

Target deletion of the AAA ATPase *PpCDC48II* in *Physcomitrella patens* results in freezing sensitivity after cold acclimation

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CDC48 is a highly conserved protein in eukaryotes and belongs to the AAA (ATPase associated with a variety of cellular activities) superfamily. It can interact with many different co-factors and form protein complexes that play important roles in various cellular processes. According to the *Physcomitrella patens* database, one member of the ATPases, the cell cycle gene *PpCDC48II*, was cloned. *PpCDC48II* contains two typical ATPase modules and is highly homologous to AtCDC48A. *PpCDC48II* was up-regulated in mRNA levels after incubation at 0°C for 36 and 72 h. To further elucidate protein function, we disrupted the *PpCDC48II* gene by transforming *P. patens* with the corresponding linear genomic sequences. When treated to the same freezing stress, it was found that *PpCDC48II* knockout plants were less resistant to freezing treatment than wild type after acclimation. This suggested that *PpCDC48II* was an essential gene for low-temperature-induced freezing tolerance in *P. patens* cells.

***PpCDC48II*, *Physcomitrella patens*, gene knockout, cold acclimation, freezing tolerance**

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Physcomitrella patens represents an excellent system to study gene function because the generation of targeted knockout plants is facilitated by high homologous recombination frequency [1,2]. In addition, *P. patens* was originally chosen to study plant differentiation processes, which were facilitated by the haploid status of the moss protonema. The moss *P. patens* has become accessible to state-of-the-art molecular and genetic analyses, which have promoted the system for analyzing almost all aspects of plant biology.

Low temperature is considered one of the most important environmental factors that limit growth, development, and distribution of plants. However, some temperate zone plants can adapt to the onset of low temperatures by producing chemical and physiological metabolites during late autumn and early spring, thus improving their resistance to low

temperature during winter. This indicates that plants evolve a set of perfect mechanisms to respond to low temperature.

Some results demonstrated that cold treatment clearly enhanced freezing tolerance in *P. patens* protonema cells. *P. patens* treated at low temperatures exhibited not only an increased tolerance to freezing but also higher expression levels of genes present in cold-treated cells [3]. Therefore, elucidating the intracellular functions of cold-regulated genes is important for understanding cold acclimation mechanism.

In a recent study, we used two powerful and 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* [4]. Of the 768 independent clones from the SSH cDNA library, approximately 50.9% were differentially regulated during cold acclimation. We identified 201 up-

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regulated, and 190 down-regulated clones using subtracted PCR product probes. These clones were then sequenced for further analysis. In all, we sequenced 420 EST clones from the two libraries for further analysis. According to EST analysis, we found that cold acclimation resulted in changes in *PpCDC48* genes expression levels. We further confirmed that *PpCDC48II* displayed up-regulated mRNA levels after incubation at 0°C for 36 and 72 h using RT-PCR.

According to these results, we cloned *PpCDC48II* from the *P. patens*. The gene contains two typical ATPase modules and is highly homologous to *AtCDC48A*. CDC48 is a member of the AAA (ATPases associated with various cellular activities) protein family. In zebrafish cells, cold-inducible expression of the cell cycle gene *CDC48* promoted cell proliferation during cold acclimation [5]. In addition, yeast CDC48 has been implicated in a plethora of functions that include cell-cycle regulation, membrane fusion, stress response, and endoplasmic reticulum (ER)-associated degradation (ERAD) [6]. Its highly conserved mammalian counterpart, p97, has been further implicated in the reformation of the nucleus [7], organelle biogenesis [8], myofibril organization [9], and protein degradation of Hif1- α [10], and HMG-CoA reductase [11]. Two underlying properties of Cdc48/p97 contribute to its myriad functions: its ATPase activity and the ability to bind ubiquitin (Ub) [12].

Compared to the extensive studies of CDC48 homologs in yeasts and mammals, the understanding of CDC48 in higher plants is rudimentary. The *Arabidopsis* genome encodes three *CDC48* isoforms: *AtCDC48A* (At3g09840), *AtCDC48B* (At3g53230), and *AtCDC48C* (At5g03340) [13]. These isoforms are predicted to share 91% (*AtCDC48B*) and 95% (*AtCDC48C*) amino acid identity with *AtCDC48A* [14]. Expression of *AtCDC48A* mRNA is highest in proliferating cells of the vegetative shoot, the root, and the flowers in rapidly growing plants [15–17]. Using immunofluorescence microscopy, *AtCDC48A* has been shown to be localized to the cytoplasm, the nucleus, and the phragmoplast mid zone during cytokinesis [14,15]. Loss-of-function and dominant negative mutant studies indicated multiple roles for *AtCDC48* in cell division, growth, and development in *Arabidopsis* [18]. At the subcellular level, *NgCDC48*, a tobacco CDC48 homolog, resided in the endoplasmic reticulum (ER) of protoplasts. Transgenic tobacco plants (*35S:anti-NgCDC48*), in which the *NgCDC48* gene was suppressed using antisense RNA method, exhibited severely aberrant development of both vegetative and reproductive organs, resulting in arrested shoot and leaf growth, and sterile flowers. These results indicate the tantalizing possibility that CDC48-mediated degradation of an as-yet unidentified protein(s) in the ER might be a critical step for cell growth and expansion in tobacco leaves [19].

