Cathepsin B degrades RbcL during freezing-induced programmed cell death in Arabidopsis

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Abstract

Programmed cell death (PCD) has been well documented in both development and in response to environmental stresses in plants; however, PCD induced by freezing stress and its molecular mechanisms remain poorly understood. In the present study, we characterised freezing-induced PCD and explored its mechanisms in *Arabidopsis*. PCD induced by freezing stress was similar to that induced by other stresses and senescence in *Arabidopsis* plants with cold acclimation. Inhibitor treatment assays and immunoblotting indicated that cathepsin B mainly contributed to increased caspase-3-like activity during freezing-induced PCD. Cathepsin B was involved in freezing-induced PCD and degraded the large subunit, RbcL, of Rubisco. Our results demonstrate an essential regulatory mechanism of cathepsin B for Rubisco degradation in freezing-induced PCD, improving our understanding of freezing-induced cell death and nitrogen and carbohydrate remobilisation in plants.

Highlight

This study demonstrated an important role of cathepsin B that degrades the Rubisco large subunit RbcL in freezing stress.

Key Message

Cathepsin B playa an important role that degrades the Rubisco large subunit RbcL in freezing stress.

Introduction

Programmed cell death (PCD) is a genetically controlled cell death that is both ubiquitous and conserved in eukaryotes. PCD plays vital roles in morphogenesis, cell homeostasis, and elimination of unprofitable cell components (Kabbage et al. 2017). PCD widely participates in plant growth and development, such as gametophyte development, fertilisation, seed development, leaf morphogenesis, xylem formation, and senescence (Daneva et al. 2016). In addition, PCD has emerged as a survival strategy for plants to fight various biotic and abiotic stresses (Coll et al. 2011; Petrov et al. 2015). Cold stress, particularly freezing stress (< 0ºC), is a widespread extreme environmental disaster that results in fatal damage to plant tissues and eventually catastrophes in crop production (Thomashow 1999; Chinnusamy et al. 2007). Hallmarks such as protoplast shrinkage, DNA fragmentation, increased levels of reactive oxygen species, and cytochrome *c* release have been identified in suspension cultures of winter wheat subjected to freezing stress, indicating the induction of PCD in the freezing stress response (Lyubushkina et al. 2014). However, the regulatory networks underlying freezing-induced PCD in plant cells remain unclear. A better understanding of the mechanisms by which plant cells exert PCD during freezing stress is of great importance.

Caspase family proteins are crucial regulators of several modes of PCD, such as apoptosis, pyroptosis, and necroptosis in mammals; among these, caspase-3 is the main executor (Shalini et al. 2015; Julien

and Wells 2017). Unfortunately, the orthologous genes of caspases are absent in known plant genomes (Salvesen et al. 2016). However, caspase-3-like enzymatic activities have been extensively detected using synthetic substrates and inhibitors during plant PCD processes triggered by distinct stimuli such as UV irradiation, pathogen infection, and self-incompatibility (Del Pozo and Lam 1998; Bonneau et al. 2008; Van Durme and Nowack 2016; Sueldo and van der Hoorn 2017). Two types of proteases, PBA1 and cathepsin B, have been identified to exhibit caspase-3-like activity in plant PCD. PBA1 is the β1 subunit of the 20S proteasome that participates in xylem development (Han et al. 2012) and in plant PCD induced by *Pseudomonas* or ER stress (Pajerowska-Mukhtar and Dong 2009; Cai et al. 2017). Cathepsin B is a papain-like cysteine protease localised in plant vacuoles. Three paralogues, *AtCathB1*, *AtCathB2*, and *AtCathB3*, have been identified in the *Arabidopsis* genome (McLellan et al. 2009). However, cathepsin B enzymatic activity is mainly provided by *AtCathB2* and *AtCathB3*, because *AtCathB1* undergoes alternative splicing in the 5th intron and generates three major splice variants that translate into non-functional proteins (Chemistry et al. 2018). In animals, cathepsin B plays an important role in triggering apoptosis (Salvesen et al. 2016) and in the degradation of autophagic bodies in lysosomes (Man and Kanneganti 2016; Araujo et al. 2018). The importance of plant cathepsin B in seed germination, hypersensitive responses, and senescence-related PCD has been well documented. Nevertheless, little is known about proteolytic cascades, particularly the *in vivo* substrates of cathepsin B during plant PCD (McLellan et al. 2009; Ge et al. 2016; Cai et al. 2017).

In this study, we demonstrate that PCD in *Arabidopsis* induced by freezing stress was accompanied by upregulation of cathepsin B. *Arabidopsis* cathepsin B was involved in freezing-induced PCD and degraded the Rubisco large subunit RbcL. Thus, our study revealed the important roles of cathepsin B in freezing-induced PCD and the degradation mechanism of RbcL for nitrogen and carbohydrate remobilisation in plants.

Materials and Methods

Plant materials and growth conditions

Wild-type (WT) and mutant plants used in this study were of the Col-0 background of *Arabidopsis thaliana*. The triple mutant line *cathb#62* was a gift from Professor Patrick Gallois. The *Arabidopsis* seedlings were grown on1/2 MS medium plates or in pots under 16 h light and 8 h dark cycle at 22°C.

Freezing stress treatment assays

For freezing stress treatment assays, 12 days-old WT *Arabidopsis* seedlings grown on 1/2 MS medium plates or 3–4 weeks-old WT *Arabidopsis* plants grown in pots were placed in an incubator (GXZ-500, Ningbo Jiangnan) and grown for 3 days at 4°C for cold acclimation. The plants were then exposed to a cryogenic circulator or a freezing incubator that was programmed to drop temperature by 1°C per hour from 0°C to -10°C, and treatment was continued for 6 h. All plants were recovered in the dark at 4°C for 12 h, and finally were transferred to a growth house at 22°C (16 h light and 8 h dark) for 7 days of recovery.

Trypan blue staining

Trypan blue staining was performed as previously described (Chen et al. 2015). In brief, the leaves were immersed in 1 mL trypan blue staining solution (80 mL 95% ethanol, 10 mL phenol, 10 mL lactic acid, 10 mL glycerol, 10 mL ddH_2O, and 0.02 g trypan blue) in 2 mL tubes and boiled for 2 min. The staining solution was discarded and the cells were destained with 70% chloral hydrate. The leaves were analysed and photographed using a stereo fluorescence microscope (Discover v20; Zeiss, Germany).

Ion leakage assay

Ion leakage assays were performed as previously described (Liu et al. 2017). *Arabidopsis* seedlings or leaves were shaken for 30 min at room temperature in 5 mL tubes containing 4 mL deionised water (S_0), and the electrical conductivity (S_1) was detected. The samples were then boiled for 10 min in an autoclave, and the electrical conductivity (S_2) was measured after shaking the samples for 30 min at room temperature. The electrolyte leakage was calculated as $[(S_1 - S_0)/ (S_2 - S_0)] \times 100\%$.

