Dehydrin MtCAS31 promotes autophagic degradation under drought stress

Xin Li**, Qianwen Liu*, Hao Feng†, Jie Deng‡, Rongxue Zhang*, Jiangqi Wen*, Jiangli Dong*, and Tao Wang*

*State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, China; †Plant Biology Division, Samuel Roberts Noble Research Institute, Ardmore, OK, USA

ABSTRACT

Drought stress seriously affects crop yield, and the mechanism underlying plant resistance to drought stress via macroautophagy/autophagy is not clear. Here, we show that a dehydrin, *Medicago truncatula* MtCAS31 (cold acclimation-specific 31), a positive regulator of drought response, plays a key role in autophagic degradation. A GFP cleavage assay and treatment with an autophagy-specific inhibitor indicated that MtCAS31 participates in the autophagic degradation pathway and that overexpressing MtCAS31 promotes autophagy under drought stress. Furthermore, we discovered that MtCAS31 interacts with the autophagy-related protein ATG8a in the AIL-like motif YXXXI, supporting its function in autophagic degradation. In addition, we identified a cargo protein of MtCAS31, the aquaporin MtPIP2;7, by screening an *M. truncatula* cDNA library. We found that MtPIP2;7 functions as a negative regulator of drought response. Under drought stress, MtCAS31 facilitated the autophagic degradation of MtPIP2;7 and reduced root hydraulic conductivity, thus reducing water loss and improving drought tolerance. Taken together, our results reveal a novel function of dehydrins in promoting the autophagic degradation of proteins, which extends our knowledge of the function of dehydrins.

**Abbreviations:** AIM: ATG8-interacting motif; ATG: autophagy-related; ATI1: ATG8-interacting protein 1; BiFC: Biomolecular fluorescence complementation; CAS31: cold acclimation-specific 31; ConCA: concanamycin A; DSK2: dominant suppressor of KAR2; ER: endoplasmic reticulum; ERAD: ER-associated degradation; NBR1: next to BRCA1 gene 1; PM: plasma membrane; PIPs: plasma membrane intrinsic proteins; TALEN: transcription activator-like effector nuclease; TSPO: tryptophan-rich sensory protein/translocator; UPR: unfolded protein response; VC: vector control

Introduction

The development of plants is deeply affected by abiotic stresses, such as salt, drought and cold stress. As a type of environmental stress, drought stress negatively affects the growth and development of plants [1–3]. To minimize the effects of stresses, plants have developed a protein quality control system to maintain homeostasis. When subjected to stresses, plants respond by protein folding and processing [4–6]. Misfolded proteins and protein aggregates accumulate in the endoplasmic reticulum (ER) lumen, a condition known as ER stress. To maintain ER homeostasis, plants exhibit an elaborate protein quality control system that includes the unfolded protein response (UPR), ER-associated degradation (ERAD), and autophagy [7,8]. UPR caused by the accumulation of aggregated misfolded proteins was shown to activate ERAD and autophagy, the 2 main degradation pathways [9]. A recent study showed that autophagy can efficiently degrade protein aggregates and proteins that are not efficiently processed by the canonical ERAD machinery due to their structure [7]. Studies have also shown that chaperones, such as Bip, participate in the UPR and ERAD pathways by regulating the endoplasmic reticulum stress (ERS) sensor [10].

Autophagy, a process that delivers proteins and organelles to lysosomes for degradation and recycling, functions in many biological processes, such as pollen germination [11], nutrient recycling [12] and abiotic and biotic stress in plants. Over 30 ATG (autophagy-related) genes, which are activated by nutrient starvation and inhibited by MTOR (mechanistic target of rapamycin) signaling, have been demonstrated to participate in autophagy [13]. In the past, autophagy was thought to be a housekeeping mechanism, but recent data have shown that autophagy is an adaptive process that is involved in biotic and abiotic stress adaptation [14,15]. The autophagic degradation pathway acts in a selective manner in which ATG8s (autophagy-related 8) play critical roles [16,17]. Nine ATG8 isoforms have been identified in *Arabidopsis thaliana*, each of which can be used as an indicator of the autophagosome [18–20]. In selective autophagic degradation, proteins targeted for degradation are recognized by a cargo receptor that interacts with ATG8 and are then degraded through autophagy [21–23]. The cargo receptor ATI1 (ATG8-interacting protein 1) recognizes chloroplast proteins and interacts with ATG8f, which mediates the autophagic degradation of chloroplast proteins [24]. TSPO (tryptophan-rich sensory protein/translocator) functions as a cargo receptor that...
mediates the autophagic degradation of PIP2 (plasma membrane intrinsic protein 2) in *Arabidopsis* under stress conditions [17]. NBR1 (next to BRCA1 gene 1) functions as a cargo receptor and interacts with ATG8 to mediate protein degradation [23]. Exploring the new cargo receptor and cargo under drought stress is a significant research project.

Dehydrins are classified as group 2 LEA (late embryogenesis abundant) proteins and are widely distributed in many plant species [25,26]. The accumulation of dehydrins improves abiotic stress tolerance [27,28]. Because they exhibit both hydrophilic and hydrophobic characteristics, dehydrins lack a stable secondary structure [26,29] and easily bind to biomolecules, such as DNA, RNA, proteins, ions and membrane components [25,26]. Previous studies have suggested that dehydrins exhibit various types of potential biochemical activities, including ion isolation, membrane stabilization, and chaperone activities [30,31]. In addition, dehydrins protect their target proteins from degradation under abiotic stress in vitro [29]. The in vivo mechanism of dehydrins in the drought response was first characterized in 2012 [27]. Heterologous expression of the *Medicago truncatula* dehydrin MtCAS31, a Y_{2}K_{4}-type dehydrin, in *Arabidopsis* decreases stoma density and improves drought tolerance [27]. In addition, MtCAS31 was shown to protect the leghemoglobin MtLb120-1 under drought stress to aid symbiotic nitrogen fixation in *M. truncatula* [32]. However, the function of MtCAS31 in protein quality control under severe drought stress has not been reported. Further studies addressing the functions of dehydrins in protein quality control under drought stress are needed.