We knocked out the *PpCDC48II* gene through homologous recombination. The mutant plants were less resistant to freezing stress than wild type after acclimation. It is suggested that cold-induced expression of *PpCDC48II* is essen-

tial for low-temperature-induced freezing tolerance in *P. patens* cells.

1 Material and methods

1.1 Plant material and growth condition

The moss *P. patens* was maintained axenically in PPNH₄ medium as described previously [20], supplemented with 7 g L⁻¹ agar when required. Plants were grown either in biofermenters or in jars in a phyto-chamber with a discontinuous white light cycle (16 h light at 25°C/8 h dark at 18°C) at quantum irradiance of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Moss cultures were propagated by fragmenting old cultures in sterile water and transferring aliquots of this suspension to fresh medium.

1.2 RNA extraction and cDNA synthesis

Total RNA was obtained from two-week-old gametophores using a phenol-based extraction method. Poly(A) RNA was isolated from total RNA using the Qiagen Oligotex mRNA Kit (Qiagen, Germany). For RT-PCR analyses, cDNA was synthesized using an RNA PCR Kit (Takara, Japan).

1.3 Recombinant DNA techniques

Manipulation of nucleic acids was performed using standard protocols or, when appropriate, according to the manufacturer's instructions. DNA sequencing was performed in an automated ABI PRISM 3100 DNA sequencer (Applied Biosystems, <http://www.appliedbiosystems.com>). PCR amplifications of *PpCDC48II* cDNA fragments were carried out on double-stranded cDNA synthesized from total RNA using the cDNA Synthesis System Kit (Takara, Japan) and primers: RTII-f: 5'-ATTCTAGAATGGCGACTCCCGCAG-3' (*PpCDC48II* forward) and RTII-r: 5'-GCCGTCGACGCTATACAAATCATCA-3' (*PpCDC48II* reverse) designed using EST sequences identified in databases. The resulting PCR fragments were cloned into the pGM-T Vector Cloning Kit (Takara Japan).

1.4 Semi-quantitative RT-PCR (SQRT-PCR) analysis

For cDNA synthesis, 1 μg of total RNA was first treated with DNase I (Sigma-Aldrich, USA) for 15 min at room temperature, and then the enzyme was inactivated by heating at 70°C for 10 min. Reverse transcription was performed with the cDNA Synthesis System Kit (Takara, Japan). Synthesized cDNAs were purified using the Gel Extraction Kit. Semi-quantitative RT-PCR analysis for *PpCDC48II* cold-inducible expression was performed using 1 μL of the cDNA, and ExTaq polymerase with the supplied buffer and dNTP (Takara-Bio, Japan). The PCR conditions

were as follows: 30 (*PpCDC48II*) or 26 (*Ppactin3*) cycles at 95°C for 30 s, 59°C (*PpCDC48II*) or 55°C (*Ppactin3*) for 30 s, and 72°C for 15 s. A 5 µL aliquot of each PCR reaction was separated on an agarose gel. We performed SQR-PCR analysis with primers 5'-ATGGCGACTCCCGCAGCTGAA-3' and 5'-AAACGGAAGAACATGAATTC-3' for *PpCDC48II*.

