Arabidopsis ubiquitin-specific protease 6 (AtUBP6) interacts with calmodulin

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Abstract Calmodulin (CaM), a key Ca^{2+} sensor in eukaryotes, regulates diverse cellular processes by interacting with many proteins. To identify Ca^{2+}/CaM-mediated signaling components, we screened an *Arabidopsis* expression library with horseradish peroxidase-conjugated *Arabidopsis* calmodulin2 (AtCaM2) and isolated a homolog of the UBP6 deubiquitinating enzyme family (AtUBP6) containing a Ca^{2+}-dependent CaM-binding domain (CaMBD). The CaM-binding activity of the AtUBP6 CaMBD was confirmed by CaM mobility shift assay, phosphodiesterase competition assay and site-directed mutagenesis. Furthermore, expression of AtUBP6 restored canavanine resistance to the Δ*ubp6* yeast mutant. This is the first demonstration that Ca^{2+} signaling via CaM is involved in ubiquitin-mediated protein degradation and/or stabilization in plants.

Keywords: Calmodulin; Calmodulin-binding protein; Ubiquitin–proteasome pathway; Deubiquitination; Ubiquitin-specific protease

1. Introduction

In eukaryotic organisms, Ca^{2+} is an important second messenger that mediates stimulus response coupling to regulate diverse cellular functions triggered by external stimuli [1–3]. Four major families of proteins that sense changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) have been identified in plants [3,4]. Of these Ca^{2+} sensors, calmodulin (CaM) is highly conserved among all eukaryotes and transduces various Ca^{2+}-mediated signals through the regulation of CaM-binding proteins (CaMBPs) [4,5].

Plants control the level and activity of their constituent proteins via selective synthesis and degradation. These processes are specified and regulated by diverse cellular stimuli such as cell division, development, biotic and abiotic stresses and hormones [6–8]. Accumulating evidence indicates that the selective breakdown of cellular proteins by the ubiquitin (Ub)–proteasome pathway is an important regulatory mechanism in plants [9–11].

Proteins for specific degradation by the Ub–proteasome pathway are tagged by covalent attachment of Ubs via an ATP-dependent cascade. The resulting Ub–protein conjugates are then recognized by the proteasome and degraded to small peptides and amino acids [12]. However, Ub itself is not degraded but is disassembled by a class of deubiquitinating enzymes (DUBs). DUBs help regulate the Ub–proteasome pathway by generating free Ub moieties from their initial translation products, recycling Ubs during breakdown of the Ub–protein conjugates, and/or removing Ubs from specific targets and thus preventing their degradation by the 26S proteasome [13]. DUBs are classified into the Ub C-terminal hydrolase (UCH) family or the Ub-specific protease (UBP) family, on the basis of their structural features [13]. The *Arabidopsis* genome contains 32 DUB genes that can be organized into 16 distinct subfamilies [11,14]. Six members of the *Arabidopsis* UBP have been characterized genetically and biochemically [15–18]. However, many aspects of their biological function and regulation, including the nature of their in vivo substrates and interacting partners, remain poorly defined.

In this study, we isolated from *Arabisopsis* a CaM-binding DUB, AtUBP6, which exhibits significant sequence identity with yeast and human UBP6 family members. AtUBP6 contains an Ub-like (UBL) domain, as well as highly conserved Cys- and His-boxes that are essential for UBP6-type protease activity. Furthermore, AtUBP6 restores growth resistance to canavanine in yeast Δ*ubp6* cells. We also demonstrate the direct interaction of CaM and AtUBP6, and propose its involvement in Ub-dependent protein degradation and/or stabilization in plants.

2. Materials and methods

2.1. CaM-overlay screening and CaM-binding mapping

To isolate CaMBPs, horseradish peroxidase (HRP)-conjugated *Arabidopsis* calmodulin2 (AtCaM2) was used as a probe to screen an *Arabidopsis* cDNA expression library [19].

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Abbreviations: CaM, calmodulin; AtCaM2, *Arabidopsis* calmodulin2; CaMBD, CaM-binding domain; CaMBP, CaM-binding protein; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; PDE, phosphodiesterase; Ub, ubiquitin; UBP, ubiquitin-specific protease; DUB, deubiquitination enzyme

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To map the CaM-binding domain (CaMBD) of AtUBP6, nine variant constructs (D0; ^{1}M–^{482}M, D1; ^{1}M–^{373}K, D2; ^{1}M–^{296}L, D3; ^{1}M–^{202}W, D4; ^{1}M–^{105}G, D5; ^{105}G–^{136}S, D6; ^{136}S–^{169}V, D7; ^{169}V–^{202}W and D8; ^{202}W–^{482}M) were prepared in a pGEX-2T vector with *BamH*I and *Sma*I. To identify the critical residue(s) in the interactions between CaM and AtUBP6, we introduced several point mutations into the GST::AtUBP6 (D0) clones as described [20]. The resulting crude recombinant proteins (1–5 μg) were assayed for CaM-binding ability as described [19].