In this study, we determined that a dehydrin, MtCAS31, functions as a positive regulator of drought stress in *Medicago*. MtCAS31 participates in drought-induced autophagic degradation as a cargo receptor. We further identified a cargo protein of MtCAS31, aquaporin MtPIP2;7. This study is the first to show that dehydrin plays an essential role in the autophagic degradation pathway, which enriches our understanding of the function of dehydrins in protein quality control.

**Results**

**MtCAS31 is expressed in the water transport system**

In a previous study by our group, heterologous expression of MtCAS31 are-GUS in *A. thaliana* and *Nicotiana benthamiana* revealed that MtCAS31 was expressed in stomata and vascular bundles [27]. To characterize the expression patterns of MtCAS31 in *M. truncatula*, we generated transgenic *M. truncatula* plants expressing MtCAS31 are-GUS construct. β-glucuronidase (GUS) staining was detected in vascular bundles and guard cells (Figure 1A), which was the same as that in *Arabidopsis*.

To determine the cellular localization of MtCAS31, cellular fractionation of *N. benthamiana* leaf cells transiently expressing the CaMV35S-MtCAS31-FLAG protein was performed. The leaf protein extract was immunoblotted with an anti-FLAG antibody to explore the localization of MtCAS31 as well as with anti-cFBPase (cytosolic fraction marker), anti-H^{+}-ATPase (membrane marker), and anti-Histone H3 (nuclear marker). Immunoblot analysis showed that MtCAS31 was detected in the membranes (a mixture of ER, tonoplasts, plasma membrane [PM] fragments and Golgi) as well as in nuclear and soluble cytoplasmic fractions (Figure 1B).

To further verify these results, we co-expressed CaMV35S: MtCAS31-GFP and CaMV35S:HDEL-RFP in *Arabidopsis* protoplasts. Fluorescence signals were detected using confocal laser scanning microscopy. The green fluorescence signals showed colocalization with red fluorescence signals, indicating that MtCAS31 was localized at the ER lumen (Figure 1C, i). Additionally, MtCAS31 partly colocalized with GmMAN1, an ER- and Golgi-localized protein (Figure 1C, ii), and MtCAS31-GFP colocalized with DAPI (Figure 1C, iii), which was used for nuclear staining. These results suggested that the vast majority of MtCAS31 localized to the ER and nucleus.

**MtCAS31 is a positive regulator of the drought response in *M. truncatula***

Dehydrin expression is generally considered a potential marker of drought stress [27,33]. Dehydrins are mainly described as cryo- and dehydro-proteostants in vitro under stress conditions [34]. For further study, qRT-PCR was used to explore the expression pattern of MtCAS31 in different tissues. *Actin* (Medtr9g095530) was employed as the reference [35,36]. The highest MtCAS31 expression was detected in the roots (45-fold compared to leaves and 50-fold compared to stems) (Figure 2A). Next, 2-week-old seedlings were treated with PEG 8000 at different concentrations to simulate moderate (30%, w:v) and severe (50%, w:v) dehydration. The expression of MtCAS31 was highly induced in both the roots (Figure 2B, i) and aerial parts (leaves and stems) (Figure 2B, ii).

In addition to drought stress, MtCAS31 was induced by high salinity (150 mM NaCl) (Fig. S1A). Under chilled (4°C) conditions, the expression level of MtCAS31 was elevated in the roots, but no obvious change was observed in the aerial parts (Fig. S1B). Because the effect of chilling on MtCAS31 expression was weak, the treatment time was extended to 72 h. The expression of MtCAS31 was also induced by abscisic acid (ABA) in both the roots and aerial parts (Fig. S1C).

To explore the function of MtCAS31 in *M. truncatula*, leaf disk transformation mediated by *Agrobacterium tumefaciens* EHA105 was performed to generate MtCAS31-overexpressing (MtCAS31OE) transgenic plants, in which expression was driven by the CaMV35S promoter. *M. truncatula* R108 was used as the receptor material. Eleven T1 transgenic MtCAS31OE lines were obtained. Three independent transgenic lines, MtCAS31OE-1, MtCAS31OE-8 and MtCAS31OE-9 (Fig. S2A), were used in further experiments. Immunoblot analysis with an anti-FLAG antibody showed that MtCAS31 was expressed at the protein level in MtCAS31OE plants (Fig. S2B). A Tnt1 retrotransposon insertion mutant of MtCAS31, NF5714, was also employed for analysis. The Tnt1 retrotransposon insertion was located in the third exon of MtCAS31 in NF5714 [32]. We also generated MtCAS31-knockout mutant plants using transcription activator-like effector nuclease (TALEN) technology. T1 homozygotes of cas31-27, cas31-34 and cas31-51 were used for further studies. Immunoblot analysis showed that MtCAS31 was not detectable in any of the mutants [32].
To assess the performance of the MtCAS31 OE and cas31 mutants under water-deficient conditions, 2-week-old seedlings were exposed to drought stress by withholding water for 7 d and then rewatered (i.e., a drought-rewatering cycle). Survival rates were scored after 3 drought-rewatering cycles, and the survival rates of the mutants (21.6% in cas31-27, 18.3% in cas31-34 and 23.3% in cas31-51) were significantly lower than that of wild-type seedlings (48.3%) (Figure 2C, i and ii). The survival rate of NF5714 (28.3%) was significantly lower than that of the wild-type plants (51.6%) (Figure 2D, i and ii). Consistent with this finding, MTCAS31OE plants showed lower stoma conductivity than VC plants. These results indicate that MtCAS31 functions as a positive regulator of the drought response in M. truncatula.

MtCAS31 participates in autophagy under drought stress

To explore the function of MtCAS31 under consistent drought stress, M. truncatula R108 was subjected to dehydration (30% PEG 8000) to simulate drought stress. Immunoblotting analysis with anti-MtCAS31 was employed to detect the protein level of MtCAS31 roots under drought stress. MtCAS31 was barely detectable in the absence of stress (0 h). After 2 h of dehydration, MtCAS31 could be detected; its expression peaked at 4 h of dehydration and was thereafter highly reduced (Figure 3A). The expression level of MtCAS31 mRNA was continuously upregulated from 0 h to 8 h under 30% PEG 8000 dehydration (Figure 2B, i), but MtCAS31 protein expression was significantly downregulated from 4 h to 8 h (Figure 3A), suggesting that MtCAS31 may be degraded from 4 h to 8 h.