1.5 Generation of the *PpCDC48II* knockout lines

For the *PpCDC48II* knockout construct, genomic DNA fragments (1565 bp) were amplified using the primers 5'-GCTTATCTAAACTCGGAAC-3' (forward) and 5'-CAAATCATCATCAGCTGCA-3' (reverse). DNA fragments were cloned into the pGMT-T vector (Takara Japan). The neomycin phosphotransferase (*Npt II*) selection cassette was amplified from pTN182 vector using *Hind III* and *Bss HII* restriction sites. The plasmids containing the *PpCDC48II* fragment were digested with *Hind III* and *Bss HII* and the excised selection cassette was inserted to yield the *PpCDC48II* knockout constructs. For transfection, 30 µg of the knockout constructs was amplified by PCR, producing a linear fragment that contained the *Npt II* selection cassette flanked by *PpCDC48II* genomic sequences. Stable antibiotic-resistant clones were selected after two rounds of incubation in PPNH4 medium supplemented with 30 µg mL⁻¹ of G418 (Sigma-Aldrich Chemie, <http://www.sigmaaldrich.com>). The first screening of putative mutant plants was carried out using three independent PCR reactions on genomic DNA purified from transformant plants. The first reaction spanned the complete targeted regions using the primers P_{1f}: 5'-GCTTATCTAAACTCGGAAC-3' and P_{1r}: 5'-CAAATCATCATCAGCTGCA-3'. The second reaction amplified part of the marker cassette using primers N_{fwd}: 5'-CACTATCCTTCGCAAGACCCTTCCTC-3' and N_{rev}: 5'-ATCCTTCCTTGCTTCCTTCCTTCCTCCGT-3'. The final reactions amplified part of the 5' and 3' gene regions outside the knockout construct using the primers P_{2f}: 5'-TATGTTTTGGTTTTGACGTG-3', P_{2r}: 5'-TGAGGAGCCACCTTCCTTTTC-3', P_{3f}: 5'-TTTTGTTCCCTTTAGTGAGGGT-3' and P_{3r}: 5'-AAGCAGCTCAATTCATTACAG-3'. These amplifications produced the expected fragments. The basic defects of *Ppcdc48II* mutants were studied in two independent lines. For the expression analysis of *Ppcdc48II* plants, 1 µg of total RNA from wild type and *Ppcdc48II* protonema were used. Reverse transcription was done according to the manufacturer's protocol. RT-PCR was performed to confirm the *PpCDC48II* knockout.

1.6 Freezing tests

For freezing tests, two-week-old gametophores cold-acclimated at 0°C for 0, 12, 24, 48, and 72 h were frozen for 90

min at -6, -4, and -10°C, and then incubated overnight in the dark at 4°C. The next day, gametophores were placed in fresh medium and incubated at 25°C under a 16 h light/8 h dark cycle for 2 d to observe the phenotype and 10 d to calculate survival efficiency.

1.7 Measurement of freezing tolerance

Two-week-old gametophores tissues of wild type and mutant *Ppcdc48II* were treated for freezing tests. Freezing tolerance was determined by measuring electrolyte leakage from cells, as previously described [21], and the survival efficiency.

1.8 Measurement of gametophores chlorophyll contents

Two-week-old gametophores tissues treated for freezing tests were harvested and chlorophyll was extracted from each colony using dimethylformamide. Chlorophyll contents (Chl a+Chl b=17.67A646.8+7.12A663.8) were measured using a spectrophotometer, and calculated according to previous description [22]. Chlorophyll contents were conventionally used for growth quantification of moss.

2 Results

2.1 Isolation and characterization of *CDC48*-like genes in *Physcomitrella*

We searched for genes encoding *CDC48* in the *P. patens* EST database [23], as well as in the genome database (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html), and identified six genes containing two highly conserved ATPase domains. The number of *CDC48*-like genes is more than that of *Arabidopsis*. Among them, we cloned the *PpCDC48II* gene. The coding region of full-length *Physcomitrella PpCDC48II* cDNA is 2448 bp long, which encode a 816 amino acid protein with a predicted molecular mass of 90.4 kD, similar to that of yeast (92.0 kD), human (89.3 kD), and *Arabidopsis* (89.4–90.4 kD) *CDC48* proteins (Figure 1A). As in other *CDC48* homologs, the *PpCDC48II* protein contains two conserved ATPase domains, in which the Walker A and Walker B motifs are well conserved. Multiple alignments of the deduced *PpCDC48* amino acid sequence with those of previously identified *CDC48* proteins are shown in Figure 1B. *PpCDC48II* is identical to the flowering plant *CDC48* proteins in *Arabidopsis* (88%), rice (89%), and tobacco (89%). Furthermore, *PpCDC48II* shares a significant degree of sequence identity with yeast (67%) and human (78%) proteins (Figure 1A and B). Therefore, the isolated cDNA clone was found to encode *Physcomitrella* homologs of *Arabidopsis AtCDC48*.