2.2. CaM mobility shift assay and phosphodiesterase competition assay with a synthetic peptide

A peptide (172-SQFWMVLRKKYPQFSQLQNG-191) corresponding to a stretch of 20-amino acids in the CaMBD of AtUBP6 was synthesized commercially (Peptron, Korea). A CaM mobility shift assay was performed with 303 pmol AtCaM2 and increasing concentrations of this peptide in the presence or absence of Ca^{2+}, as described previously [20].

Cyclic nucleotide phosphodiesterase (PDE) assays were performed in an initial 500 μl reaction volume with increasing concentrations of AtCaM2 (1–200 nM) in the presence (100 nM) or absence of peptide, as described [20]. The dissociation constant (K_d) of AtCaM2 for the peptide was calculated in either the presence (100 nM) or absence of peptide, as described [21].

2.3. Complementation of a yeast Δubp6 mutantΔ

The yeast strains BY4741 wild-type and Δ*ubp*6 (*Mat a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0; *YFR010w::kanMX4*) were obtained from Euroscarf (Frankfurt, Germany) and transformed with the vector pYX (Novagen) harboring AtUBP6 or AtUBP6C113S. Canavanine sensitivity assays were performed essentially as described [16] with modifications. Transformed cells normalized by optical density were harvested and resuspended in the S.D. medium without arginine. To test for canavanine sensitivity, resuspended cells were incubated in liquid medium containing several different concentrations of canavanine without arginine and their optical densities were measured at A_{600}nm after a 48–60-h incubation at 30 °C. To further confirm the results obtained, 10-fold serial dilutions were spotted onto the S.D. plates lacking arginine with or without 5 μM canavanine and incubated for 3–5 days at 30 °C.

3. Results

3.1. Isolation of Arabidopsis UBP6

To identify molecular components of Ca^{2+}/CaM-mediated signaling pathways, we screened an *Arabidopsis* cDNA expression library using HRP-conjugated AtCaM2 as a probe [19]. Fifty positive clones were obtained from about 5×10^5 recombinant phages. cDNA sequencing of the clones and comparisons to known sequences in GenBank™ revealed that they included several known plant CaMBPs [22,23], some previously unknown function of CaMBPs and several novel CaMBPs. Among the novel CaMBP clones, we identified two cDNA clones that encode a full-length UBP, which had previously been suggested to be AtUBP6 (AF302660) [15]. Like most other UBPs, AtUBP6 contains the conserved Cys- and His-boxes that are essential for their activity (Fig. 1A) and the Q, G, L and F domains that are of unknown function [15]. AtUBP6 also contains an UBL domain that functions as an N-terminal regulatory domain in other UBP6 family members specifically yeast UBP6 and human USP14 (Fig. 1A and B) [24–26]. Interestingly, a putative CaMBD and its hydrophobic residues of AtUBP6 were conserved in UBP6 family DUBs in *Arabidopsis*, yeast and human (Fig. 1C). These results suggest that AtUBP6 is a member of the UBP6 DUB family and may be the functional homolog of yeast UBP6 and/or mammalian USP14.

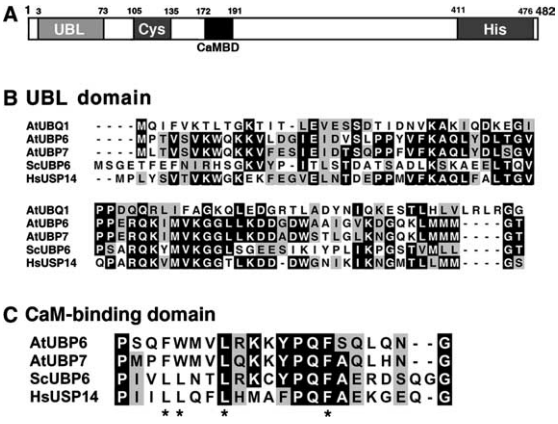

Fig. 1. Structure of AtUBP6 and sequence alignment of UBL and putative CaMBD domain of UBP6 family. (A) Schematic diagram of AtUBP6 structure. Locations of the UBL domain, putative CaMBD, Cys- and His-boxes denoted Cys and His, respectively, are indicated. The numbers indicate amino acid residues. (B) Alignment of the *Arabidopsis* Ub and UBL domain of AtUBP6 with that of other UBP6 family members. (C) Alignment of a putative CaMBD of AtUBP6 with that of other UBP6 family members. Asterisks (*) indicate the position of the conserved hydrophobic residues. Dashes represent gaps that were introduced to maximize alignment. GenBank™ Accession Nos.: AtUBQ1, J05507; AtUBP6, AF302660; AtUBP7, AF302661; yeast UBP6, P43593; human USP14, P54578.