To examine whether MtCAS31 plays a role in drought-induced autophagic degradation, we generated stable transgenic plants mediated by Agrobacterium tumefaciens to
express MtCAS31\textsubscript{pro}\textsuperscript{-}\textit{MtCAS31-GFP}. Treatment with 30% PEG 8000 was applied to explore the change in MtCAS31-GFP under drought-induced autophagy. Concanamycin A (ConcA), a vacuolar (V)-type H\textsuperscript{+}-ATPase inhibitor that blocks hydrolase activity by increasing the vacuolar pH to block the autophagic degradation pathway, was added to Figure 2.

(A) Relative expression of MtCAS31 in leaves, stems and roots of \textit{M. truncatula} in the absence of stress. The values were normalized to Actin expression. The data represent the mean±SD of 3 replicates. (B) Using qRT-PCR, the relative expression of \textit{MtCAS31} under dehydration (30% and 50% PEG 8000 treatment, w:v) in roots (i) and aerial parts (ii) was determined at the indicated time points. The values were normalized to \textit{Actin} expression. The data represent the mean±SD of 3 replicates. (C) Phenotypes of \textit{cas31} mutant and wild-type plants after 3 drought-rewatering cycles (i). The survival rate was scored (ii), and relative electrolyte leakage was calculated (iii). The data represent the mean±SD of 2 replicates with 15 plants each. Asterisks indicate statistically significant differences between the wild-type and \textit{cas31} mutant plants. *P < 0.05, **P < 0.01, Student’s t-test. (D) After 3 drought-rewatering cycles, photographs of the NF5714 and wild-type phenotypes were collected (i). The survival rate (ii) and relative electrolyte leakage (iii) were calculated. NF5714, the Tnt1-insertion mutant of \textit{MtCAS31}. The data represent the mean±SD of 2 replicates with 15 plants each. Asterisks indicate statistically significant differences between wild-type and NF5714. *P < 0.05, **P < 0.01, Student’s t-test. (E) \textit{MtCAS31}\textsubscript{OE} and VC plants were exposed to drought stress, and the phenotype (i), survival rate (ii) and relative electrolyte leakage (iii) were scored. VC, vector control. The data represent the mean±SD of 2 replicates with 15 plants each. Asterisks indicate statistically significant differences between wild-type and \textit{MtCAS31}\textsubscript{OE} plants. *P < 0.05, **P < 0.01, Student’s t-test. (F) Stoma conductivity was measured with a photosynthesis system (LI-6400TX). (i) Wild-type, \textit{cas31} mutant, (ii) VC and \textit{MtCAS31}\textsubscript{OE} plants after 3 drought-rewatering cycles. The data represent the mean±SD of 2 replicates with 15 plants each. Asterisks indicate statistically significant differences. *P < 0.05, **P < 0.01, Student’s t-test. (G) The transpiration rates of wild-type, \textit{cas31} mutant, VC and \textit{MtCAS31}\textsubscript{OE} plants under 3 drought-rewatering cycles were determined with a photosynthesis system (LI-6400TX). (i) Wild-type and \textit{cas31} mutant. (ii) VC and \textit{MtCAS31}\textsubscript{OE}. The data represent the mean±SD of 2 replicates with 15 plants each. Asterisks indicate statistically significant differences. *P < 0.05, **P < 0.01, Student’s t-test.
block autophagy for 4 h in the middle of the 8-h dehydration treatment in all of the following experiments. GFP fluorescence was detected by confocal microscopy. Little GFP fluorescence was detected in the absence of stress (dehydration for 0 h) (i), under dehydration treatment for 2 h (ii), under dehydration treatment for 4 h (iii), under dehydration treatment for 8 h (iv) and a under a combined 4 h ConcA with 8-h dehydration treatment (v). Bar: 35 μm. No fluorescence signal was detected in the absence of stress, probably because MtCAS31-GFP was driven by the MtCAS31 native promoter, which is induced by dehydration. Consistent with the results shown in Figure 3A. When ConcA (1 μM) was added to block autophagy for 4 h in the middle of the 8-h treatment, punctate GFP fluorescence accumulated (Figure 3B, v), suggesting that MtCAS31 is degraded via the autophagy pathway.

A GFP cleavage assay was also performed to verify the autophagic degradation of MtCAS31. MtCAS31 pro: MtCAS31-GFP transgenic plants were treated with dehydration at different time points, as shown in Figure 3B. Immunoblotting with anti-GFP was used to detect the cleavage of MtCAS31-GFP. After 2 h of dehydration, MtCAS31-GFP was detectable and accumulated...
more at 4 h. At 8 h of treatment, MtCAS31-GFP was decreased (Figure 3C, up lane). However, free GFP accumulated as the dehydration progressed (Figure 3C, down lane). After combining dehydration with ConA treatment, the ratio of free GFP to MtCAS31-GFP was largely reduced (Figure 3C), indicating that MtCAS31 was degraded via the autophagic degradation pathway.

To further investigate the function of MtCAS31 in autophagic degradation, MtATG8f, an autophagy indicator in plants, was fused with GFP and transformed into wild-type, cas31 mutant, and MtCAS31OE plants mediated by Agrobacterium rhizogenes. After a combination treatment with dehydration and ConA, punctate GFP fluorescence was significantly reduced in the cas31 mutant and more accumulated in MtCAS31OE compared to that in wild-type (Figure 3D iv and Figure 3E). After treatment with only ConA, there was no significant difference in the number of punctate GFP in these materials, which was regarded as the control (Figure 3D i-iii and Figure 3E). To confirm the effect of MtCAS31 on the autophagic degradation pathway, a GFP cleavage assay was performed. NPTII was used as the reference, which was expressed in the same vector as GFP-MtATG8f but from a different expression cassette. When treated with dehydration, the ratio of free GFP to GFP-ATG8f was increased in MtCAS31OE and reduced in cas31 (Figure 3F), indicating that MtCAS31 functions during drought-induced autophagy pathway, and it can be degraded by autophagy. In the absence of stress, no significant difference in these materials was observed, but free GFP was detected, which may have resulted from A. rhizogenes growth with these plants at all times.