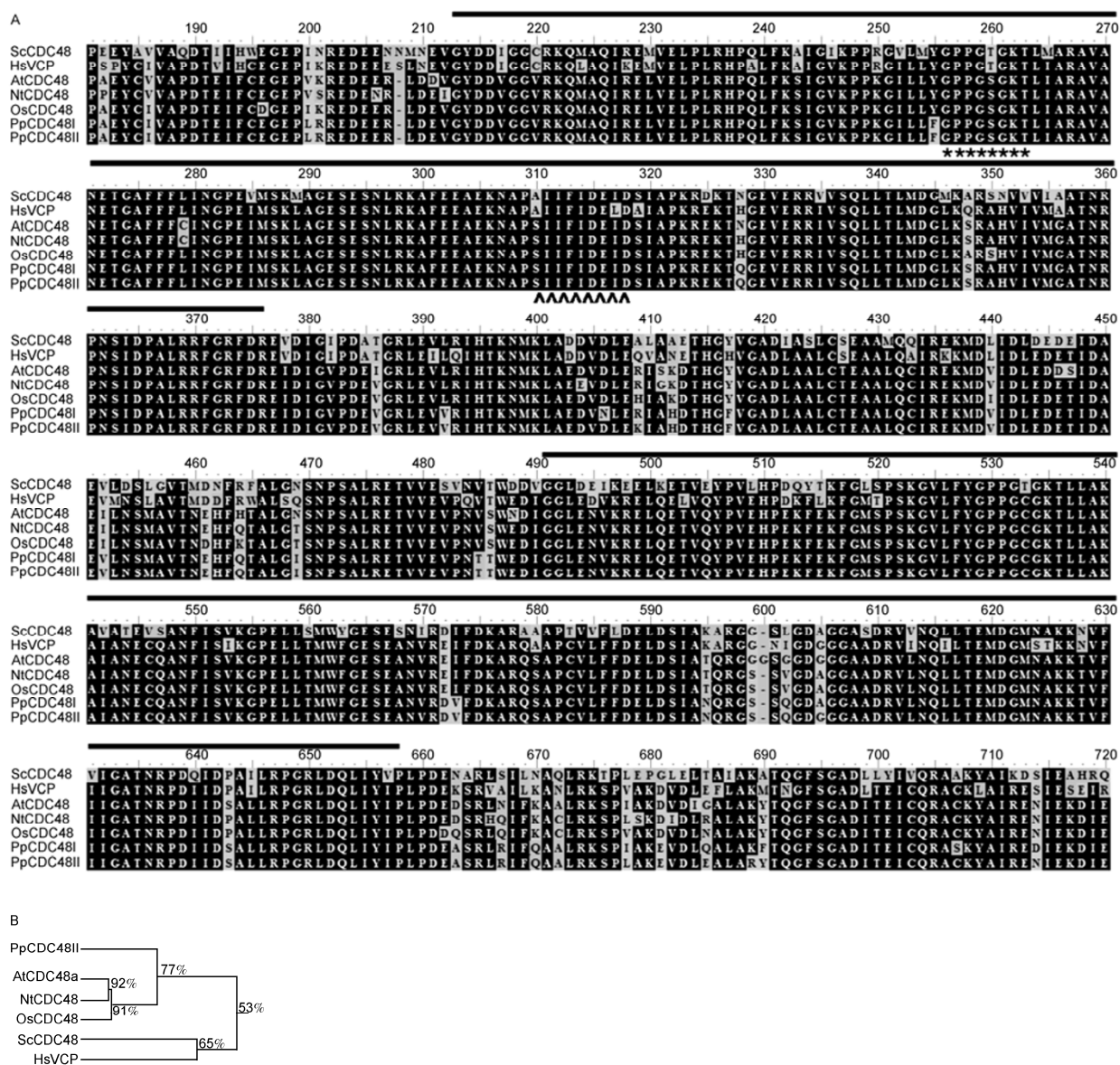


Figure 1 Sequence analysis of *Physcomitrella* PpCDC48II. A, Comparison of the deduced amino acid sequence of *Physcomitrella* PpCDC48II with CDC48 homologs from *Arabidopsis* (AtCDC48a), rice (OsCDC48), yeast (ScCDC48), tobacco (NiCDC48), and human (HsVCP). Amino acids that are identical in all seven proteins are shown in the same color. Solid lines represent two highly conserved AAA cassette domains. The Walker A and Walker B motifs are indicated by asterisk and arrowheads, respectively. Dashes show gaps introduced into the amino acid sequences for optimal sequence alignment. B, Phylogenetic analysis of CDC48 proteins from tobacco (NiCDC48), *Arabidopsis* (AtCDC48a), rice (OsCDC48), yeast (ScCDC48p), and human (HsVCP). The dendrogram was constructed using DNAMAN software with the neighbor-joining method. The tree is drawn to scale, with branch lengths representing units of evolutionary distance.