3.2. Mapping of the CaMBD in AtUBP6

Based on the structural characteristics of known CaMBDs, a putative CaMBD was predicted to reside between the Cys- and His-boxes of AtUBP6. To confirm and map this CaMBD, full-length AtUBP6 (D0) and eight truncated proteins (D1–D8) were expressed in *Escherichia coli* as glutathione *S*-transferase (GST)-fusion proteins and a CaM-overlay assay was performed (Fig. 2). Five recombinant proteins (D0–D3 and D7) containing the putative CaMBD interacted with CaM::HRP in a Ca^{2+}-dependent manner, whereas GST and four proteins lacking this region (D4–D6 and D8) did not (Fig. 2B). These results demonstrated that CaM binds to the predicted AtUBP6 CaMBD in a Ca^{2+}-dependent manner.

3.3. Characterization of the AtUBP6 CaMBD

While the CaM-binding sequence of the AtUBP6 CaMBD (^{172}Ser to ^{191}Gly) does not constitute a classical CaM-binding motif [27], it does form a basic, amphiphilic helical structure whose hydrophobic residues are segregated from hydrophilic residues along the helix (Fig. 3A). Thus, to further confirm that CaM binds to the 20-amino acid stretch from ^{172}Ser to ^{191}Gly of AtUBP6, a peptide corresponding to this region was synthesized and used for a CaM mobility shift assay under non-denaturing conditions [21]. As predicted, the intensity of a higher molecular mass band, representing the peptide-CaM complex, was enhanced with increasing concentrations of the synthetic peptide in the presence of Ca^{2+}, whereas this higher molecular weight complex was undetectable when EGTA was substituted for Ca^{2+} (Fig. 3B).

We further analyzed the binding of the synthetic peptide to CaM by performing a competition assay with PDE, a known Ca^{2+}/CaM-dependent enzyme. To determine K_d values of the peptide for the activation of PDE by CaM, PDE activation was monitored either in the presence (100 nM) or absence of the peptide with increasing doses of CaM. The activation

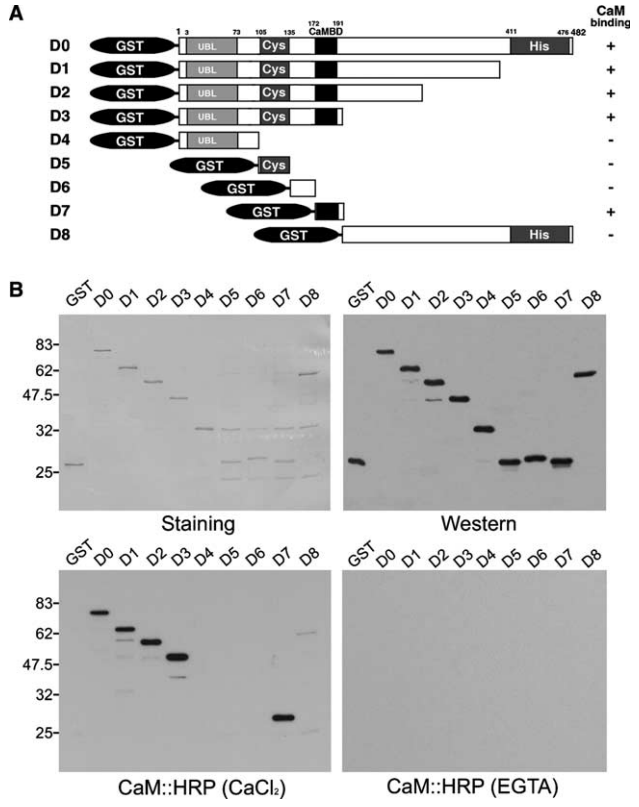

Fig. 2. Identification of a CaMBD in AtUBP6 CaMBD. (A) Nine AtUBP6 fragments (D0–D8) were expressed as GST fusion proteins. The CaM-binding abilities are shown in the right-hand column (+, binds CaM; –, does not bind CaM). (B) CaM-binding analysis of AtUBP6. The GST and GST-fusion proteins (D0–D8) were analyzed by Coomassie Brilliant Blue staining, Western blotting with an anti-GST antibody and CaM-overlay using CaM::HRP in the presence or absence of Ca^{2+}.

curves shifted to the right in the presence of the peptide, indicating that competition had occurred between PDE and the peptide for binding to CaM (Fig. 3C). The concentrations of AtCaM2 needed to achieve half-maximal activation of PDE activity in the absence and presence of the 20-mer peptide were 11.2 and 72.1 nM, respectively, representing a 6.4-fold difference. The K_d value of the peptide for activation of PDE by AtCaM2 was determined to be 7.2 nM. These results show that the 20-mer peptide, representing residues ^{172}Ser to ^{191}Gly of AtUBP6, is sufficient for Ca^{2+}-dependent CaM-binding.