**MtCAS31 specifically interacts with the autophagy-related protein 8a**

Because dehydrins are intrinsically disordered proteins and easily bind with other biological molecules, we investigated whether CAS31 is functional as a cargo receptor in the autophagy pathway. Generally, ATG8-interacting proteins contain the ATG8-interacting motif (AIM; W/F/YX,L/I,V). Hence, we analyzed the sequence of MtCAS31 and found 2 AIM-like motifs (YGNPL22, YGNP135) (Figure 4B). We examined the interaction between MtCAS31 and ATG8 proteins. MtCAS1 interacted with MtATG8a, as determined by a yeast two-hybrid assay and a bimolecular fluorescence complementation (BiFC) assay (Figures 4A and D), but not with other ATG8 isoforms (Fig. S3A). We replaced YGNPL22 with AGNPA and found that ATG8a could still interact with MtCAS1 (Fig. S3C). However, when we replaced YGNPL35 with AGNPA, MtCAS31 could no longer interact with ATG8a, as determined by yeast two-hybrid and BiFC assays (Figures 4C and D). Furthermore, when YGNPL35 was replaced with AGNPA35, MtCAS31 could not interact with MtATG8a in the coimmunoprecipitation (Co-IP) assay (Figure 4E). These results suggested that MtCAS31 specifically interacts with ATG8a in the AIM-like motif YGNP135.

**The aquaporin MtPIP2;7 interacts with MtCAS31 and plays a negative role in the drought response**

To identify MtCAS31 cargo, we used MtCAS31 as the bait to screen an M. truncatula cDNA library. We identified 35 proteins, including metabolic enzymes, abiotic stress response proteins, and other proteins (Table S1). Among these proteins, we were particularly interested in the plant aquaporin MtPIP2;7 (Medtr2g094270). In phylogenetic analyses, MtPIP2;7 clustered with a group of proteins belonging to the aquaporin PIP2 subfamily (Fig. S4), which has previously been reported to participate in abiotic stress [37–39].

To further confirm the interaction between MtCAS31 and MtPIP2;7, we first employed a split-ubiquitin system yeast two-hybrid (SuY2H) assay. When co-transformed with NubG-MtCAS31 and MtPIP2;7-Cub, the yeast strain Saccharomyces cerevisiae NY52 grew in synthetic dropout (SD) medium (-Trp/-Leu/Ade/-His), and the reporter gene LacZ was activated (Figure 5A). A GST affinity-isolation assay was also performed to confirm the interaction between MtCAS31 and MtPIP2;7 (Figure 5B). This result was further examined in the plants with a BiFC assay. Full-length MtCAS31 was fused with the N terminus of YFP (MtCAS31-YFP[N]), and MtPIP2;7 was fused with the C terminus of YFP (MtPIP2;7-YFP[C]). These constructs were co-transformed into Arabidopsis protoplasts via PEG-mediated transformation. HDEL was fused with RFP and co-transformed into Arabidopsis protoplasts. GFP fluorescence colocalized with HDEL-RFP in protoplasts co-transformed with MtCAS31-YFP[N] and MtPIP2;7-YFP[C], whereas no green fluorescence was detected in the negative controls (Figure 5C), indicating that MtCAS31 interacted with MtPIP2;7 in the ER. Co-IP was also performed to verify the results. MtCAS31-FLAG and MtPIP2;7-MYC were co-expressed in Nicotiana benthamiana. Protein was immunoprecipitated with anti-FLAG beads and detected with anti-MYC. MtCAS31 and MtPIP2;7 were coimmunoprecipitated (Figure 5D). These results indicate that MtCAS31 interacts with MtPIP2;7 both in vitro and in vivo.

To investigate the characteristics and function of MtPIP2;7, we analyzed the expression pattern of MtPIP2;7 in M. truncatula. The highest expression was detected in the roots by qRT-PCR (Figure 6A), which was consistent with a previous study in Arabidopsis [40]. Moreover, GUS activity was detected mainly in vascular bundles in the leaves (Figure 6B, i), stems (Figure 6B, ii) and roots (Figure 6B, iii) by GUS staining of MtPIP2;7pro::GUS transgenic M. truncatula. The examination of subcellular localization showed that MtPIP2;7 localized to both the ER and PM (Figure 6C). After dehydration (PEG 8000 treatment, 30% and 50%, w:v), the relative expression of MtPIP2;7 was not altered in aerial parts (Fig. 6Dii) but was substantially decreased in the roots (Fig. 6Dii). Under other abiotic stresses, such as high salinity and chilling (4°C), the relative expression of MtPIP2;7 was not obviously changed, and ABA also had no significant effect on MtPIP2;7 (Fig. S5). Many PIP2s have been reported to play roles in the drought response [41–45]. To understand the biological function of MtPIP2;7, we generated MtPIP2;7 knockout mutants using TALEN technology (Fig. S6A). Three independent T1-generation lines, pip2;7–59, pip2;7–69 and pip2;7–77, in which MtPIP2;7 was not detected by immunoblot analysis (Fig. S6B) were selected for further experiments. The water transduction ability of roots is reflected by the root hydraulic conductivity (Lp), which mainly relies on the content of PIP2s in the PM [46–48]. Lp was significantly lower in pip2;7 mutants than in wild-type plants, indicating that MtPIP2;7 contributes to...
water conduction and affects the hydraulic conductivity of roots (Figure 7A). To further understand the function of MtPIP2;7, wild-type and pip2;7 mutant plants were exposed to drought stress. After 3 drought-rewatering cycles, the survival rates of pip2;7 mutants (78.3%–59, 80.0%–69, and 70.0%–77) were significantly higher than that of wild-type plants (48.3%) (Figures 7B and C), and electrolyte leakage was significantly lower (Figure 7D), indicating that MtPIP2;7 plays a negative role in the drought response.

