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Long-chain base kinase1 affects freezing tolerance in *Arabidopsis thaliana*

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ABSTRACT

Long-chain base kinases (LCBKs) phosphorylate sphingolipid-derived long-chain base lipids and participate in the regulation of stress responses in plants. Here, we isolated a novel *Arabidopsis thaliana* mutant, *lcbk1-2*, which was extremely sensitive to freezing temperatures with or without cold acclimation. Physiological assays revealed that concentrations of osmolytes (proline and soluble sugars) and the activity of superoxide dismutase were significantly decreased in the *lcbk1-2* mutant, compared with wild type. Also, the balance of reactive oxygen species (ROS) was disrupted in the *lcbk1-2* mutant with or without cold treatment and, consistent with this, gene expression profiling analysis showed that the expression of cold-responsive ROS-scavenging genes was substantially decreased in the *lcbk1-2* mutant. The expression of membrane lipid-related genes, which are linked to freezing tolerance in plants, was also impaired in the *lcbk1-2* mutant. Furthermore, transgenic lines overexpressing *LCBK1* showed enhanced freezing tolerance with over-accumulation of osmolytes. Collectively, our results suggested that LCBK1 functions as a novel positive regulator of freezing tolerance in *Arabidopsis* and may participate in the accumulation of osmolytes, the regulation of ROS homeostasis and lipid metabolism.

1. Introduction

Temperature restricts plant geographical distribution, growth, and productivity [1]. Plant cold acclimation involves the CBF- (C-repeat/dehydration responsive element-binding factor) dependent regulatory cascade [2,3]. Cold stress rapidly induces *CBF* expression, which activates a set of cold-regulated (*COR*) genes by directly binding to the CRT/DRE (C-repeat/drought responsive element) *cis*-elements of their promoters [4,5]. Studies in *Arabidopsis* used *cbf*s triple mutants and *CBF* overexpression lines to uncover the predominant roles of CBFs in regulation of the plant response to cold temperatures [6]. However, *Arabidopsis* CBFs only regulate 7% of *COR* genes, suggesting the existence of CBF-independent pathways [6,7]. Indeed, several components of CBF-independent pathways have been identified [8,9]. For example, the *Arabidopsis eskimo1* mutant exhibited increased freezing tolerance compared to the wild-type plants without affecting the expression of *COR* genes [10]. *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE9* encodes a putative homeodomain transcription factor that mediates cold tolerance without disrupting the expression of *CBF* regulons [11]. SENSITIVE TO FREEZING2 modulates freezing tolerance via lipid remodeling of the outer chloroplast membrane [12].

Plants adapt to low temperatures by triggering a sophisticated cold-activated molecular program to initiate various biochemical and physiological processes that protect the plant against damage at low temperature [9,13–17]. For example, transcriptome analysis indicates that the transcripts of various cold-response genes involved in fatty acid metabolism and reactive oxygen species (ROS) scavenging, and key enzymes for biosynthesis of osmolytes such as proline, sugars, and late embryogenesis abundant proteins, increase in abundance in response to cold temperatures [14–16]. Among these plant responses, the biosynthesis and modification of cell membrane lipids play important roles in plant resistance to chilling or freezing temperatures [18,19]. Therefore, the regulation of lipid metabolism in temperature signalling has been extensively studied [20–25]. For example, *acyl-lipid desaturase 2* (*ADS2*)

Abbreviations: ACBP6, acyl-coenzyme A-binding protein 6; ADS2, acyl-lipid desaturase 2; APX1, ascorbate peroxidase 1; CAT1, catalase 1; CBFs, C-repeat/dehydration-responsive element binding factors; CCS1, copper chaperone for superoxide dismutase 1; COR, cold-regulated; CRT/DRE, C-repeat/drought-responsive element; CSD, copper/zinc superoxide dismutase; DAB, 3,3′-diaminobenzidine; DEGs, differentially expressed genes; FER1, ferritins 1; GUS, β-glucuronidase; LCBKs, long-chain base kinases; MS, Murashige and Skoog; NBT, nitroblue tetrazolium; PHS-P, phytosphingosine-1-phosphate; PLD, phospholipase D; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcription-PCR; SOD, superoxide dismutase; TH8, thioredoxin H8; WT, wild type

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is a cold-inducible gene that encodes a desaturase for plastid lipids and the *ads2* mutants exhibit sensitivity to chilling and freezing stress [20]. PHOSPHOLIPASE D δ (PLDδ), which functions in membrane lipid hydrolysis, acts as a positive regulator of freezing tolerance [22], whereas PHOSPHOLIPASE D α1 (PLDα1) has a negative role in freezing tolerance [23]. In addition, ACYL-COA-BINDING PROTEIN 6 (ACBP6) potentially plays a role in the cytosolic trafficking of phosphatidylcholine. Knockout of *ACBP6* results in decreased phosphatidylcholine content and reduced freezing tolerance and overexpression of *ACBP6* enhances freezing tolerance [26].

Sphingolipids (SLs) are complex molecules derived from phospholipids and are major components of membrane lipids in eukaryotic cells [27]. The long-chain base (LCB) SL metabolites and their phosphorylated derivatives (LCBPs) are critical regulators of many physiological processes and stress responses [28–36]. The cellular levels and bioactivity of LCBPs are strictly controlled by specific LCB kinases (LCBKs). In *Arabidopsis thaliana*, four putative *LCBK* isoforms, *LCBK1* (*At5g23450*), *LCBK2* (*At2g46090*), *SPHK1* (*At4g21540*), and *SPHK2* (*At4g21534*), have been proposed to function as LCB kinases [34]. LCBKs function as important signal transducers in stress responses [37–39]. In *Arabidopsis*, abscisic acid stimulates the enzymatic activity of SPHK1 and SPHK2 during stomatal closure [34–36]. Cold triggers an increase in LCBK2 kinase activity to catalyze the production of phytosphingosine-1-phosphate (PHS-P) [40]. In rice (*Oryza sativa* L.), OsLCBK1 and OsLCBK2 modulate the plant disease resistance and cell death [41]. However, the precise mechanisms that LCBKs mediate in the response to abiotic stresses remain unknown.