Total RNA extraction and RT-qPCR assays

Total RNA was extracted from *Arabidopsis* leaves using an RNA prep Pure Plant Kit (TIANGEN). Total RNA (1 μg) was used to synthesise first-strand cDNA using the Hifair® III 1st Strand cDNA Synthesis SuperMix kit (YEASEN, 11141ES10). RT-qPCR assays were performed using an ABI Real-Time PCR Detection System (ABI, Q5) with the Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (YEASEN, 11202ES08). Three biological replicates and four or three technical replicates were established for each sample. *AtUBC21* (At5g25760) was used as a reference control. The RT-qPCR primer sequences are listed in Supplementary Table S3.

Enzymatic activity assays

Enzymatic activity assays were performed as previously described (Ge et al. 2016; Cai et al. 2017). For caspase-3-like activity assay, *Arabidopsis* leaves were ground in liquid nitrogen and total proteins were extracted in extraction buffer (5 mM DTT and 1× cocktail) after centrifugation at 4°C, 16 000 × *g* for 10 min. Protein extracts (50 μL) were incubated in 50 μL 2× DEVDase assay buffer (50 mM NaAc, 200 mM NaCl, 2 mM EDTA, pH 5.5) with 0.5 μL 10 mM (Z-DEVD) -R110 (Bachem, M-2615). For inhibitor treatment assays, protein extracts were incubated in DEVDase assay buffer containing 200 μM Ac-DEVD-CHO (Bachem, H-2496), 200 μM β-lactone (Abcam, ab141412), 200 μM CA-074 (Bachem, N-1475), and the same volume of DMSO for 30 min at 30°C before adding substrates. For cathepsin B activity, the ProCathB2 and ProCathB3 (3 mg/mL) were self-activated in active buffer (0.1 M NaAc, 2.5 mM DTT, 20 mg/mL dextran sulphate, pH 4.5) for 60 min at 37°C. The activated cathepsin B was incubated in reaction buffer (0.1 M NaAc, 5 mM DTT, pH 4.5) containing 10 μM Z-Arg-Arg-4-methylcoumaryl-7-amide (MCA) (GenicBio). All reactions were performed in black 96-well plates at 30°C using a Thermo Scientific Microplate Reader (Varioskan Flash). The fluorescence of R110 (Ex 485 nm, Em 538 nm) and MCA (Ex 355 nm, Em 460 nm) was detected every 10 min at least six times. Enzymatic activity was calculated using the slope (fluorescent units/min), sample weight (g), or protein concentration (mg).

Activity labelling, SDS-PAGE, and immunoblotting

Activity labelling assays were performed as previously described (Ge et al. 2016; Cai et al. 2017). Total proteins from *Arabidopsis* leaves were extracted in extraction buffer (5 mM DTT and 1× cocktail), and the protein extracts were incubated with 100 μM biotin-DEVD-FMK in DEVDase assay buffer for 1 h at 37°C. The labelled proteins were visualised with immunoblotting using horseradish peroxidase (HRP)-labelled streptavidin (1/5000) (A0303, Beyotime). For SDS-PAGE and immunoblotting assays, the total proteins or proteins labelled with 1× protein loading buffer were boiled in water and separated by SDS-PAGE (12%). The gels were then subjected to Coomassie Brilliant Blue staining or transferred to a PVDF membrane (0.22 μm) at 200 mA for 1.5 h in transfer buffer. The PVDF membrane was blocked with 5% non-fat powdered milk in TBST buffer for 1 h at room temperature. After incubation with primary and secondary antibodies, the target proteins were detected using an enhanced chemiluminescent reagent (P10200, NCM Biotech) with a chemiluminescence imaging analysis system (BG-gdsAUTO 710 MINI, Baygene Biotech). PBA1 was used as loading control.

LC-MS/MS

LC-MS/MS analysis was performed as previously described, and mainly included alkylation, tryptic digestion, mass spectrometry, and database searching (Yang et al. 2022).

Yeast two-hybrid assays

Yeast two-hybrid assays were performed as previously described (Yang et al. 2022). The CDS of RbcL was cloned into the pGADT7-GW plasmid, and the CDSs, CDSs without signal peptides, and mature fragments of cathepsin B were cloned into the pGBKT7-GW plasmid using gateway cloning system. The constructed plasmids were co-transformed into the AH109 strain and the interactions between RbcL and cathepsin B were determined by observing the growth status of positive clones in media lacking leucine (Leu), tryptophan (Trp), histidine (His), and adenine (Ade).

Purification of recombinant RbcL

The CDS of RbcL containing EcoR1 and Xho1 restriction sites at the N-terminal and C-termini, respectively, was amplified by PCR. The CDS of RbcL was ligated into pET30a after being digested with EcoR1 and Xho1, which generated RbcL-pET30a. *Escherichia coli* BL21 cells transformed with RbcL-pET30a were grown and induced by isopropyl β-D-thiogalactoside (IPTG) in 300 mL LB medium to express recombinant RbcL. The cultures were pelleted, dissolved in 50 mL 20 mM PBS buffer (50 mM NaCl,10 mM imidazole, pH 7.4), and lysed by sonication. After centrifugation at 4°C, 16 000 × *g* for 30 min, the precipitate was dissolved in 10 mL 8M urea solution (50 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, 5% glycerol, 5 mM DTT). The denatured recombinant RbcL was renatured through 12 h dialysis at 4°C against 6, 4, 2, and 0 M urea solutions. The renatured recombinant RbcL was clarified by centrifugation at 4°C, 12000 × *g* for 5 min and purified by Ni-affinity chromatography.

Purification of recombinant cathepsin B

The CDS of ProCathB2 and ProCathB3 were ligated into pPICK9K-His after they were cut using restriction enzymes EcoR1 and Not1, which generated ProCathB2-His-pPICK9K and ProCathB3His-pPICK9K. *Pichia pastoris* strain GS115 cells, which were transformed with ProCathB2-His-pPICK9K and ProCathB3His-pPICK9K, were grown and induced by methanol in 300 mL BMMY medium to secrete recombinant ProCathB2 and ProCathB3. The recombinant ProCathB2 and ProCathB3 were purified by Ni-affinity chromatography from culture supernatants that were obtained by centrifugation at 4°C, 12000 × *g* for 1 min.

Microscale thermophoresis (MST) assays

The recombinant RbcL was fluorescently labelled using a Monolith™ RED-NHS protein labelling kit (Cat# MO-L011), followed by mixing with a range of concentrations of ProCathB and activated CathB. The mixtures were loaded into a Monolith NT.115 (Nanotemper) using hydrophilic capillaries (Nanotemper) to measure the binding affinities at 40% MST power and 20% excitation power. K_D was calculated using triplicate F_{norm} measurements at each concentration using NanoTemper software. GraphPad8.0 was used to draw the binding curves.

Degradation of RbcL in vitro

The recombinant RbcL (0.3 mg/mL) was treated with cathepsin B active buffer and self-activated cathepsin B (0.6 mg/mL) in reaction buffer (0.1 M NaAc, 2.5 mM DTT, pH 4.5) at 37°C. After incubation for 0, 30, 60, 90, 120, and 240 min, the samples were boiled with 1× protein-loading buffer and detected by immunoblotting using Anti-RbcL (AS03037, Agrisera).

