. patens* gametophores. With the increase of the amount of time which the plants were acclimated, so was the freezing tolerance. Minami *et al.* (2005) have used *P. patens* protonema and demonstrated freezing tolerance (LT50) changes ranging from approximately −2.0°C for non-acclimated plants to −3.5°C for plants cold acclimated at 0°C for 7 d. Based on these results, it is clear that gametophores are more freezing tolerant than protonema in *P. patens*. In addition, there are different types of changes in *LEA*-like gene expression in cold-acclimated protonema and

Table 6. Gene ontology (GO) mappings: Molecular functions (140 clusters)

Categories and subcategories	Representation	% of Total
Antioxidant activity	3	2.1
Binding	2(46)	1.4(32.9)
Amino acid binding	2	1.4
Carbohydrate binding	1	0.7
Metal ion binding	(10)	(7.1)
Calcium	3	2.1
Magnesium	2	1.4
Transition metal	5	3.6
Nucleic acid binding	1(12)	0.7(8.6)
DNA binding	5	3.6
RNA binding	6	4.3
Nucleotide binding	(17)	(12.1)
Purine nucleotide(adenyl/guanyl)	17	12.1
Protein binding	2	1.4
Catalytic activity	1(106)	0.7(75.7)
Helicase activity	1	0.7
Hydrolase activity	7(29)	5(20.7)
acting on acid anhydrides	9	6.4
acting on cardon-nitrogen(not peptide)	1	0.7
acting on ester bonds	2	1.4
acting on glycosyl bonds	4	2.9
peptidase activity	6	4.3
Isomerase activity	3	2.1
Kinase activity	2(8)	1.4(5.7)
Protein Kinase activity	6	4.3
Ligase activity	4	2.9
Lyase activity	11	7.9
Oxireductase(Ored) activity	13(33)	9.3(23.6)
disulfide Ored activity	1	0.7
Monooxygenase activity	3	2.1
Ored activity-CH-OH donors	7	5
Ored activity-acting on NADH/NADPH	2	1.4
Ored activity-acting on paired donors	1	0.7
Ored activity-peroxide acceptor	1	0.7
Ored activity-single donor	1	0.7
Ored activity-sulfur group donors	1	0.7
Ored activity-CH-NH2 group donors	3	2.1
Small protein conjugating enzyme activity	2	1.4
Transferase activity	14	10
Chaparone activity	5	3.6
Enzyme regulator activity	2(4)	1.4(2.9)
Enzyme activator activity	2	1.4
Unknown molecular function	1	0.7
Nutrient reserve activity	1	0.7
Signal Transducer activity	(2)	(1.4)
Receptor activity	2	1.4
Structural Molecule activity	(11)	(7.9)
Structural component of ribosome	11	7.9
Transcription Regulator activity	(1)	(0.7)
Transcription factor	1	0.7
Translation Regulator activity	(4)	(2.9)
Nucleic acid binding	4	2.9
Transporter activity	9(20)	6.4(14.2)
Carbohydrate transport	1	0.7
Carrier activity	3	2.1
Electron transport	4	2.9
Ion transport	1	0.7
Protein transport	2	1.4

gametophores in *P. patens* (data not shown). For these reasons, we chose the *P. patens* gametophores for further analyses.

Identifying the molecular mechanisms that control the cold-induced freezing tolerance in plants is a major prerequisite for improving crop yield. In particular, it is important to learn the functions and interactions of the genes involved in the cell signaling and regulatory pathways that mediate cold acclimation. As a first step toward realizing this goal, we applied cDNA-AFLP and SSH techniques to identifying genes that are differentially expressed under cold acclimation in *P. patens*. In all, we identified 510 differentially expressed ESTs, which were assigned to 365 EST clusters. We selected 29 of these EST clusters for relative expression analysis by real-time RT-PCR during cold acclimation. Nine of them were transcribed from early responsive genes that encoded components of cell signaling pathways, indicating that in *P. patens*, similar to what has been observed in higher plants, a change in the expression levels of cell signaling components is an early step in cold acclimation.