2.2 Targeted disruption of *PpCDC48II* caused decreased freezing tolerance

To confirm *PpCDC48II* function, we performed a loss-of-function analysis and disrupted *PpCDC48II* via homologous recombination (Figure 2A). Two independent disruptants (lines 2 and 3) were selected after PCR analysis confirmed the complete deletion of the *PpCDC48II* coding sequence (Figure 2B). In addition, RT-PCR analysis confirmed that both transgenic lines lost *PpCDC48II* expression while retaining *PpCDC48II* expression in the wild type (Figure 2C).

To elucidate that it is low temperature itself that enhanced freezing tolerance in gametophytes, we treated the wild type and mutant at -6°C for various time courses. Survival of the freeze-thawed cells was determined by measuring electrolyte leakage (Figure 3B). The results indicated both the wild type and the mutant gametophytes were freezing hypersensitive. We next evaluated freezing sensitivities of wild type and disruptants after cold acclimation at 0°C . The gametophores grown at 25°C for 14 d were transferred to fresh agar plates and incubated at 0°C for 0, 12, 48, and 72 h. The cold

acclimated gametophores, including wild type and *Ppcdc48II* mutants, were frozen at -6°C for 90 min and thawed at 4°C overnight, and then the treated plants were transferred to normal condition for 2 d. Freezing tolerance of *P. patens* gametophores was examined by measuring electrolyte leakage after treatment (Figure 3E). The results indicated that incubation temperature had differential effects on the freezing tolerance of wild type and mutant gametophores (Figure 3C). Most wild type gametophores cold acclimated for 48 h at 0°C increased freezing tolerance and survived. In contrast, the survival of *Ppcdc48II* plants markedly decreased even with cold acclimation for 72 h. The *P. patens* gametophores freezing tolerance during different temperature after 72 h cold acclimation is shown in Figure 3F and Table 1. Both wild type and *Ppcdc48II* acclimated at 0°C for 72 h hardly survived at -10°C . About 70% of acclimated wild type gametophores can survive at -6°C , while the survival efficiency of mutants was only 10%. In addition, the growth of the frozen-thawed gametophores was also evaluated by their chlorophyll content, which is conventionally used for growth quantification of moss (Figure 3D). The results were consistent with the freezing tests. The data demonstrated that disruption of *PpCDC48II* led to freezing-hypersensitivity after cold acclimation, providing concrete evidence for the role of PpCDC48II as a positive regulator of cold-induced freezing tolerance in moss. The data also suggested that the acquisition of freezing tolerance in *P. patens* involved a

PpCDC48II-mediated signaling pathway.

2.3 Expression of *PpCDC48II* after cold-treatment

It is well documented that CDC48 proteins participated in multiple cellular processes. We examined the expression of *PpCDC48II* gene during cold treatment in *P. patens* (Figure 3A). The results indicated that the *PpCDC48II* gene encoding AAA ATPase was regulated by low temperatures. The expression level of *PpCDC48II* was slightly up-regulated at 24 h, distinctly up-regulated at 36 h, and peaked at 72 h after incubation at 0°C , a cold acclimated temperature.

3 Discussion

Gene inactivation by targeted gene replacement or by RNA interference, together with the nearly completed sequencing of the *Physcomitrella patens* genome, opens the way for detailed functional study of genes involved in both development and metabolism.

It is known that low-temperature treatment causes various biochemical and physiological changes in plant cells, including expression of numerous genes, accumulation of various proteins, increase of antioxidants, and activity of various metabolic enzymes [24–27]. Many results demonstrated that cold-induced, highly hydrophilic proteins such as LEA proteins play important roles in the enhancement of