Statistical analysis

All values with means ± SD were calculated using GraphPad 8.01 software. The statistical significance of differences was assessed using a one-way ANOVA or t-test.

Accession numbers

Sequence data from this study can be found in the Arabidopsis Information Resource (http://www.arabidopsis.org/) with accession numbers *CathB1* (AT1G02300), *CathB2* (AT1G02305), *CathB3* (AT4G01610), and *RbcL* (ATCG00490).

Results

Freezing stress induces PCD in Arabidopsis

To explore whether freezing stress induces PCD in *Arabidopsis* seedlings, 3-week-old wild-type seedlings with 3 days of cold acclimation were exposed to -10 ºC for 6 h and then analysed during the 7-day recovery period. After freezing stress, *Arabidopsis* seedlings with previous cold acclimation showed segmental water-soaking and wilting (Fig. 1A, 1B). Although cell death was not observed in rosette leaves with trypan blue staining (Fig. 1C), the ion leakage ratio in the leaves increased significantly ($p < 0.001$)

(Fig. 1D). The seedlings restored a normal appearance on day 3 during the recovery period (Fig. 1A, 1B), and cell death and ion leakage reduction were both detected in the leaves (Fig. 1C, 1D). Leaf chlorosis was accompanied by a significant increase in cell death and ion leakage ratio ($p < 0.001$) on days 5 and 7 during recovery (Fig. 1A-1D). Interestingly, the severity of chlorosis and cell death in senescent leaves was higher than that in tender leaves (Fig. 1B, 1C), suggesting that senescent leaves are particularly sensitive to freezing stress. In addition, increased caspase-3-like activity, an essential indicator of PCD in plant cells, was detected using a synthetic fluorescent substrate after freezing stress. Caspase-3-like activity was significantly elevated on day 3 and culminated on day 5 after freezing stress (Fig. 1E). Moreover, the transcriptional level of *SAG12*, a marker of senescence, was significantly upregulated after freezing stress ($p < 0.001$) (Fig. 1F). These results indicate that freezing stress induces PCD in *Arabidopsis* seedlings.

Cathepsin B acts as caspase-3-like proteases in freezing-induced PCD

As caspase-3-like activity increased significantly in freezing-induced PCD ($p < 0.001$) (Fig. 1E), we identified proteases with caspase-3-like activity. Previous studies have shown that PBA1 (Pajerowska-Mukhtar and Dong 2009; Han et al. 2012) and cathepsin B (Ge et al. 2016; Cai et al. 2017) possess caspase-3-like activity in *Arabidopsis* and poplar. Therefore, we used inhibitors of these proteins to investigate whether they contribute to caspase-3-like activity during freezing-induced PCD. The results show that elevated caspase-3-like activity in leaves after freezing stress was reduced by only 25% after using the PBA1 inhibitor β-lactone, and by 75% after using cathepsin B inhibitor CA-074. The inhibitory effect on cathepsin B was similar to that of the generic caspase-3 inhibitor Ac-DEVD-CHO (Fig. 2A), implying that cathepsin B is the major caspase-3-like protease required in freezing-induced PCD. We further used the biotinylated caspase-3 inhibitor biotin-DEVD–FMK to label caspase-3-like proteases in freeze-stressed leaves. Three protein bands with remarkably elevated levels were found in WT *Arabidopsis* leaves after freezing stress by immunoblotting, and their sizes were consistent with those of the precursor (P), mature form 1 (M), and mature form 2 (m) of *Arabidopsis* cathepsin B (Fig. 2B). However, PBA1 abundance was not different in leaves of the same weight before and after freezing stress (Fig. 2B). In addition, the transcriptional levels of two *Arabidopsis* cathepsin B orthologues, *CathB2* and *CathB3*, were significantly upregulated in *Arabidopsis* leaves after freezing stress ($p < 0.001$); *CathB3* expression increased by more than 60 times (Fig. 2D, 2E). In addition, PBA1 transcription increased slightly after freezing stress (Fig. 2F). Taken together, these results indicate that cathepsin B mainly contributes to increased caspase-3-like activity during freezing-induced PCD.

Depletion of cathepsin B induces compensatory expression of cathepsin L homologues in freezing stress

Although we showed the important role of *Arabidopsis* cathepsin B in the proteolytic cascade during freezing-induced PCD as the major caspase-3-like protease, there were no significant phenotypic differences, cell death levels and ion leakage rates, between WT and cathepsin B knockout (*cathb#62*) seedlings (Supplementary Fig. S1A-S1D). Both WT and *cathb#62 Arabidopsis* plants displayed severe

leaf chlorosis on day 7 during growth recovery after freezing stress. Furthermore, 12 days-old WT and *cathb#62 Arabidopsis* seedlings grown on 1/2 MS medium plates were used to investigate the phenotypes after treatment with a cryogenic circulator to avoid temperature instability in the freezing incubator. The results similarly showed that there was no significant difference in phenotype, survival rate, or ion leakage rate between WT and *cathb #62 Arabidopsis* seedlings after freezing stress (Supplementary Fig. S1E-S1G).

As reported in mammalian cells, cathepsin B and S depletion results in a compensatory upregulation of cathepsin Z (Akkari et al. 2016). Thus, we hypothesised that the failure to achieve distinct cell death phenotypes in *cathb#62 Arabidopsis* seedlings might result from the compensatory upregulation of the expression of other cysteine cathepsin proteases, which also play vital roles in the freezing stress response. Herein, we examined alterations in the transcription levels of 30 members of the papain-like cysteine protease (PLCPs) family in the WT and *cathb#62* mutants before and after freezing stress. We found that 12 members of 30 PLCPs genes were significantly upregulated in both WT and *cathb#62* mutants at day 5 of recovery growth after freezing stress (Fig. 3 and Supplementary Fig. S2). Notably, the transcription levels of *SAG2*, *SAG12*, *CEP1*, *CEP2*, and *RD21A*, which are all cathepsin L homologues, were more than twice upregulated in *the cathb#62* mutant than those in WT seedlings (Fig. 3). The important roles of *SAG2*, *SAG12*, CEP1, and RD21A have been demonstrated in plant senescence (Díaz-Mendoza et al. 2014), tapetum degeneration, xylem element cell death (Cheng et al. 2019; Han et al. 2019), necrotrophic fungal pathogen *Botrytis cinerea* (Lampl et al. 2013), and sphingolipid-induced cell death (Thuleau et al. 2018).

Cathepsin B degrades RbcL in freezing stress-induced PCD

The investigation of *in vivo* cathepsin B substrates in plant PCD is of great importance for the elucidation of cell death signalling pathways. We performed an *in vitro* cell-free degradation assay to identify potential substrates of *Arabidopsis* cathepsin B during freezing stress-induced PCD. Caspase-3-like activity peaked on day 5 after freezing stress in *Arabidopsis* leaves. Thus, we pulled down caspase-3-like protease, most of which was cathepsin B, using biotin-DEVD-FMK from total soluble protein extract. A DMSO solution was used as the control group. Two protein bands, whose abundance was reduced remarkably as shown in SDS-PAGE, were selected and identified as Rubisco large subunit (RbcL) and fructose-bisphosphate aldolase (FBA) by LC-MS/MS (Fig. 4B-4E). Moreover, our *in vitro* degradation assays using recombinant AtCathB3 and *Arabidopsis* total protein extracts also suggested that RbcL is a potential substrate (unpublished data). Therefore, we investigated whether RbcL is a substrate of cathepsin B.