Cell signaling is an early step under cold acclimation in *P. patens*. In plants, cold stress induces the expression of a large set of genes, leading to the accumulation of specific stress-associated proteins that are involved in low-temperature signaling and regulatory pathways. *CBF/DREB1* family transcription factors are master regulatory switches that control the expression of many *COR* genes under cold acclimation (Thomashow, 1999; 2001). CBF/DREB1 proteins, which are encoded by members of the *AP2/EREBP* multi-gene family, regulate the transcription of several *COR* genes in response to cold and water stress (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998; Seki *et al.*, 2001). One class of *COR* genes belongs to the LEA protein family (Welin *et al.*, 1995; Thomashow, 1999). In higher plants, *CBF/DREB1* genes are induced early and transiently by cold stress (Liu *et al.*, 1998). Liu and his fellows (2007) found that *PpDBF1* (*P. patens* DRE-binding Factor1) transcripts were accumulated under cold stresses. This result, combine with our results, the expression profiles of *Pp-SSH20* and *Pp-SSH7*, imply that similar regulating systems are conserved in moss and higher plants.

Whether ABA plays a fundamental role in activating the cold-acclimation response is unresolved. Recent genetic evidence suggests that the stress-signaling pathways that activate *LEA*-like genes depend on the ABA-inducible bZIP transcription factors ABF/AREB in higher plants (Yamaguchi-Shinozaki and Shinozaki, 2006). However, in our study, the relative expression levels of the ABA regulated transcription factors, *Pp-SSH89* and *Pp-SSH215*, showed only slight increases in expression under cold acclimation (Fig. 4B, Table 2). Results from a recent study on LEA proteins also indicate that bryophytes have an ABA-independent cold-signaling pathway that can mediate acquisition of freezing tolerance (Minami *et al.*, 2005). Together with our results, these suggest that ABA may play a minor role in *P. patens* cold acclimation. However,