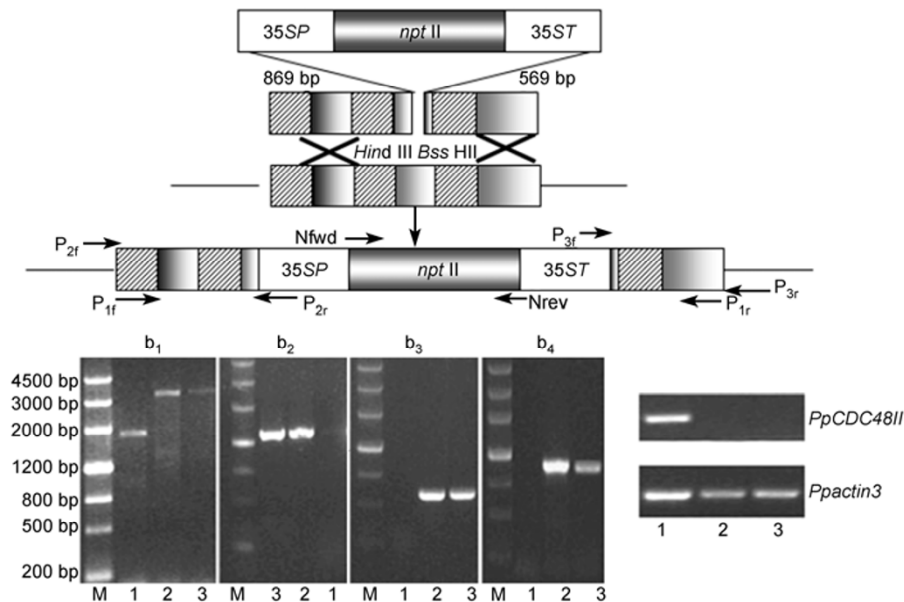


Figure 2 Generation and molecular analysis of *PpCDC48II* knockout lines. A, Insertion of a *Npt II* selection marker cassette into *Hind III* and *Bss HII* restriction site in exon 7 of the *PpCDC48II* gene (top); the resulting *PpCDC48II* knockout construct (middle); genomic structure after integration of the *PpCDC48II* knockout construct via homologous recombination (bottom). Primers used for the analysis of transgenic lines are indicated by arrows and Roman numerals. Dark gray box, *Npt II* cassette; boxes with fine lines, exon sequences within the knockout construct; gray boxes, introns flanking the knockout construct. B, PCR analysis to confirm 5' and 3' integration of the *PpCDC48II* knockout construct using primer P_{1f} and P_{1r} (b_1); *Npt II* identification using primer N_{fwd} and N_{rev} (b_2); 5' integration analysis using primers P_{2f} and P_{2r} (b_4); 3' integration analysis using primers P_{3f} and P_{3r} (b_3); PCR products obtained from wild type line 1 and *Ppcdc48II* mutant line 3 and line 2. C, RT-PCR analysis using primers $RTII-f$ and $RTII-r$ (*PpCDC48II*) and primers for the control gene *Ppactin3*. 1, wild type; 2 and 3, *Ppcdc48II* knockout line 2 and line 3.