In yeast two-hybrid (Y2H) assays, we confirmed the interaction between RbcL and the activated forms of AtCathB2 or AtCathB3 (Fig. 5B and Supplementary Fig. S3). We performed microscale thermophoresis (MST) assays to validate the interaction between cathepsin B and RbcL. Recombinant AtCathB3 was produced in *Pichia pastoris* with the expected enzymatic activities (Supplementary Fig. S4A-S4G). Recombinant RbcL was harvested from the prokaryotic expression system *of E. coli* strain BL21 (Supplementary Fig. S4H, S4I). Consistent with Y2H assays, MST assays showed that activated

AtCathB2 and AtCathB3 have high binding affinities for RbcL, and the dissociation constants (K_D) were 2.29 μM and 1.57 μM, respectively (Fig. 5C, 5D).

Next, we investigated RbcL degradation *in vitro*. Equally activated AtCathB2 or AtCathB3 was added to each reaction containing equal amounts of RbcL protein, and the buffer was used as a control. Recombinant RbcL protein was degraded dramatically in reactions in which activated CathB2 and CathB3 were added (Fig. 5E-5H). These results indicate that cathepsin B degraded RbcL.

Discussion

PCD is a pro-survival strategy against freezing stress

In the present study, the model plant *A. thaliana* was employed as the research object to demonstrate that freezing stress induces PCD in plants via morphological, cell biology, molecular biology, and genetic methods, which may be a sacrificial protective mechanism for plants under freezing stress. Plants, as sessile organisms, cannot avoid environmental stress owing to their lack of mobility. Therefore, they have adapted the ability to deal with various stimuli and ensure their survival and proliferation (Zhu 2016). Cold stress is generally divided into chilling and freezing stresses and is a global environmental factor that affects plant growth, development, productivity, and geographic distribution (Thomashow 1999; Chinnusamy et al. 2007). PCD is a survival mechanism that occurs in plants and can be driven by a genetically programmed signalling pathway. It has been widely studied in biotic and abiotic stresses (Coll et al. 2011; Petrov et al. 2015). However, PCD induced by freezing stress is not well understood. Although a previous study has shown that suspension cultures of winter wheat display features of PCD after freezing stress (Lyubushkina et al. 2014), there is no other evidence to confirm that freezing stress induces PCD in plants.

In this study, we found that WT *Arabidopsis* plants with cold acclimation showed phenotypes such as gradually turning yellow and increased staining area of cell death after freezing stress (Fig. 1A-1C), which are similar to those of senescence and other stress-induced PCD (Li et al. 2012; Coll et al. 2014). Freezing-induced PCD was further confirmed by detecting ion leakage, activation of caspase-3-like activity, and transcriptional activation of *SAG12* (Fig. 1D-1F), which are important hallmarks of PCD and senescence. Moreover, it was found that old leaves were particularly vulnerable to cell death compared to young leaves after freezing stress, and we hypothesise that this may be the biological significance of freezing-induced PCD in that the death of old leaves may protect young leaves from death or provide a material source for young leaves.

Cathepsin B is involved in freezing-induced PCD

Although there are no orthologues of caspases in plants, caspase-like activities have been detected in many plant PCD processes using synthetic caspase-specific fluorescent substrates (Van Durme and Nowack 2016; Sueldo and van der Hoorn 2017). In addition, the presence of these caspase-like activities

was found to be critical for the occurrence of PCD in plants in caspase inhibitor treatment experiments (Van Durme and Nowack 2016; Sueldo and van der Hoorn 2017), suggesting that there are proteases in plants that participate in the regulation of plant PCD. Therefore, the identification of proteases with caspase-like activity has long been a research focus in this field. Several plant proteases responsible for various caspase activities have been identified over the past decade. For example, VPE has been found to possess caspase-1 like activity (Hatsugai 2004), saspases possess caspase-1, -6, and – 8-like activities (Coffeen and Wolpert 2004), phytaspase possesses caspase-6 like activity (Chichkova et al. 2010), and the 20S proteasome β1 subunit PBA1 (Hatsugai et al. 2009) and cathepsin B (Ge et al. 2016) possess caspase-3-like activity. Application of the cathepsin B inhibitor CA074 reduced caspase-3-like activity by 30% in ER-stress-induced PCD, and the PBA1 inhibitor β-lactone reduced caspase-3-like activity by 60%, suggesting that both cathepsin B and PBA1 are responsible for the increased caspase-3-like activity during ER stress (Cai et al. 2017). In contrast to ER stress-induced PCD, applying β-lactone reduced caspase-like activity by only approximately 25%, whereas it reduced caspase-like activity by more than 70% in freezing-induced PCD (Fig. 2A). In addition, immunoblotting showed that the content of PBA1 in *Arabidopsis* leaves of the same quality did not change significantly before and after freezing stress, whereas the content of the proteins labelled by biotin-DEVD-FMK increased significantly after freezing stress. The sizes of the labelled proteins were consistent with those of the precursor (P), mature form 1 (M), and mature form 2 (m) of cathepsin B (Fig. 2B). Moreover, the transcriptional levels of *CathB2* and *CathB3* were significantly increased in *Arabidopsis* leaves after freezing stress; in particular, the transcriptional level of *CathB3* increased by more than 60 times, whereas the transcriptional level of PBA1 showed a slight increase after freezing stress (Fig. 2C-2F). Therefore, cathepsin B was mainly responsible for the increased caspase-3-like activity in freezing-induced PCD.

As an important member of PLCPs, cathepsin B has been found to be involved in many biological processes, such as senescence and immune response (McLellan et al. 2009) before it was identified to possess caspase-3-like activity. The triple mutant of cathepsin B, *cathb#62*, shows a significant delay in dark-induced senescence and a significant reduction in cell death induced by pathogens, UV-C, MV, and ER stresses (McLellan et al. 2009; Ge et al. 2016; Cai et al. 2017). However, there was no significant difference in the phenotype between *cathb#62* and WT *Arabidopsis* after freezing stress (Supplementary Fig. S1). A recent study on animals identified that there is a compensatory mechanism in the cathepsin family: combined deletion of cathepsin B and cathepsin S can lead to the compensatory expression of cathepsin Z (Akkari et al. 2016). In this study, the transcriptional levels of 12 genes of the cathepsin family in *Arabidopsis* were significantly activated in the WT and *cathb#62* mutants after freezing stress (Fig. 3). Interestingly, the expression levels of *SAG2*, *SAG12*, *CEP1*, *CEP2*, and *RD21A* in *the cathb#62* mutant were significantly higher than those in the WT, and these five genes belong to cathepsin L. *SAG2* and *SAG12* are plant senescence-associated genes (Díaz-Mendoza et al. 2014), especially *SAG12*, which is highly expressed during leaf senescence and is located in SAVs to degrade chloroplast proteins (Carrión et al. 2013). *CEP1* is involved in tapetal cell death during pollen development (Zhang et al. 2014) and the tracheary elements cell death during xylem development (Cheng et al. 2019; Han et al. 2019). *RD21A* is involved in immunity against the necrotrophic fungal pathogen *Botrytis cinerea* and