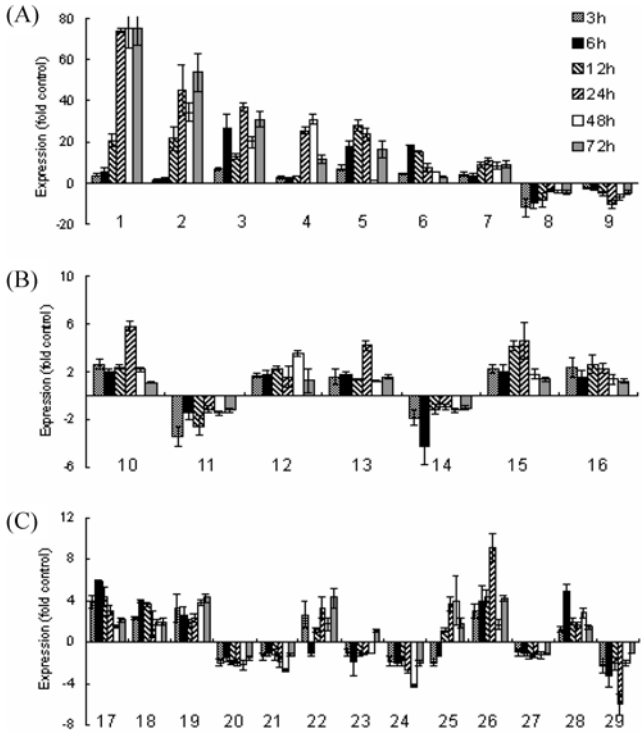

Fig. 4. Relative expression profiles of the 29 target transcripts analyzed by real-time RT-PCR in the cold-acclimated *P. patens*. The transcript levels of each cluster in cold-acclimated plants were plotted as the relative expression (fold) of the control plants exposed to cold for 3, 6, 12, 24, 48, or 72 h. (A) Nine target transcripts showed dramatically changed relative expression levels at their peaks, at least a 10-fold change from levels seen in control plants. 1: *Pp-SSH7*, 2: *Pp-cAFLP254*, 3: *Pp-SSH251*, 4: *Pp-SSH-cAFLP2*, 5: *Pp-SSH18*, 6: *Pp-SSH125*, 7: *Pp-SSH108*, 8: *Pp-SSH213*, *9: Pp-cAFLP62*. (B) Relative expression profiles of seven target transcripts related to cold acclimation signaling. 10: *Pp-SSH20*, 11: *Pp-SSH89*, 12: *Pp-SSH215*, 13: *Pp-SSH216*, 14: *Pp-SSH94*, 15: *Pp-cAFLP72*, 16: *Pp-SSH129*. (C) Relative expression profiles of target transcripts associated with membrane structure, cell division, and unknown proteins. 17: *Pp-SSH294*, 18: *Pp-SSH321*, 19: *Pp-cAFLP361*, 20: *Pp-SSH32*, 21: *Pp-cAFLP354*, 22: *Pp-cAFLP299*, 23: *Pp-SSH153*, 24: *Pp-SSH318*, 25: *Pp-SSH152*, 26: *Pp-SSH208*, 27: *Pp-cAFLP291*, 28: *Pp-SSH238, 29: Pp-SSH119*.

we found that *Pp-SSH213* expression was dramatically repressed after 3 h of cold stress, lasting through at least 72 h. *Pp-SSH213* encodes a violaxanthin de-epoxidase, which is the xanthophyll cycle enzyme that converts violaxanthin to zeaxanthin (Latowski *et al.*, 2002). The catalyzed reaction degrades the ABA intermediate violaxanthin, so down-regulation of this gene may increase ABA biosynthesis. The clearly role of ABA in *P. patens* cold acclimation requires further investigation.

Among other *P. patens* cell signaling components, the expression of two calmodulin-like proteins, *Pp-SSH18* and *Pp-SSH125*, was dramatically increased under early cold

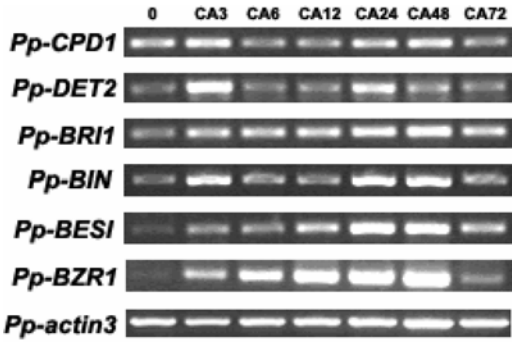

Fig. 5. Expression of genes encoding BR-related proteins during cold acclimation. The mRNA abundance of *Pp-CPD1*, *Pp-DET2*, *Pp-BRI1*, *Pp-BIN*, *Pp-BES1* and *Pp-BZR1* was analyzed by sQRT-PCR. RNAs were isolated from *P. patens* gametophores that had been cold-acclimated at 0°C for 3 h (CA3), 6 h (CA6), 12 h (CA12), 24 h (CA24), 48 h (CA48), or 72 h (CA72). *Ppactin3* was used as an endogenous control.

acclimation (Fig. 4A, Table 2). Calcium is required for the expression of some *COR* genes as well as increases freezing tolerance in higher plants (Knight *et al.*, 1996). Our results indicate that calcium also has an important role in cold acclimation in *P. patens*. Several identified differentially expressed transcripts also play important roles in cold acclimation. The expression of *Pp-SSH216*, *Pp-cAFLP72* and *Pp-SSH129* was slightly increased by cold acclimation (Fig. 4B, Table 2). Expression of *Pp-SSH94,* which encodes a phosphatidylinositol 4-kinase (PI4K), a key enzyme in phosphatidylinositol (PI) metabolism, was repressed by cold acclimation, reaching a minimum value after 6 h of cold stress (Fig. 4B). Similarly, expression of the *Arabidopsis* isoforms PI4Kα2 and PI4Kβ2 also decreases in response to cold stress (Lin *et al.*, 2004). PI may serve as a precursor for the generation of the second-messenger molecules, suggesting a role in cold signaling pathways in *P. patens*.