MtCAS31 facilitates the autophagic degradation of MtPIP2;7

We wanted to determine whether MtPIP2;7, a negative regulator of drought stress, is degraded by autophagy. We used immunoblotting analysis to detect the protein level of MtPIP2;7 in wild-type roots that were dehydrated for 8 h (30% PEG 8000), revealing reduced MtPIP2;7 expression after dehydration compared to that in the absence of stress (Figure 8A, i). However, the decrease in MtPIP2;7 was inhibited by ConcA (Figure 8A, ii), suggesting that MtPIP2;7 can be degraded via the autophagy pathway.

To explore whether MtPIP2;7 is a cargo protein in the MtCAS31-ATG8a-mediated autophagic degradation pathway, the autophagy inhibitors Conca (1 µM) and spautin-1 (1 µM) were used on the wild-type and cas31 mutant plants, respectively. Spautin-1 is a USP10 and USP13 (ubiquitin-specific peptidases) inhibitor that blocks the autophagic degradation pathway. After combining dehydration with ConcA or spautin-1 treatment, no significant difference in the MtPIP2;7 protein level was observed between the cas31 mutant and wild-type plants (Figure 8B). These results indicated that MtCAS31 affects MtPIP2;7 degradation via the autophagy pathway.
To further verify the above results, MtPIP2;7-GFP driven by the CaMV35S promoter was transformed into wild-type and cas31 mutant plants by hairy root transformation. GFP fluorescence was detected with confocal laser scanning microscopy. MtPIP2;7-GFP was mainly localized in the PM in both the wild-type and cas31 mutant plants without dehydration (Figure 8C, i and iv). After 8 h of dehydration, the GFP fluorescence in wild-type plants was mainly detected in vacuoles (V) (Figure 8C, ii), implying that MtPIP2;7 was degraded. However, some GFP fluorescence remained in the PM in the cas31 mutant (Figure 8C, v). After 8 h of dehydration and ConcA combination treatment, punctate GFP fluorescence was significantly increased in wild-type plants, while more MtPIP2;7 remained in the PM in cas31 mutants (Figure 8C, iii and vi, Figure 8D), indicating that MtCAS31 promoted MtPIP2;7 degradation via the autophagic degradation pathway.

Because high MtPIP2;7 expression indicates a higher Lp, we measured Lp in wild-type and cas31 mutant plants. Mutants of cas31 exhibited a significantly higher Lp than wild-type plants under dehydration, but no difference was observed in the absence of stress (Figure 8E). The phenotypic experiments proved that MtCAS31 promoted MtPIP2;7 degradation.

**MtCAS31 functions as a cargo receptor in drought-induced autophagic degradation**

We found that the dehydrin MtCAS31 specifically interacts with ATG8a in the AIM-like motif YGNP135 (Figure 4) and interacts with MtPIP2;7 to facilitate its autophagic degradation (Figure 5, 8). However, MtPIP2;7 could not interact with the ATG8 proteins directly (Fig. S7A). *M. truncatula* was subjected to Co-IP analysis to verify the interaction of MtCAS31-MtPIP2;7 using MtCAS1-FLAG and Medtr6g012980-FLAG transgenic *M. truncatula* plants. Medtr6g012980, which could not interact with ATG8 (Fig. S7B), was employed as the negative control. Total proteins were extracted from the leaf, which was co-transformed with the indicated constructs and incubated with FLAG beads to immunoprecipitate the target protein. Coprecipitated proteins were analyzed by immunoblotting using anti-FLAG and anti-MYC antibodies.
indicated that the MtPIP2;7-MtCAS31-MtATG8 complex exists in *M. truncatula* (Figure 9A).

Taken together, our results demonstrate that the dehydrin MtCAS31 participates in drought-induced autophagic degradation as a cargo receptor. In the absence of stress, MtPIP2;7 in roots was translocated to the PM to transport water from the soil to plant cells. Under drought stress, MtCAS31 was highly induced in the ER and interacted with the cargo protein MtPIP2;7 to facilitate autophagic degradation. These phenomena will result in less water loss under drought stress and thus improve the drought tolerance of *M. truncatula* (Figure 9B).

**Discussion**

**Dehydrins play roles in protein quality control under persistent drought stress**

The development of plants is substantially influenced by abiotic stresses, such as heat, salt and drought stress. Drought is the most common type of environmental stress and triggers the misfolding of proteins that are toxic to plants. Hence, protein quality control is critical for plant survival. To relieve stress in plants, protective proteins, such as chaperones and LEA (late embryogenesis abundant) proteins, prevent proteins from aggregating under mild and moderate stress [49]. In addition, protein degradation systems remove damaged proteins to protect plants under severe stress. Dehydrins have previously been considered molecular protectors that protect the proteins with which they interact, such as lactate dehydrogenase and lysozyme, from degradation or aggregation under abiotic stress [29,30]. We previously demonstrated that the dehydrin MtCAS31 interacts with ICE1 (Inducer of CBF expression 1) and affects stomatal development to improve drought tolerance [27]. Moreover, we found that MtCAS31 can protect the leghemoglobin MtLb120-1 under drought stress [32]. However, whether MtCAS31 plays a role in protein degradation and recycling remains unknown.

In this study, we defined a new dehydrin function, demonstrating that they play an important role in the autophagic...
degradation pathway under severe drought. During drought stress, MtCAS31 first accumulated and then became degraded (Figure 3A). A GFP cleavage assay and treatment with the ConcA indicated that MtCAS31 participates in the autophagy pathway and that overexpression of MtCAS31 promotes autophagy under drought stress (Figure 3B–F). Further experiments proved that MtCAS31 interacted with MtATG8a in the AIM-like motif YGNPI35, supporting its function in autophagic degradation (Figure 4). Because subcellular localization analysis of MtCAS31 showed that the vast majority of MtCAS31 localized to the ER and nucleus (Figure 1B, C) and previous studies showed that autophagosome membranes are derived from the ER or PM [13,50], we hypothesized that MtCAS31 from the ER participates in the autophagy pathway. The autophagic cargo receptors identified thus far in plants include AT11/2, NBR1/ Joka2, orosomucoid ORM1/2, DSK2, TSPO and ubiquitin receptor RPN10 [14,17,21,22,51]. Among these proteins, the first 5 all interact with ATG8 in the typical AIM motif except ubiquitin receptor RPN10, which interacts with ATG8 in the ubiquitin-interacting motif (UIM) motif [52]. Unlike these reported cargo receptors, MtCAS31 interacts with MtATG8a in the AIM-like motif YXXXI, which enriches our understanding of proteins that interact with ATG8 in different regions.