sphingolipid-induced cell death (Lampl et al. 2013; Thuleau et al. 2018). These studies have shown that these five genes play an important role in plant PCD and freezing-induced PCD. The double expression of these five cathepsin L genes in the *cathb#62* mutant indicates that plant cathepsin also has a compensatory mechanism. This may be the main reason why the *cathb#62* mutant displayed the same phenotype as the WT after freezing stress. Although knockdown of cathepsin B did not reduce freezing-induced PCD, cathepsin B was greatly induced after freezing stress and mainly provided increased caspase-3 like activity. Furthermore, the loss of cathepsin B induced the expression of five genes of cathepsin L, suggesting that cathepsin B is involved in freezing-induced PCD.

Cathepsin B degrades RbcL

Although cathepsin B is involved in PCD during plant development, biological stress, and abiotic stress (McLellan et al. 2009; Ge et al. 2016; Cai et al. 2017), its molecular function in PCD has not been reported. In this study, RbcL and FBA were identified as candidate substrates for cathepsin B, using an *in vitro* cell-free degradation assay (Fig. 4). We further demonstrated that RbcL is a substrate for cathepsin B using yeast two-hybrid, MST, and *in vitro* degradation assays (Fig. 5). The relationship between cathepsin B and FBA was not further confirmed in this study, mainly because we were interested in the degradation of RbcL. Rubisco is the most abundant enzyme in plants, and the content of its large subunit, RbcL, accounts for more than 50% of the total protein in *Arabidopsis* leaves according to SDS-PAGE (Fig. 4A). Rubisco is not only an important source of nitrogen that is mobilised to young organs and developing seeds from senescent leaf (Irving and Robinson 2006), but also a source of carbohydrates for respiration under environmental stresses that reduce photosynthesis (VA 1978). However, the mechanisms underlying Rubisco degradation remain largely unclear. The DNA-binding protease, CND41, is the first protease found to be involved in the degradation of Rubisco during leaf senescence induced by nitrogen-depletion experiments in tobacco (Kato et al. 2004). CND41 mainly degrades denatured Rubisco in acidic environments; however, it is unclear how CND41 degrades Rubisco in chloroplasts in acidic environments. Recently, it was suggested that the chloroplast Clp protease may be involved in Rubisco degradation (Majeran et al. 2019), which was only found in mutants of the catalytic subunit of the Clp protease ClpP without direct evidence of *in vitro* degradation. Have *et al.* found through proteomics that some PLCPs may be involved in the degradation of RbcL in the autophagy mutant *atg5* (Havé et al. 2017), but there is a lack of direct evidence. In this study, we also found that the content of Rubisco small subunit RbcS and other proteins was not dependent on caspase-3 activity (Fig. 4), which suggests that cathepsin B has substrate specificity and that different proteases are responsible for the degradation of Rubisco subunits in plants.

Declarations

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Author contributions

HZ and L-ZA conceived the project. GY, TC, T-TF, X-YL, Y-QC and W-CD performed the experiments. GY analysed the data and wrote the manuscript. HZ, and L-ZA examined the data and manuscript.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information fles).

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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Figures