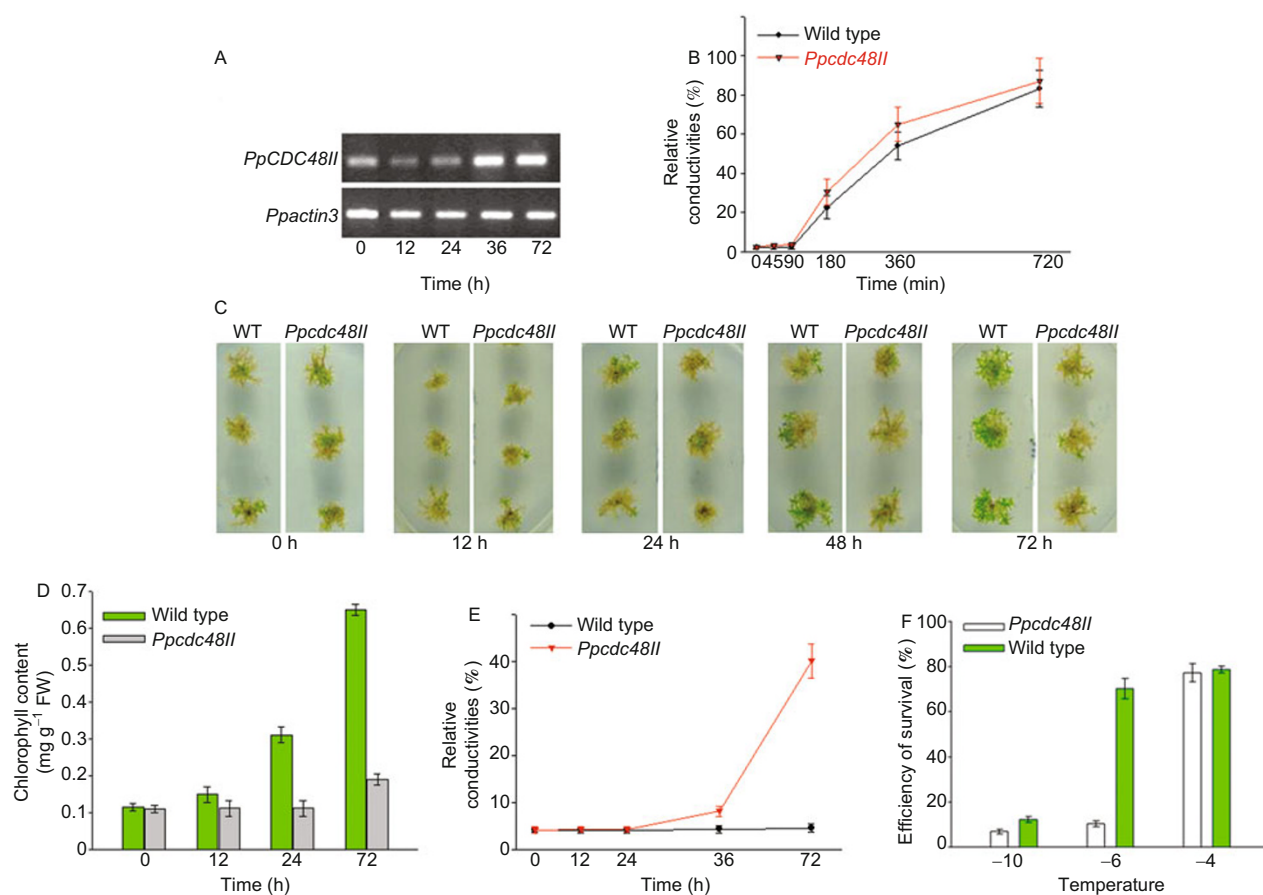


Figure 3 *Ppdc48II* mutant lines were more susceptible to freezing stress after cold acclimation. A, Expression of *PpCDC48II* during cold acclimation. Relative *PpCDC48II* mRNA expression was analyzed using SQRT-PCR. RNAs were isolated from *P. patens* gametophores that were cold-acclimated at 0°C for 12, 24, 36, and 72 h. *Ppactin3* was used as an endogenous control. B, Time course changes of *P. patens* gametophores freezing tolerance after treatment at -6°C. Two-week-old gametophores tissues were incubated on PPNH₄ medium at -6°C for various time points. Cell survival was determined by measuring electrolyte leakage after freeze-thawing. Error bars indicate standard errors of three independent experiments. C, The freezing sensitivity of *Ppdc48II* mutants after cold acclimation. D, Measurement of gametophores chlorophyll contents after freezing tests. E, Time course of cold-induced freezing tolerance of wild type and *Ppdc48II* gametophore cells after acclimation. F, The survival efficiency after 10 d of refreshing growth. Two-week-old wild type and *Ppdc48II* gametophores were incubated on PPNH₄ medium at 0°C for the indicated time periods and subjected to equilibrium freezing to -6°C for 90 min. After thawing, the tissues were grown on fresh PPNH₄ medium at 23°C for 2 d. The growth quantification was determined by measuring gametophores chlorophyll content (D), and survival was determined by measuring electrolyte leakage after thawing (E). Two-week-old wild type and *Ppdc48II* gametophores that were acclimated at 0°C for 72 h, were frozen at -4, -6, and -10°C for 90 min. After thawing, the tissues were grown on fresh PPNH₄ medium at 23°C for 10 d. The survival efficiency was calculated (F), and the values are shown in Table 1 (*N*=50).

Table 1 Freezing tolerance of *P. patens* gametophores after cold acclimation^{a)}

	<i>Ppdc48II</i>		Wild type	
	Survival efficiency (%) [#]	SD	Survival efficiency (%) [#]	SD
-4°C	77.30	0.042	78.6	0.014
-6°C	10.32	0.015	70.27	0.045
-10°C	6.91	0.011	12.31	0.013

a) SD, standard deviation. #, this test was repeated three times. For each test, the number of plants used in each line was greater than 50.

freezing tolerance after low-temperature treatments in *P. patens* [28], as reported in higher plants [29,30]. Therefore, elucidating the intracellular functions of cold-regulated genes is important for understanding the cold acclimation mechanism.

One cold-inducible protein was first identified in *Physcomitrella*. Like other CDC48 homologs, PpCDC48II

contained two highly conserved AAA-ATPase domains (Figure 1A), including the Walker A and B motifs, which were remarkably homologous not only to plant proteins, but also to yeast and human proteins. Therefore, because of the high degree of homology in amino acid sequence and domain structure, the cloned protein named PpCDC48II is the *Physcomitrella* homolog of *Arabidopsis* AtCDC48s.