Studies have also shown that AT11 plays a dual role in the autophagy pathway under carbon starvation and salt stress and during senescence, as it localizes at ER-associated bodies or plastid bodies to transport plastid proteins to the vacuole [24]. NBR1 was shown to promote the selective autophagic degradation of ubiquitinated targets via interacting with AtATG8 [51]. Orosomucoid ORM1/2 can maintain the function of the plant immune system by interacting with FLS2 (flagellin-sensing 2) and ATG8 [53]. Under drought and starvation stress, DSK2 is phosphorylated by BIN2 (a GSK3-like kinase) to promote the DSK2-ATG8 interaction and then enhances BES1 (BRI1-EMS suppressor 1) autophagic degradation [14]. Ubiquitin receptor RPN10 also functions as a selective autophagy cargo receptor that facilitates the degradation of inactive 26S proteasomes by interacting with ATG8 in the UIM [21]. Under ABA treatment, TSPO is highly induced and interacts with AtPIP2;7 in the ER and Golgi. At the same time, TSPO acts as a cargo receptor and interacts with AtATG8 to promote AtPIP2;7 autophagic degradation. In our work, we found that MtCAS31 interacts with MtPIP2;7 and MtATG8 to form the MtPIP2;7-MtCAS31-MtATG8 complex in the ER and facilitate MtPIP2;7 degradation under drought stress. We also found that PIP2;7 has another cargo receptor in addition to TPSO, MtCAS31, that participates in the autophagy pathway, extending our knowledge of the relationship between cargo and cargo receptors.

We speculated that the functions of MtCAS31 under moderate and severe stress are different. Under mild and moderate drought stress, MtCAS31 is upregulated and accumulates [13] in the membrane, nucleus, and soluble cytoplasm. MtCAS31 functions as a molecular protector, preventing enzymes such as leghemoglobin from inactivation [29,32]. When stress becomes more severe, MtCAS31 promotes the autophagic degradation of proteins, such as aquaporin MtPIP2;7, to resist drought stress (Figure 8). According to the above hypothesis...
and observations, we speculated that dehydrins play roles in protein quality control under persistent drought stress.

Aquaporins can be degraded via different pathways under drought stress

Aquaporins, membrane proteins that belong to the MIP (major intrinsic protein) superfamily [54], play key roles in the drought response in plants. According to their structure and function, aquaporins are mainly divided into 5 groups: PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (NOD26-like intrinsic proteins), SIPs (small basic intrinsic proteins), and XIPs (X intrinsic proteins) [55]. Among these 5 groups, studies have primarily focused on PIPs, which include 2 subgroups: PIP1 and PIP2 [56,57]. PIP2 proteins play important roles in water transport in the...
PM [46–48] and have been reported to participate in abiotic stress responses [40,45,58,59]. Overexpression of PIP2s can improve water use efficiency and enhance drought tolerance in rice, soybean and barley [42,46,59]. In this study, we found that MtPIP2;7 was downregulated and degraded under drought stress, playing a negative role in the drought response, which is consistent with previously reported results [17,43,60].

In plants, aquaporin expression is regulated at both the transcript and protein levels under environmental stress. Salt stress induces the dephosphorylation and internalization of PIPs, and drought stress induces the ubiquitylation of PIPs [48], which are then degraded in the proteasome. The level of PIPs directly affects water transport activity. Under drought stress, AtPIP2;1 was found to be degraded by the 26S proteasome pathway [43]. AtPIP2;7 can be recognized by the stress-induced cargo receptor AtTSPO and is degraded via the autophagic degradation pathway [17]. According to the present study, both the transcript and protein levels of the aquaporin MtPIP2;7 are reduced under dehydration conditions (Figure 6D ii and 8A i). Our results suggest that MtCAS31 interacts with MtPIP2;7 and facilitates the autophagic degradation of MtPIP2;7 (Figure 5, 8). In the cas31 mutant, the autophagic degradation of MtPIP2;7 was reduced under drought stress (Figure 8), but MtPIP2;7 could not interact with MtATG8s (Fig. S7A), identifying MtCAS31 as a new cargo receptor of MtPIP2;7 in M. truncatula. Elucidation of whether MtCAS31, as a cargo receptor, promotes the autophagic degradation of other proteins requires further study.

In conclusion, the dehydrin MtCAS31 acts as a cargo receptor to form the MtPIP2;7-MtCAS31-MtATG8 complex, which facilitates MtPIP2;7 autophagic degradation, thus reducing water loss under drought stress and improving drought tolerance. This report is the first to demonstrate that a dehydrin is involved in the autophagic degradation pathway in the drought response and enriches our knowledge regarding the molecular mechanisms of dehydrins.

**Materials and methods**

**Plant materials**

MtCAS31OE, pip2;7-TALEN and MtCAS31pro-GUS transgenic plants were generated via the leaf disk transformation method [61].

The full-length MtCAS31 fused with 3x FLAG was inserted into pMDC32 vector to generate MtCAS31OE, and pMDC32 vector was modified from pCAMBIA1300 (Cambia) with double CaMV35S. The vector used to generate pip2;7-TALEN was modified from pCAMBIA1300 and driven by a double CaMV35S promoter. The vector employed to generate MtCAS31pro-GUS was pCAMBIA1381 (Cambia). Transgenic plants were obtained through Agrobacterium-mediated transformation of M. truncatula R108 (UMR 1097, INRA, Montpellier, France). The plants were grown at room temperature after vernalization at 4°C for 16 h. Thereafter, the plants were grown in a 24°C chamber under 16-h light (light intensity of 200 μmol m⁻² s⁻¹)/8-h dark conditions and 70% relative air humidity.