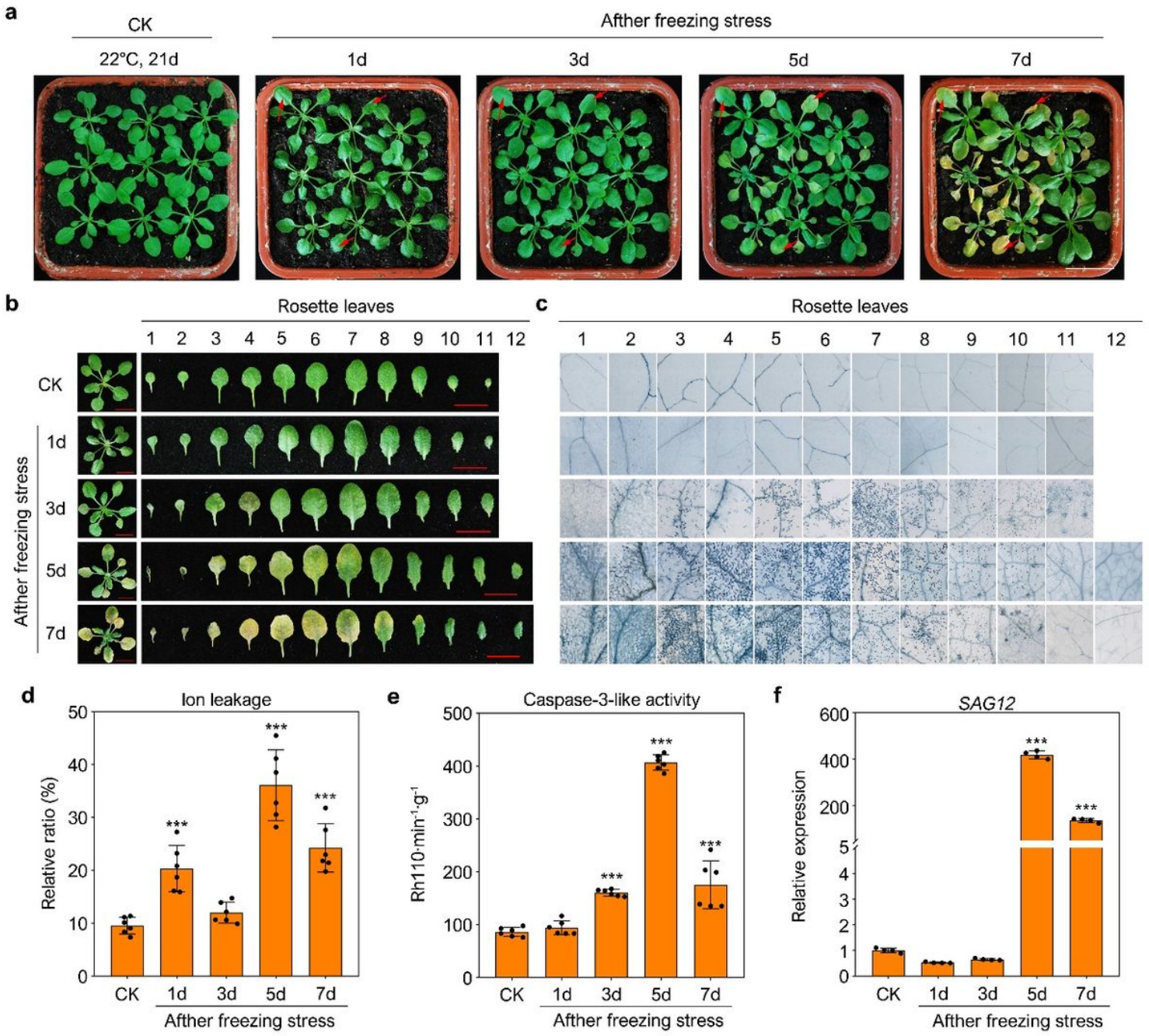


Figure 1

Freezing stress induces PCD in cold acclimated *Arabidopsis* plants.(**a**) The phenotypes of wild-type *Arabidopsis* plants recovered for 7 days after freezing stress. Twenty-one days old plants of wild-type *Arabidopsis* were placed in an incubator and grown for 3 days at 4 ºC for cold acclimation. The wild-type *Arabidopsis* were then exposed to a freezing incubator that was programmed to drop temperature by 1 ºC per hour from 0 ºC to -10 ºC, and treatment was continued for 6 h. All plants were recovered in the dark at 4 ºC for 12 h, and all plants were transferred to the growth house at 22 ºC (16 h light and 8 h dark cycle) for 7 days of recovery. Scale bar, 2 cm. (**b**) Phenotype of each leave of wild-type *Arabidopsis* before and after freezing stress. Scale bar, 1 cm. (**c**) Trypan blue staining of wild-type *Arabidopsis* leaves before and after freezing stress. Scale bar, 200 μm. (**d**) Ion leakage of wild-type *Arabidopsis* leaves before and after

freezing stress. The values are mean ± SD (***P < 0.001, one-way ANOVA, n = 6). (**e**) Caspase-3-like activity of wild-type *Arabidopsis* leaves before and after freezing stress. The values are mean ± SD (***P < 0.001, one-way ANOVA, n = 6). (**f**) Transcriptional levels of *SAG12*, a marker gene for senescence, in leaves of wild-type *Arabidopsis* before and after freezing stress. *UBC21*was used as a control gene, and the transcription level of *SAG12* in leaves at 22 ºC was set to 1 as the CK. The values are mean ± SD (***P < 0.001, one-way ANOVA, n = 4). All experiments were performed as three independent replicates with similar results.

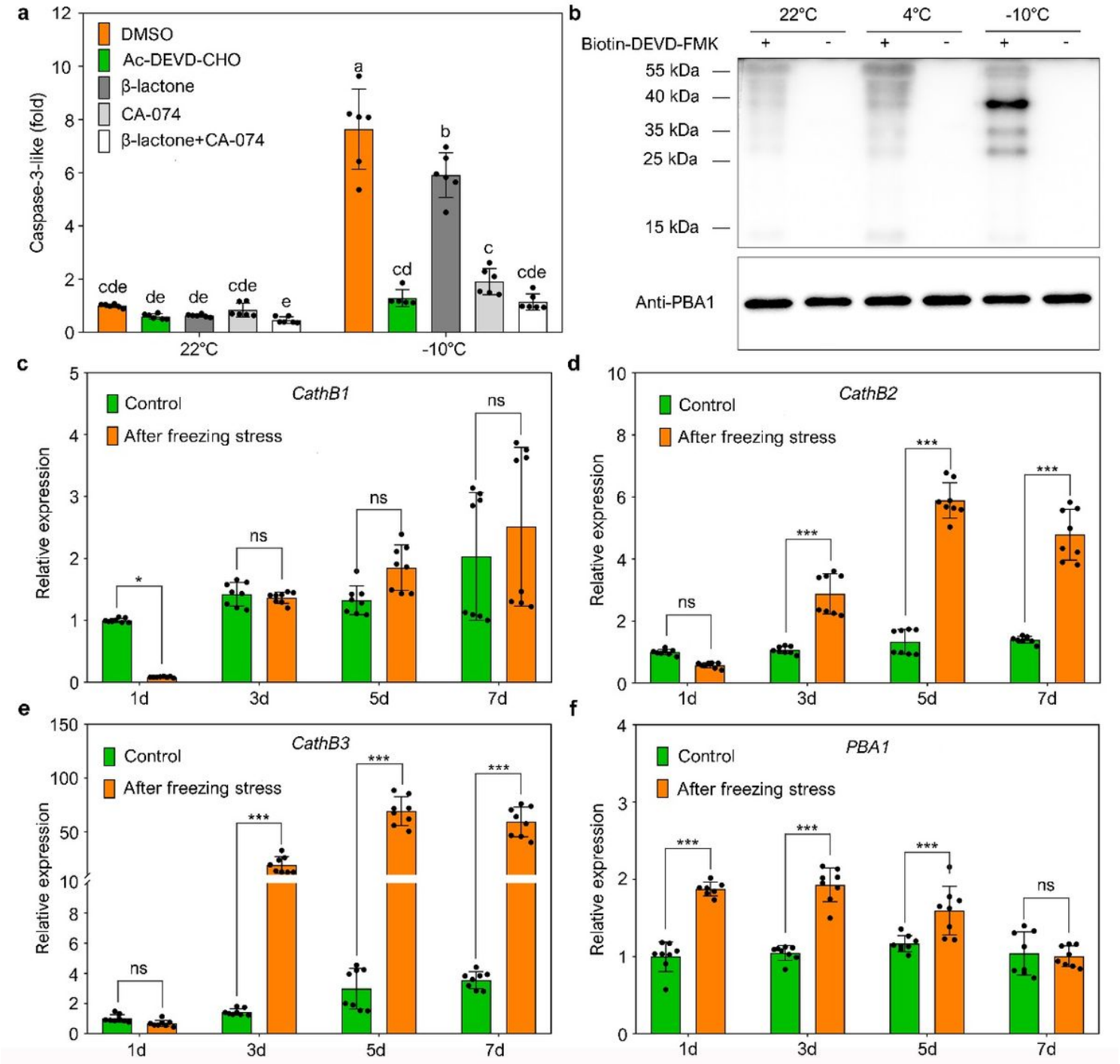


Figure 2

Cathepsin B contributes to increased caspase-3-like activityduring freezing-induced PCD. (**a**) Caspase-3 activities after treatment with inhibitors. Total proteins were extracted from *Arabidopsis* leaves grown at 22 ºC or recovered for 5 days after freezing stress. DMSO (control), caspase-3 general inhibitor AC-DEVD-CHO, PBA1 inhibitor β-lactone, cathepsin B inhibitor CA-074, and both β-lactone and CA-074 were added to the total protein. The samples were incubated in water for 30 min, and caspase-3-like activity was detected. The values are means ± SD (different letters indicate significant difference among samples at 0.05 level, one-way ANOVA, n = 6). (**b**) Immunoblotting was used to detect biotin-DEVD-FMK labeled proteins with caspase-3-like activity and PBA1 in *Arabidopsis* leaves before and after freezing stress. (**c**-**f**) Transcriptional levels of *CathB* and *PBA1* in *Arabidopsis* leaves before and after freezing stress. *UBC21* was used as a control gene, and the values are mean ± SD (***P < 0.001, two-way ANOVA, n = 8). All experiments were performed as three independent replicates with similar results.