Brassinosteroids (BRs) are a group of plant steroidal hormones that have potential roles in protecting plants from environmental stresses. Several studies suggest that BRs increase freezing resistance in higher plants (Krishna, 2003). However, recent evidence (Lee *et al.*, 2005) suggest that BRs level might be decreased under cold stress or that cold signaling might interfere with BRs regulation. *Pp-SSH-cAFLP2* encodes a 3-β hydroxysteroid dehydrogenase that catalyzes the transformation of teasterone to 3-dehydroteasterone or 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone, which is involved in the biosynthesis of BRs (Fujioka and Sakurai, 1997). Surprisingly, we found that *Pp-SSH-cAFLP2* expression level increased dramatically between 3 h and 72 h after cold acclimation (Fig. 4A, Table 2). Ehanced *Pp-SSH-cAFLP2* expression under cold acclimation was found both in cDNA-AFLP and in SSH methods. *Pp-SSH-cAFLP2* was also one of the most abundant differentially expressed transcripts (Table 3). These clues implied that BRs may function in cold acclimation. In order to verify this, we carefully analyzed the mRNA abundance of transcripts for 6 genes, *Pp-CPD1*, *Pp-DET2*, *Pp-BRI1*, *Pp-BIN*, *Pp-BES1* and *Pp-BZR1*. The orthologous genes of the 6 transcripts in other species are involved in BRs biosynthesis and the BRs signaling pathway, respectively (Noguchi *et al.*, 2000; Li *et al.*, 2001; He *et al.*, 2002; Yin *et al.*, 2002; Kinoshita *et al.*, 2005; Yin *et al.*, 2005). In response to BRs, BRI1 inhibits BIN2; active BIN2 phosphorylates BES1 and BZR1. BZR1 then represses the expression of other genes, such as *CPD*, a key enzyme in the BRs biosynthesis pathway (Sablowski and Harberd, 2005). Our results show that all 6 transcripts were induced under cold acclimation (Fig. 5). *Pp-BES1* and *Pp-BZR1* were obviously up-regulated; the expression of *Pp-CPD1* increased initially, then decreased. Overall, our results suggest that BRs play an important role in *P. patens* cold acclimation.

Cold acclimation has a great effect on cell membrane systems. Multiple forms of membrane damage can occur as a consequence of freezing-induced cellular dehydration in higher plants. Therefore, it is not surprising that a key function of cold acclimation is to stabilize membranes against freezing injury. Many mechanisms appear to be involved in this stabilization. The best-documented are changes in lipid composition (Thomashow, 1999). Results from several past studies indicate that a high level of unsaturated fatty acids in membranes increases resistance to chilling (Murata *et al.*, 1992; Wolter *et al.*, 1992). Our results show that lipid composition is also an important factor in *P. patens* cold acclimation. For example, the expression of *Pp-SSH294*, which encodes an ω-6 fatty acid desaturase, a critical enzyme in biosynthesis of the polyunsaturated fatty acids 18 : 2 and 18 : 3 (Hitz *et al.*, 1994), increased starting at 3 h after cold acclimation, ultimately achieving 5.8-fold increased expression level (Fig. 4C, Table 2). In addition, one of the nine transcripts that showed a dramatic change in relative expression levels, *Pp-cAFLP62,* encodes a cytochrome P450 78A11 that catalyzes 12-monooxygenation of lauric acid, a step in fatty acid synthesis (Miyoshi *et al.*, 2004); after cold acclimation *Pp-cAFLP62* expression decreased, reaching its lowest level of expression at 24 h (Fig. 4, Table 2).