**Hairy root transformation**

The constructs that were needed for hairy root transformation (MtPIP2;7-GFP and NGFP-MtATG8f driven by a double CaMV35S [dCaMV35S] promoter) were introduced into Agrobacterium rhizogenes ARqua1 strain. M. truncatula hairy root transformation was conducted as described and modified by Boisson-Dernier et al. [62]. In brief, wild-type, cas31 mutant and MtCAS31 OE plants were germinated on 0.8% agar (BD, 214,010) until the length of the roots reached 1 cm. The root tips of the seedlings were cut, and the wounds were wrapped with ARqua1 expressing dCaMV35S;MtPIP2;7-GFP.
or dCaMV35S::GFP-MtATG8f. The treated seedlings were plated on half-strength Murashige and Skoog (MS) medium (Phytotech Labs, M519) with 25 mM kanamycin (Genthiold, 25,389–94-0) at 20°C for 7 d and then cultured at 24°C for 14 d until hairy roots emerged.

**Dehydration treatment and gene expression analysis**

To examine the expression patterns of MtCAS31 and MtPIP2;7 under dehydration, *M. truncatula* plants were cultured on half-strength MS medium in bottles under 16-h light (light intensity was 200 μmol m⁻² s⁻¹)/8-h dark conditions and 70% relative air humidity for a week. Then, the seedlings were soaked in 30% (w:v) PEG 8000 (VWR, 25,322–68-3) and 50% (w:v) PEG 8000 for the indicated times. The relative expression of the selected genes was detected by qRT-PCR using a CFX-96 real-time system (Bio-Rad) and SYBR Premix Ex Taq (TaKaRa, RR420A). RNA was extracted with TRIzol reagent (Ambion, 15,596,018), and cDNA was obtained by reverse transcription using M-MLV reverse transcriptase (Promega, M1701). The relative expression of the target genes was normalized to Actin expression.

**Drought treatment, survival rate and electrolyte leakage measurement**

Plants (VC, MtCAS31OE, wild-type, cas31 mutant and pip2;7 mutant) were exposed to drought stress to study their performance in response to drought stress. The seedlings were grown in chambers with an equal weight of dry soil:vermiculite (1:3, v:v) at 24°C under 16-h light (light intensity was 200 μmol m⁻² s⁻¹)/8-h dark conditions and 70% relative air humidity. Two-week-old seedlings were exposed to drought for 7 d and then re-watered with 200 mL of water, which represented a drought-rewatering treatment cycle. After 3 drought treatment cycles, the survival rate and electrolyte leakage were determined. To calculate the survival rate, plants with extended and green leaves after 3 drought-rewatering cycles were considered to be alive. In contrast, plants with withered leaves were considered dead. The survival rate was calculated as the number of surviving plants: total plants.

Electrolyte leakage was measured with an ion leakage meter (Hanna, HI8733) at 3, 7, 14 and 21 d after the resumption of watering after the last cycle. In the assay, the ion concentration in Milli-Q water without any treatment was measured using an ion leakage meter, and this concentration was considered S0. Then, plant material subjected to drought stress was soaked in Milli-Q water and placed under vacuum for 15 min, at which point the initial ion concentration (S1) was recorded. Next, the Milli-Q water from the treated plant material was boiled for 15 min and shaken for 30 min until it reached room temperature, and the ion concentration was measured again (S2). Electrolyte leakage was calculated as follows: (S1-S0):(S2-S0) × 100%.

**Measurement of transpiration rates and stomatal conductance**

The transpiration rate and stomatal conductance were measured using a photosynthesis system (LI-6400XT) at a 300 μmol m⁻² s⁻¹ light intensity, as described by Li et al. [63]. Wild-type, VC, cas31 mutant and MtCAS31OE plants were subjected to drought stress and used to assess transpiration rates and stomatal conductance.

**Measurement of root hydraulic conductivity**

Lp, was measured using the hydrostatic pressure method described by Ehler et al. [64]. To measure the Lp, of wild-type plants and pip2;7 mutants, 2-week-old seedlings that were grown at 24°C under 16-h light (light intensity was 200 μmol m⁻² s⁻¹)/8-h dark conditions and 70% relative air humidity were employed. The detached root systems of the plants were inserted into a glass bottle filled with half-strength MS culture medium [65] in a pressure chamber. The pressure was gradually increased from 0.2 MPa to 0.8 MPa, and the sap from the de-topped roots was collected and weighed. Finally, the slope of the flow rate-pressure curve (K) was determined, the surface area (A) of the roots was measured using ImageJ (NIH) software, and Lp was calculated as K:A.

**Split-ubiquitin yeast two-hybrid (SuY2H) assay**

Because MtPIP2;7 is a membrane protein, the interaction between MtCAS31 and MtPIP2;7 in yeast was verified with the split-ubiquitin system described by Hachez et al. [66]. The vectors pBT3-STE (Dualsystems Biotech, P03233) and pPR3-N (Dualsystems Biotech, P03234) were employed in the SuY2H assay. MtPIP2;7 was inserted into pBT3-STE and fused with Cub (MtPIP2;7-Cub), in which expression was driven by CaMV35S. MtCAS31 was inserted into pPR3-N and fused with NubG (NubG-MtCAS31), and expression was again driven by CaMV35S. Cub represents the C terminus of ubiquitin, and NubG represents the mutant N terminus of ubiquitin. MtPIP2;7-Cub and NubG-MtCAS31 were co-transformed into the *Saccharomyces cerevisiae* NMY51 strain (Dualsystems Biotech, P04005) via PEG-mediated transformation. MtPIP2;7-Cub/Alg5-NubI was used as the positive control. NubI (N terminus of ubiquitin without mutation), Alg5 (Alg5 protein from yeast), MtPIP2;7-Cub/Alg5-NubG, NubG, and Alg5-Cub/MtCAS31-NubG were employed as negative controls. The positive and negative controls were also transformed into yeast. After transformation, the yeast cells were first plated on SD/–Leu/–Trp. Next, yeast cells transformed with both vectors were dropped onto SD medium (SD/–Trp/–Leu/–Ade/–His) with 70 mM 3-AT (Sangon Biotech, 61–82-5), and the yeast were incubated for 48 h at 30°C. A 20 mg/mL concentration of X-a-gal (Inalco, 1758–0200) was used to test the expression of the LacZ reporter gene.
Subcellular localization and biomolecular fluorescence complementation (BiFC) assay