The N-terminal region of *Physcomitrella* PpCDC48II contains two putative nuclear localization signals (KKDFSTAILERKK and KGKKRKD), which are important in CDC48 protein translocation into the nucleus [15]. Amino acid sequence analysis indicated that PpCDC48II lacked membrane-spanning regions and was a soluble protein. These sequence characteristics indicated a tantalizing possibility that PpCDC48II localized to the nucleoplasm and the cytoplasm, as do AtCDC48 and ScCDC48p [31,32], may be transported between the nucleus and cytoplasm, and function in both places.

The AAA-ATPase CDC48/p97 in non-plant systems has been shown to function in various pathways. However, the cellular roles of plant CDC48 proteins are rudimental. In *Arabidopsis*, localization studies and other studies implicated a role for AtCDC48 in plant cytokinesis, cell expansion and division. The independent *Arabidopsis* T-DNA insertion alleles of *Atcdc48A* were recessive and displayed the same phenotypes, including defects in pollen tube germination and expansion embryo developmental arrest, and seeding lethality [18]. The tobacco NgCDC48, located in the endoplasmic reticulum (ER), was recently implicated in ER-associated degradation of an as-yet unidentified target protein(s) [19].

To confirm *PpCDC48II* function under cold conditions, we constructed the transgenic *Physcomitrella* plants in which *PpCDC48II* was knocked out through homologous recombination (Figure 2). Slow equilibrium freezing of *P. patens* gametophores grown under normal growth conditions to -6°C killed more than 90% of the cells, indicating that the gametophore cells are freezing-sensitive (Figure 3B). Cold treatment for 72 h dramatically increased the freezing tolerance of wild type gametophores, while the *Ppcdc48II* mutants failed to generate freezing tolerance after cold acclimation, which indicated PpCDC48II plays important roles in the enhancement of freezing tolerance during cold acclimation. In addition, cold-treatment resulted in a remarkable increase in *PpCDC48II* gene expression within 72 h (Figure 3A). These results suggested that PpCDC48II participated in the protection of gametophore cells that lead to the development of freezing tolerance. Previous studies of zebrafish CDC48 showed that the cold-inducible protein CDC48 may be regulated by transcription factors in response to cell proliferation and cell cycle progression under cold conditions [4]. Some evidences showed that CDC48 regulates cell cycle in various organisms, such as yeast, *Arabidopsis*, mouse, and human [15,31,33,34]. According to the results of cold-sensitive yeast mutants studies, cell division arrest occurs at the large budded cell stage, when there is an undivided nucleus and microtubule bundles spread throughout the cytoplasm from an unseparated spindle pole body [31]. Therefore, yeast CDC48 plays an important role in the M phase of cell cycle at low temperature, and these mutant phenotypes are consistent with defect in homotypic fusion of the inner nuclear

and ER membranes.

Nevertheless, there are several reports describing that CDC48 has a variety of biological functions, including a role in regulating apoptosis [34–37]. CDC48p/p97/VCP is involved in a number of diverse cellular pathways through interactions with different adaptor proteins [38], which can distinguish between native and non-native proteins. Some results demonstrated Cdc48/VCP to be crucially involved in several cellular functions controlled by ubiquitination [12]. The chaperone activity of CDC48 may be required to disassemble the SNARE (soluble N-ethylmaleimide-sensitive fusion attachment protein receptors) complex in membrane fusion, and to recognize proteins destined to be processed or degraded after their release from the ER [32]. In tobacco, some experiments indicated that NgCDC48 is involved in ER-associated degradation, and NgCDC48-mediated protein degradation, in turn, is required for normal growth and development of tobacco plants [19]. According to our previous study, differentially expressed ESTs analyzed under cold acclimation in *P. patens*, indicated that cold acclimation of *P. patens* reduced cell division and reprogrammed plant development, and the ubiquitin-proteasome system was proposed to be involved in this process [4]. Furthermore, our data demonstrated that disruption of *PpCDC48II* led to freezing hypersensitivity after cold acclimation, providing concrete evidence for the role of PpCDC48II as a positive regulator of cold-induced freezing tolerance in moss (Figure 3). These data also suggested that the acquisition of freezing tolerance in *P. patens* involves a *PpCDC48II*-mediated signaling pathway. Therefore, we are tempted to consider that enhanced chaperone activity by *PpCDC48II* induction at cold temperatures may recognize and degrade misfolded proteins in gametophores. Higher levels of the CDC48 protein may be required to promote cellular functions in the cold than at normal growth temperature. We are currently conducting a yeast two-hybrid screen to identify PpCDC48II-interacting proteins and targeting experiment to define PpCDC48II localization. Further experiments are required to elucidate the mode of action of PpCDC48II and its interacting proteins.

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