The biological activity of membranes depends not only on lipid composition, but also on membrane-associated proteins. *Pp-cAFLP361*, *Pp-SSH321*, and *Pp-SSH32* encode stress-inducible membrane protein, a plasma membrane intrinsic protein 1a, and an agglutinin isolectin 1 WGA1, respectively (Table 2). Expression of each of these genes was altered by cold acclimation (Fig. 4). The nature of the most abundant differentially expressed transcripts (Table 3) also provides new insight into the nature of the cell membrane systems that are associated with cold acclimation. One of these transcripts encodes a LEA protein, which have been associated with cold stress in higher plants. LEAs alleviate stress caused by dehydration by interacting with membrane surfaces or with water molecules (Thomashow, 1999; Kobayashi *et al.*, 2004).

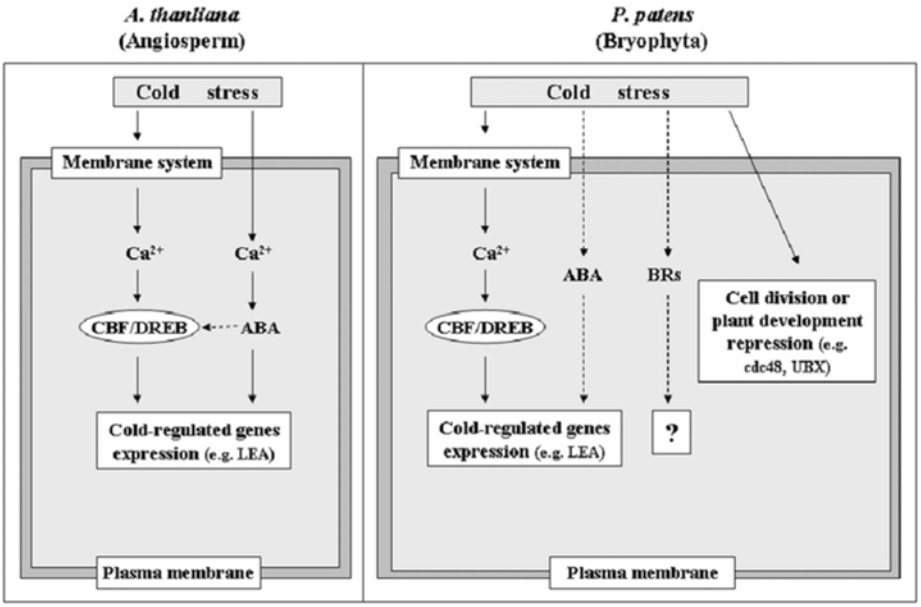

Fig. 6. Comparison of the cold acclimation processes of *A. thaliana* (Angiosperm) and *P. patens* (Bryophyta). Low temperature leads to rapid induction of the CBF/DREB1 that in turn results in expression of the cold-regulated genes (e.g. LEA). Such activation may involve alterations in calcium signaling. Action of the CBF/DREB1 regulon in cold acclimation may be similar in *A. thaliana* and *P. patens*. Stabilization of membrane system helps to increase freezing tolerance. ABA appears not to perform the same functions in both plants, whereas BRs might play an important role in cold acclimation of *P. patens*. Low temperature seems to specially interfere with cell division and plant development in *P. patens*.

Our results show that LEAs may also play a role in *P. patens* cold acclimation.

Functional annotation of transcripts suggested active changes in cellular activities and metabolisms. Furthermore, the changes we identified in the expression levels of proteins that affect membrane systems may provide useful insights into the molecular mechanisms underlying cold acclimation. Of 94 transcripts clusters mapped into the Cellular Component Classification by functional annotation, almost all of them were mapped to the Intracellular components and Membrane category (Table 5). The importance of maintaining membrane fluidity during cold acclimation may explain the advantage of clusters associated with membrane categories. Our results reinforce the notion that membrane stabilization systems are important for the stress response induced by cold acclimation in *P. patens*. The whole *P. patens* plants consist primarily of a single cell layer, and lack the complex stress response systems found in higher plants; therefore, *P. patens* evolved cellular membrane stabilization mechanisms that require stress-induced gene expression to protect membrane from freezing damage.