In this study, we report that *Arabidopsis LCBK1* plays a prominent role in freezing tolerance. The loss-of-function mutant *lcbk1-2* is hypersensitive to freezing treatment, whereas overexpression of *LCBK1* enhanced freezing tolerance in *Arabidopsis*. Alterations of *LCBK1* did not affect the expression of *CBF* genes but resulted in different contents of proline and soluble sugars. We also observed that expression of a set of cold-induced genes, which are involved in lipid metabolism and ROS scavenging, was impaired in the *lcbk1-2* mutant after cold treatment. These results suggest that *LCBK1* mediates plant freezing tolerance by regulating the accumulation of osmolytes, ROS homeostasis, as well as lipid metabolism.

2. Materials and methods

2.1. Plant growth conditions, cold treatment, and freezing treatment

Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. Col-0 and *lcbk1-2* mutant seeds were sown in soil under a photoperiod of 16-h light/8-h dark or on Murashige and Skoog (MS) medium (Sigma-Aldrich) containing 2% sucrose and 0.8% agar at 22 °C. The *lcbk1-2* (SALK_152371) mutant was obtained from The Arabidopsis Information Resource (TAIR, http://arabidopsis.org).

Two-week-old seedlings grown on MS agar plates at 22 °C were used for the cold treatment and freezing tolerance assays. For the cold treatment, two-week-old seedlings were grown on MS agar plates at 4 °C for 0, 3, 6, 12, 24 h. For cold acclimation prior to the freezing treatment, the seedlings were transferred to 4 °C for 3 days. The assays were performed as previously described [42]. Briefly, seedlings were placed in a −1 °C freezing chamber (RUMED 4001, Germany) and programmed to cool at a rate of 1 °C h^{-1}. The plates were removed at −4, −5, and −6 °C for non-acclimated seedlings or at −6, −7, and −8 °C for cold-acclimated seedlings and incubated at 4 °C for 12 h before being transferred to 22 °C for post-freezing recovery. Seedling survival was calculated after a 4-day recovery period.

2.2. Physiological analyses

An electrolyte leakage assay was performed as previously described [43]. The percentage of electrolyte leakage was calculated as the percentage of conductivity before and after autoclaving. The proline content was measured according the protocol of Bates et al. [44]. The total contents of soluble sugars (sucrose, glucose, and fructose) was measured as previously described [45].

2.3. Plasmid construction and plant transformation

For molecular complementation of the *lcbk1-2* mutant and overexpression of *LCBK1 in the wild type (WT) Col-0 background, a 2.4-kb LCBK1* cDNA fragment was amplified by PCR using the primers LCBK1-F and LCBK1-R (Table S1). The fragment was then cloned into the pCAMBIA1300 vector (CAMBIA) containing a Super promoter [46], which was used to generate *pSuper:LCBK1/lcbk1-2* complemented plants and *pSuper:LCBK1/Col-0* overexpression plants.

A 1.9-kb genomic fragment containing the sequences upstream of the *LCBK1* ATG start codon was amplified by PCR using the primers LCBK1-p1F and LCBK1-p1R (Table S1). The fragment was fused with the β-glucuronidase (GUS) reporter gene in the binary vector PZP212 [47] to obtain the construct p*LCBK1*:*GUS*. *Agrobacterium tumefaciens GV3101* was transformed with the different constructs and used to transform the WT and *lcbk1-2* mutant plants via the floral dip method [48].

2.4. RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from 14-day-old seedlings grown on MS agar plates at 22 °C or 4 °C using TRIzol (Invitrogen) and qRT-PCR was performed using the SYBR Green PCR Master Mix kit (TaKaRa, Tokyo, Japan) as previously described [49]. Values are shown as the mean ± standard deviation of three repeats. Student's *t*-test was used for statistical analysis [50]. The primers used are listed in Supplementary Table S1.

2.5. GUS, nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining

Histochemical detection of GUS activity in various plant tissues was performed as previously described with minor modifications [47,49]. In brief, tissues were incubated in staining solution (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indoyl glucuronide, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide) at 37 °C overnight. After incubation, the stained tissues were cleared of chlorophyll in an ethanol series (20%, 40%, 60%, 80%, and 100%) and then observed and photographed. NBT staining was performed to detect superoxide, while DAB staining was performed to detect hydrogen peroxide (H_2O_2) as previously described [51]. All experiments were repeated more than three times. Tissues were examined under a stereomicroscope (Leica, MZ10F).

2.6. Quantification of superoxide dismutase (SOD) enzyme activity

Two-week-old seedlings were frozen in liquid nitrogen and ground using a mortar and pestle. The ground materials were suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged to remove the cell debris (4 °C, 10 min, 12,000 rpm). SOD activity was determined with an NBT-based assay using a Power Wave XS2 (BioTek) [52,53].

2.7. Microarray analysis

Microarray experiments were performed following the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, total RNA was extracted using a Plant RNA Prep Kit (Qiagen, Hilden, Germany) from two-week-old Col-0 and *lcbk1-2* mutant seedlings grown on 1/2 MS medium containing 2% sucrose at 22 °C or 4 °C for 3 h and 24 h under a 16-h light/8-h dark photoperiod. Then, 6 μg of total RNA was used to