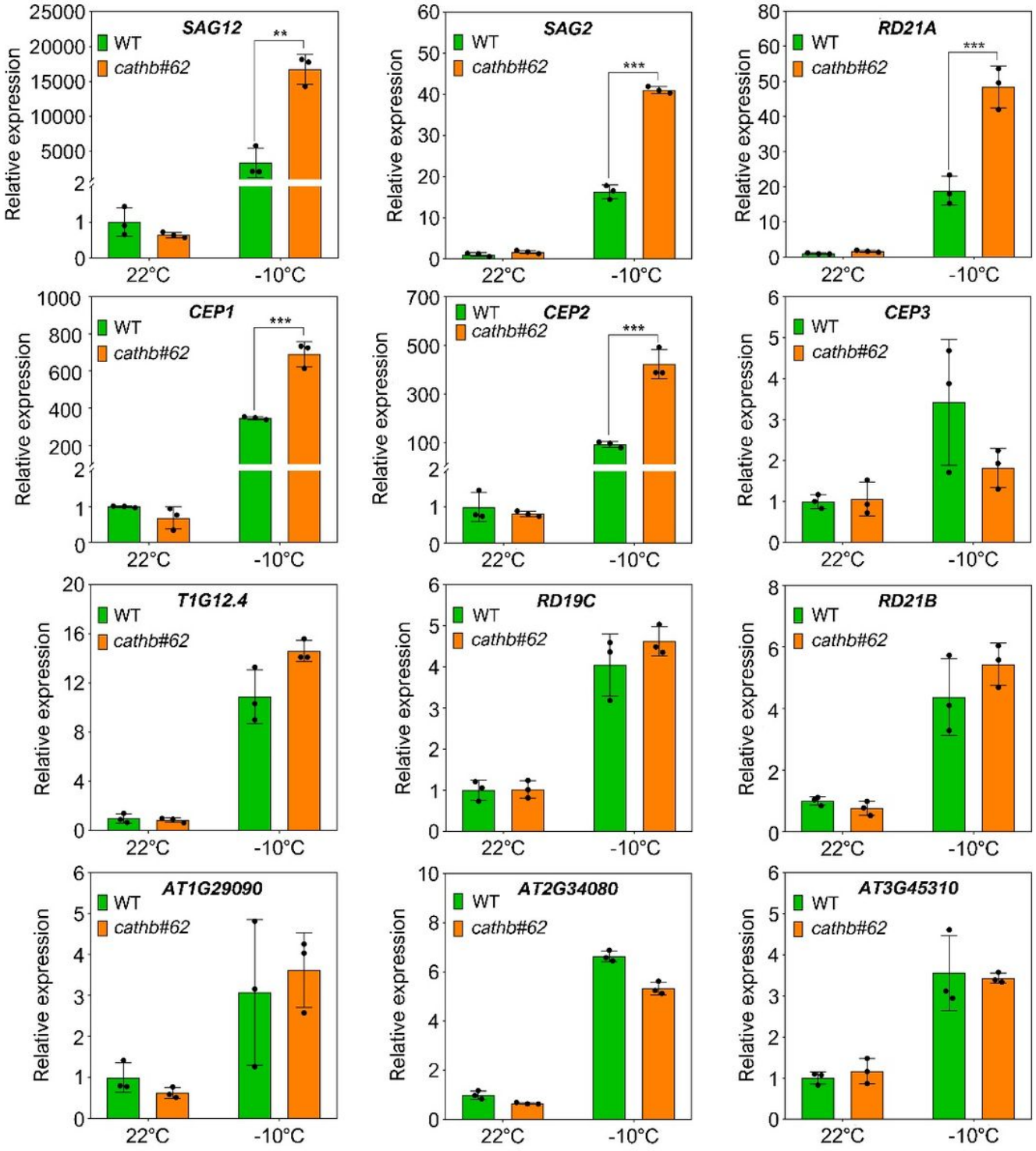
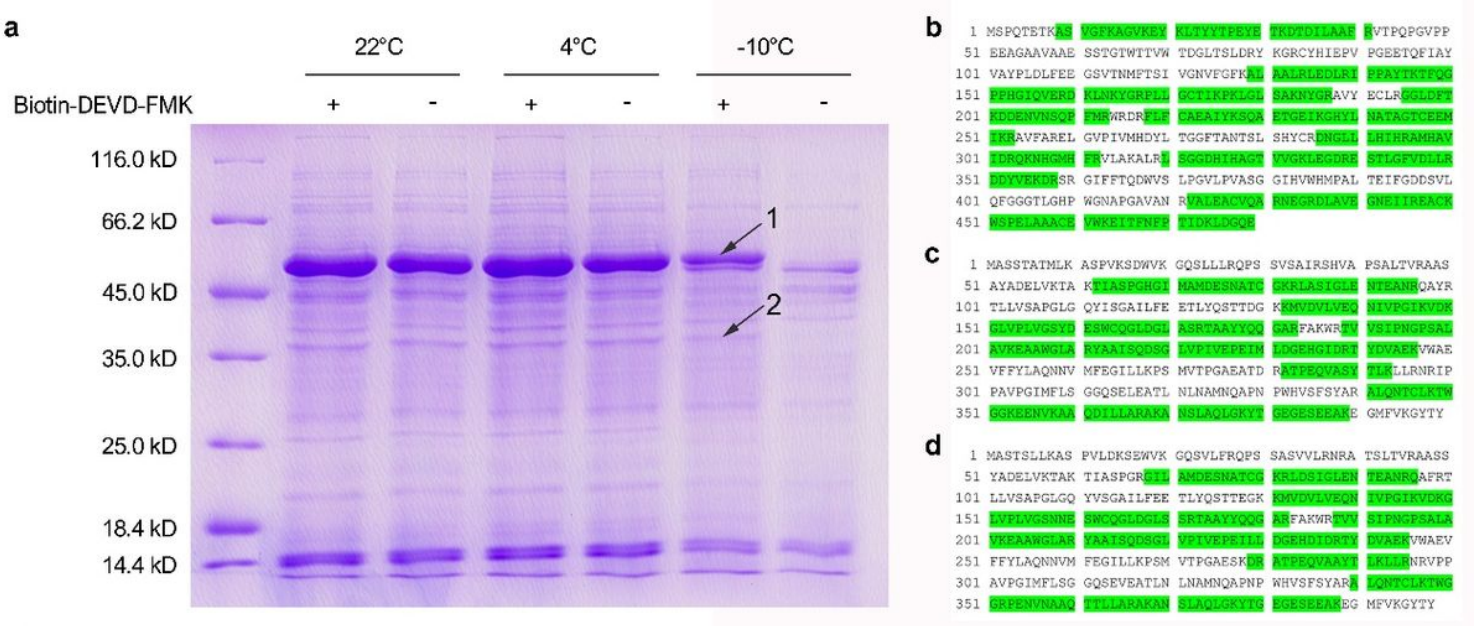