Other factors are involved in cold acclimation. Under cold conditions, plants grow slowly, occasionally suffering growth defects or damage. These cold-induced growth changes may be caused by reducing photosynthesis, and generally low metabolic activities (Kubien *et al.*, 2003). In our study, cold stress induced enhanced expression of *Pp-SSH251* (Fig. 4, Table 2), which encodes a tryptophan-rich sensory protein (TspO)-related integral membrane protein that acts as a negative regulator of photosynthesis; the *Arabidopsis* TspO homolog is involved in tetrapyrrole transport (Lindemann *et al.*, 2004). Our results indicate that cold acclimation also reduced photosynthesis in *P. patens*. The identity of the most abundant differentially expressed transcripts (Table 3) provides insight into the nature of the changes in metabolic activity that is associated with cold acclimation. Other than genes that encode LEA proteins, and signal transduction and transcription regulatory pathway components, such as calmodulin and DREB subfamily transcription factors, the most abundant differentially expressed transcripts we identified are associated with genes that play roles in carbohydrate and lipid metabolisms; these differentially expressed transcripts include hydroxysteroid dehydrogenase, fructose-bisphosphate aldolase, chloroplast protein CP12 and ribulose bisphosphate carboxylase. Our results indicate that metabolic reactions change dramatically during cold acclimation. In the Biological Processes Classification, most differentially expressed EST clusters are associated with metabolisms, cell growth and maintenance, and ion transport (Table 4). This distribution seems to reflect the nature of the cellular disturbance that results from stress, which consistent with results from a previous study in which nearly identical distributions of EST clusters were observed in cDNA collections from protonemal tissues of *P. patens* following various hormone treatments (Nishiyama *et al.*, 2003). In the Molecular Function

Supplementary Table. E and R^2 values of linear regressions for dilutions of the reference and target gene transcripts

Name	E value[a]	R^2 [b]
Pp-SSH7	114% (SD = 3.6%)	1.00000 (SD = 0.0009)
Pp-cAFLP254	111%	0.99831
Pp-SSH251	104%	0.99987
Pp-SSH-cAFLP2	98%	0.99851
Pp-SSH18	98%	0.99095
Pp-SSH125	118%	0.99875
Pp-SSH108	133%	0.99787
Pp-SSH213	119%	1.00000
Pp-cAFLP62	99%	0.99947
Pp-SSH20	109%	0.99885
Pp-SSH89	115%	0.99956
Pp-SSH215	105%	1.00000
Pp-SSH216	102%	0.99855
Pp-SSH94	109%	0.99787
Pp-cAFLP72	108%	0.99926
Pp-SSH129	94%	0.99845
Pp-SSH294	108%	0.99961
Pp-SSH321	99%	0.99997
Pp-cAFLP361	112%	0.99765
Pp-SSH32	102%	0.99903
Pp-cAFLP354	103%	0.99826
Pp-cAFLP299	107%	0.99793
Pp-SSH153	100%	0.99997
Pp-SSH318	98%	0.99944
Pp-SSH152	110%	0.99836
Pp-SSH208	103%	0.99949
Pp-cAFLP291	108%	0.99997
Pp-SSH238	107%	0.99922
Pp-SSH119	104%	0.99710

[a]: PCR efficiency; [a]: squared regression coefficient; SD: standard deviation.

Classification (Table 6), differentially expressed EST clusters were associated primarily with catalytic activity, binding activity, ored activity, and transporter activity. Although transcript abundance and functional annotation may reflect the metabolic or physiological needs of the moss under cold acclimation, it is also important to know how transcripts are regulated at the translation level. This work is currently being carried out in our laboratory.