CaMV35S: MtCAS31-GFP or CaMV35S: MtPIP2;7-GFP was expressed in Arabidopsis protoplasts to study the MtCAS31 and MtPIP2;7 protein subcellular localization. HDEL-RFP was co-transformed with MtCAS31-GFP or MtPIP2;7-GFP into the protoplasts as the ER marker, and GmMAN1-RFP was co-transformed with MtCAS31-GFP into the protoplasts as the ER/Golgi marker. DAPI (20 μg/mL, Sigma, D9542) was used to stain with the nuclear. FM 4-64 (30 μg/mL, Thermo Fisher Scientific, T13320) was used to stain with the cell membrane. BiFC was employed to study the interaction of MtPIP2;7-MtCAS31 and MtCAS31-MtATG8a in vivo. The vectors that we used in this study were pUC-SPYCE(MR) (C terminus of YFP, YFP[C]) and pUC-SPYNE(R)173 (N terminus of YFP, YFP[N]) driven by the CaMV35S promoter, and the vectors were obtained from Weihua Wu’s lab [67]. The constructs and the respective negative controls were co-transformed into Arabidopsis protoplasts via PEG-mediated transformation. After incubation for 16 h at 24°C, fluorescence was observed via confocal laser scanning microscopy (Olympus FluoView FV1000) with excitation at 488 nm (for GFP fluorescence detection), 518 nm (for DAPI detection) or 546 nm (for RFP detection). Z-stacks (1-3 μm per slice) were then captured.

GST affinity-isolation assay

For protein affinity-isolation experiments, recombinant proteins (MtCAS31-His and GST-MtPIP2;7) were purified using E. coli. MtPIP2;7 was then inserted into the pGEX-4T-1 vector (addgene, 27–4580-01) and fused with a GST tag, and MtCAS31 was inserted into the pET-30a vector (Novagen, 69,909–3) and fused with a His tag. Because MtPIP2;7 is a membrane protein, the nonionic detergent NP-40 (1% VWR, VWRCM158-50ML) was added when this protein was prepared. The target proteins (GST-MtPIP2;7) were released from the cells by sonication and further centrifuged and filtered through a 0.45-μm filter. Then, the proteins were purified with Glutathione-Sepharose beads (GE, 52–2303-00AK). Thereafter, the GST protein and GST-MtPIP2;7 were immobilized with Glutathione-Sepharose beads and incubated with MtCAS31-His protein in binding buffer (Binding buffer: 20mM Tris-HCl [pH 7.5], 150mM NaCl, 3mM MgCl2, 1mM DTT and 0.1% Triton X-1000) at 4°C for 3 h [68]. The beads were then washed 3 times with binding buffer, and the samples were boiled, separated via SDS-PAGE, and analyzed by immunoblotting with anti-His and anti-GST antibodies.

Coimmunoprecipitation

Total proteins were extracted from the plant materials that were co-transformed with the indicated constructs and incubated with FLAG beads for 3 h at 4°C to immunoprecipitate the target protein. The FLAG beads were washed with cold IP buffer 3–5 times. The lysis and IP buffer were as follows: 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10% glycerin, and 0.5% NP-40 (Amresco, E109). Before use, a protease inhibitor PMSF (2mM, Thermo Fisher Scientific, 36,978) and DTT (1mM, Beyotime, 27,565–41-9) were added to the lysis buffer.

Dehydration and autophagy inhibitor treatment

For dehydration treatment, the transformed hairy roots of wild-type and cas31 mutant plants were soaked in half-strength MS medium with 30% (w/v) PEG 8000 for 8 h. For combined dehydration with ConcA (Abcam, ab144227) or spautin-1 (Sigma, SML0440-5MG) treatment, the inhibitors were added to block autophagy for 4 h in the middle of the 8-h dehydration. After treatment, fluorescence was detected by confocal laser scanning microscopy (Olympus FluoView FV1000).

GFP cleavage assay

To test the autophagy flux, GFP-MtATG8f was expressed in wild-type, cas31 and MtCAS31OE plants by hairy root transformation. Total root protein was extracted in the absence of stress and under 30% PEG dehydration treatment. The protein extract was analyzed with anti-GFP, and GFP-MtATG8f and free GFP were detected. In this assay, NPTII was used as the reference, which was in the same vector as GFP-MtATG8f but expressed from a different cassette. Meanwhile, free GFP was also analyzed by immunoblotting with anti-GFP in M. truncatula hairy roots expressing MtCAS31 pro-MtCAS31-GFP under dehydration at different PEG 8000 treatment timepoints and a combined 4 h ConcA with 8-h dehydration treatment.

Antibody

MtCAS31 (full-length) and MtPIP2;7 (full-length) were expressed in the E. coli Rosetta strain using the pET-30a vector, purified and used to produce anti-MtCAS31 and anti-MtPIP2;7, respectively. The antibodies were produced by Beijing Huada Protein (http://proteomics.biogo.net/). The following antibodies were used: anti-FLAG (Sigma, F1804), anti-MYC (Sigma, M4439), anti-HA (Sigma, R3663), anti-GFP (Abmart, M20004S), anti-ATG8 (Abcam, ab4753), anti-His (ProteinTech, 66,005–1-Ig), anti-GST (ProteinTech, 66,001–2-Ig), anti-H+-ATPase (Agrisera, AS07-260), anti-Histone 3 (Agrisera, AS16-3968), anti-cFBPase (Agrisera, AS04-043), and anti-NPTII (Abcam, ab60018), anti-Actin (CW BIO, CW0264).

Primers

The primers used to construct the vectors in this study are provided in Data Set S1.

Accession numbers

The sequence data from this article have been deposited into the NCBI database. The accession numbers for the genes referenced in this article can be found in Data Set S2 online.
Statistical measurements

The data are presented as the mean±SD. Significant differences between two groups or multiple groups were conducted using the Student’s t-test and one-way ANOVA with the SPSS statistical software (Version 16.0). Asterisks indicate statistically significant differences, *P < 0.05 were considered significant, **P < 0.01 were considered very significant.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Jiangli Dong http://orcid.org/0000-0003-2643-6358
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