Figure 3

Transcriptional levels of papain-like Cys proteases family genes in wild-type and *cathb#62* triple mutants before and after freezing stress. Total RNAs were extracted from the leaves of wild-type and *cathb#62* triple mutant plants grown at 22 ºC for 3-4 weeks and recovered for 5 days after freezing stress. The transcriptional levels of genes were detected by RT-qPCR. *UBC21* was used as a control gene, and the transcription levels of genes in wild-type plant leaves grown at 22 ºC were set to 1. The values are means

± SD (**P < 0.01, ***P < 0.001, t test, n = 3). All experiments were performed as three independent replicates with similar results.



e

Number	Accession	Protein	Score	Coverage	Peptides	PSMs	UP	AAs	MW (kDa)	Calc. pI
1	O03042	RbcL	285.22	56.58	42	738	7	479	52.9	6.29
2	Q9SJU4	FBA1	219.88	51.63	24	183	10	399	42.9	6.58
2	Q944G9	FBA2	205.432	51.26	25	173	12	398	43	7.24

Figure 4

Identification of substrates for cathepsin B. (**a**) SDS-PAGE gel electrophoresis was used to detect proteins that were degraded dependent on caspase-3 activity. Total proteins were extracted from leaves of wild-type *Arabidopsis* plant grown at 22 °C for 3-4 weeks, cold acclimated at 4 °C for 3 days, and recovered for 5 days after -10 °C treatment. Total proteins were then incubated at 37 °Cfor 30 min with or without the caspase-3 inhibitor biotin-DEVD-FMK, and were detected by SDS-PAGE gel electrophoresis after incubation. The protein bands numbered 1 and 2 indicated by the arrow are proteins for caspase-3 activity-dependent degradation. (**b-d**) Amino acid sequences of Rbcl, FBA1, and FBA2. The sequences with green background are peptides identified by mass spectrometry in gel bands numbered 1 and 2. (**e**) Results of LC-MS/MS analysis. PSMs indicate peptide-spectrum matches, UP indicates unique peptides, AAs indicate the number of amino acids of the protein, MW indicates the molecular weight, and Calc.pI indicates the isoelectric point.

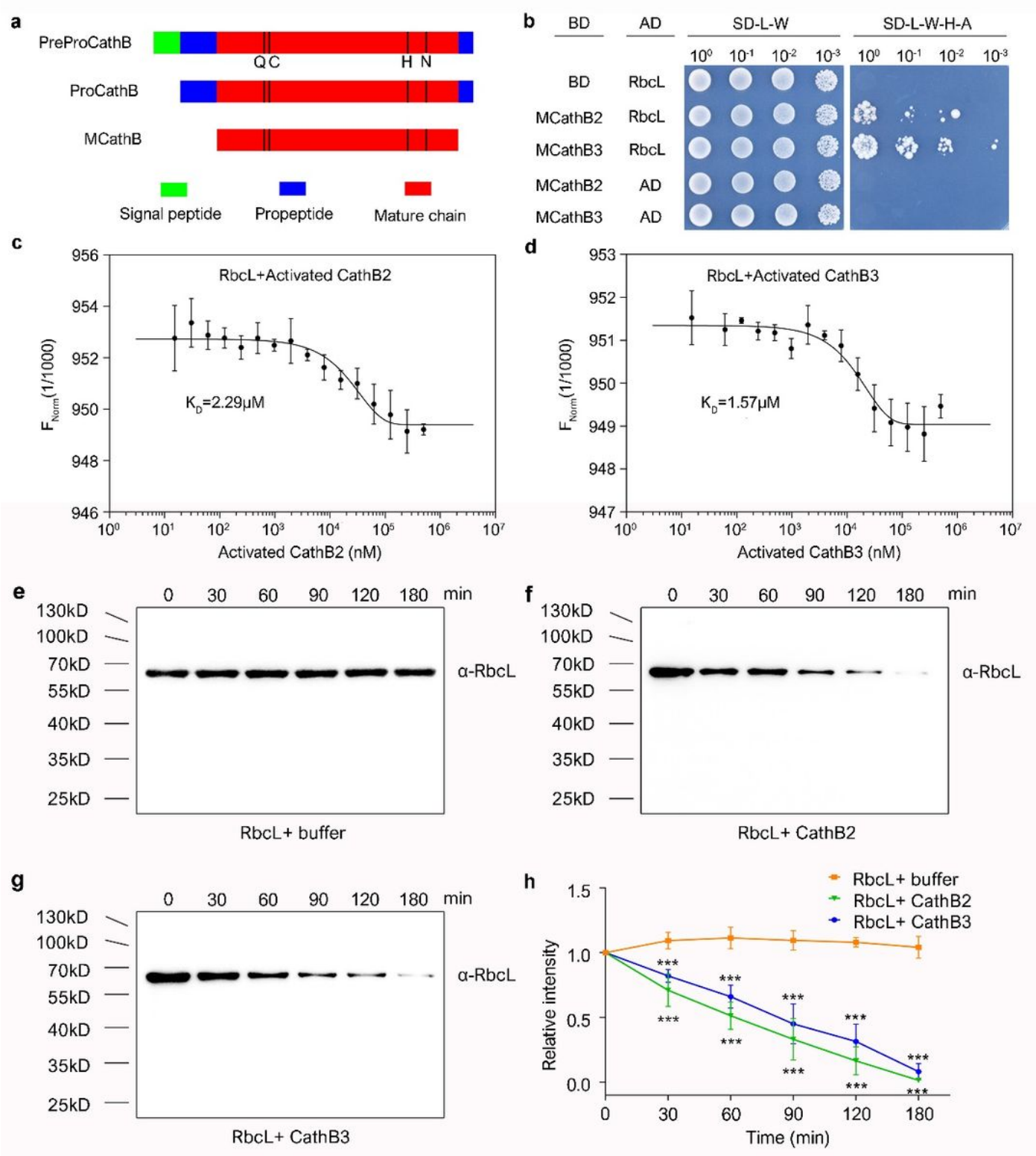


Figure 5

RbcL is a substrate of cathepsin B. (**a**) Diagram of prepro-, pro, and mature cathepsin B. Q, C, H, and N are catalytic positions. (**b**) Interaction between mature cathepsin B and RbcL in yeast. (**c**, **d**) MST binding curves for activated CathB2 and CathB3 titrated against RbcL. RbcL was labelled with a fluorescent kit and cathepsin B was used as ligand to test the affinity. Error bars show the RMSEs from a triplicate experiment. KD, dissociation constant. (**e**-**g**) Immunoblots analysis of RbcL degradation by cathepsin B.

ProCathB2 and ProCathB3 were auto-activated in the activation buffer for 30 min. RbcL was incubated at 37°C with enzyme-free protein buffer, activated CathB2, and activated CathB3 for 0, 30, 60, 90, 120, and 180 min. All samples were boiled for 10 min with protein loading buffer for immunoblot analysis wtih anti-RbcL primary antibody. (**h**) Statistical analyses of the changes in RbcL at the indicated time in above reactions. The values are mean ± SD (***P < 0.001, two-way ANOVA, n = 9). experiments were performed as three independent replicates with similar results.

Supplementary Files

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