There is evidence that factors such as the accumulation of soluble sugars and antioxidants also play important roles in alleviating freezing-induced cellular damage (Livingston and Henson, 1998; Thomashow, 1999), suggesting that the expression of genes involved in producing these sugars, or providing antioxidant protection may also be induced by cold acclimation. Differentially expressed transcripts, *Pp-cAFLP354*, *Pp-SSH153*, and *Pp-SSH318* encode pyruvate orthophosphate dikinase, peroxiredoxin and secretory peroxidase, respectively (Table 2). The up-/down-regulation of these genes may indicate that sugars and antioxidant should also have function in *P. patens* cold acclimation. *Pp-SSH152* and *Pp-SSH208,* which encode Hypersensitive-induced protein and Drought-induced protein 1, respectively (Table 2), were also regulated at the transcription level (Fig. 4C). Our results indicate that there is cross-talk among various stresses in *P. patens*.

Interestingly, we found that cold acclimation resulted in changes in the expression levels of two genes associated with cell division; the expression of *Pp-cAFLP291*, which encodes cdc48, a critical regulator of the G1 phase of cell cycle (Feiler *et al.*, 1995; Rancour *et al.*, 2002), decreased, and expression of *Pp-SSH238,* which encodes the CDC48-interacting UBX-domain protein, a negative regulator of cdc48 (Rancour *et al.*, 2004) (Table 2), increased (Fig. 4C). In addition, the expression levels of two genes involved in plant development were altered by cold acclimation. The expression of *Pp-cAFLP254*, which encodes a LIM domain-containing protein, increased dramatically under cold acclimation, peaking after 24 h of cold stress (Fig. 4A, Table 2). LIM-domain proteins regulate developmental processes by acting as an interface for protein-protein interactions (Schmeichel and Beckerle, 1994; Agulnick *et al.*, 1996). Cold acclimation affected the expression level of *Pp-cAFLP254* in a manner that was similar to how it affected expression of the LEA-related gene *Pp-SSH7*. The expression of *Pp-cAFLP299*, which encodes a hydroxyproline-rich cell wall glycoprotein (HRGP) that is believed to be essential for plant embryo development and cell shape regulation (Yoshiba *et al.*, 2001; Hall and Cannon, 2002), was also altered by cold acclimation. As noted above, the differentially expressed genes, *Pp-cAFLP62* and *Pp-SSH-cAFLP2*, are also involved in plant development. Therefore, one possibility is that cold acclimation of *P. patens* reduces cell division and reprograms plant development.

Pp-SSH108 and *Pp-SSH119* encode proteins that lack homology to any known sequences (Table 2). *Pp-SSH108* was up-regulated and *Pp-SSH119* was down-regulated (Fig. 4). These genes may represent unique cold-regulated genes in *P. patens*, indicating that complex cold-induced gene expression varies from plant to plant.

Conclusion

Figure 6 illustrates the cold acclimation processes of angiosperm (e.g. *A. thaliana*) and bryophyta (e.g. *P. patens*). In higher plants, the CBF/DREB1 family transcriptional factors and ABA have key roles in the low-temperature signaling and regulatory pathways underlying cold acclimation. In *P. patens*, we found that the expression levels of *Pp-SSH20* and *Pp-SSH7* increased dramatically in response to cold stress. Therefore the CBF/DREB1 family transcription factors may also have an important function in cold acclimation in *P. patens*. However, the expression levels of two ABA related transcripts were not altered in *P. patens*; possibly suggesting

that ABA may not act in a way similar to that in higher plants. Interestingly, BRs may play an important role in *P. patens*, which may be not consistent with *Arabidopsis*. In addition, in *P. patens*, cell division and plant developmental signaling pathways are repressed distinctly by cold acclimation.

The moss *P. patens* is an excellent model plant for the isolation and characterization of genes involved in cold acclimation. *P. patens* has become a valuable model system for the functional analysis of plant genes because its high frequency of homologous recombination allows the generation of targeted knockout mutants. The differential expression profiles we have developed for *P. patens* provide novel insights into the mechanisms that mediate cold acclimation, and the information gleaned from these studies has also identified valuable gene targets for further study of cold acclimation mechanisms. Confirmation of the specific function of each differentially expressed gene in cold acclimation will require further biochemical and genetic analyses.

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