3.4. Critical residues within the CaM-binding motif

To identify the critical residues of the CaMBD in AtUBP6, we used site-directed mutagenesis to introduce single amino acid substitutions into the GST-fused AtUBP6 (D0). Within CaMBD, hydrophobic residues important for CaM-binding were separately replaced with Arg or Lys and denoted F174R, W175R, L178K, F185R and L188K, respectively (Fig. 4A). To determine the CaM-binding of these mutants, they were subjected to a CaM-overlay assay. This revealed that the W175R and L178K amino acid substitutions completely abolished the Ca^{2+}-dependent CaM-binding ability of AtUBP6, while the F174R, F185R and L188K substitutions were without effect (Fig. 4B). Thus, ^{175}W and ^{178}L are key residues in the interaction of CaM with AtUBP6.

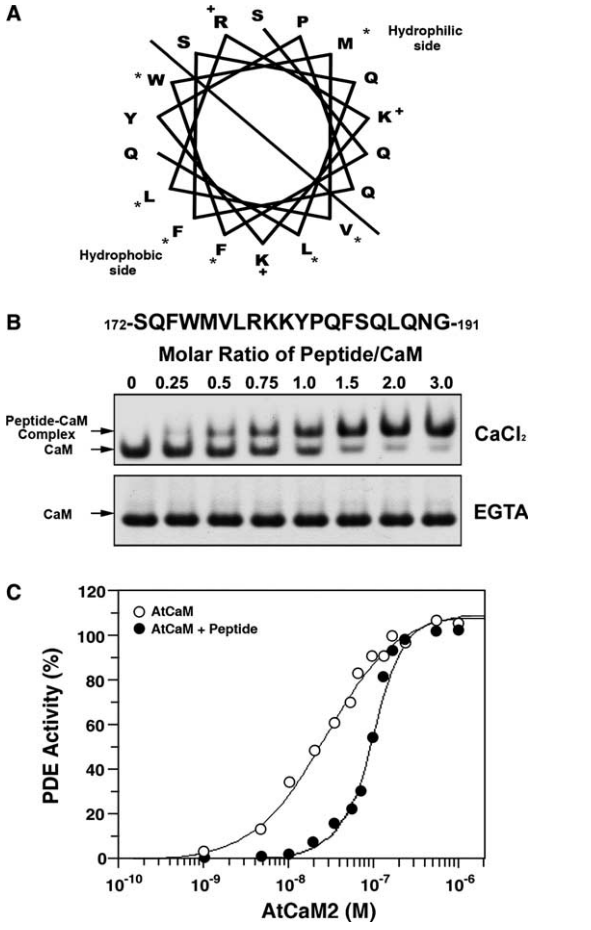

Fig. 3. Analysis of CaM-binding using a synthetic peptide of AtUBP6 CaMBD. (A) The helical wheel projection of the CaMBD of AtUBP6. The hydrophobic and basic amino acids are marked by (∗) and (+), respectively. (B) CaM mobility shift assay. AtCaM2 was mixed with the peptide at the indicated molar ratios in the presence of 0.1 mM $CaCl_2$ (upper panel) or 2 mM EGTA (lower panel). Arrows indicate the position of the free CaM and the peptide-CaM complex. (C) Peptide inhibition of AtCaM2 stimulated PDE activity. PDE activity was measured in the presence of varying concentrations of AtCaM2, either in the presence or absence of a fixed concentration (100 nM) of peptide ($n = 3$).

3.5. Complementation of AtUBP6 in a yeast mutant

To assess the enzymatic activity and substrate specificity of AtUBP6, UBP activity assays were performed using AtUBQ1, AtUBQ10, Ub–X-β-galactosidase (X = M or R) and Lys48-linked poly-Ub chains in vitro and in vivo in *E. coli*. However, it was not possible to demonstrate the activity of AtUBP6 using this approach (data not shown). Given that AtUBP6 exhibits significant sequence similarity to UBP6 enzymes and contains domains that are conserved within UBP6 family members, we changed our approach and attempted to complement a yeast Δ*ubp*6 strain with AtUBP6. To achieve this, cDNA for either wild-type AtUBP6 or an inactive mutant, AtUBP6C113S, in which the active site ^{113}Cys residue was changed to Ser, was introduced into the mutant yeast. Both forms of AtUBP6 were individually expressed in wild-type and Δ*ubp*6 cells using the constitutive yeast expression vector pYX and tested for growth sensitivity to several different concentrations of canavanine in liquid S.D. media (Fig. 5A), and afterwards on selected plates (Fig. 5B). The stable expressions

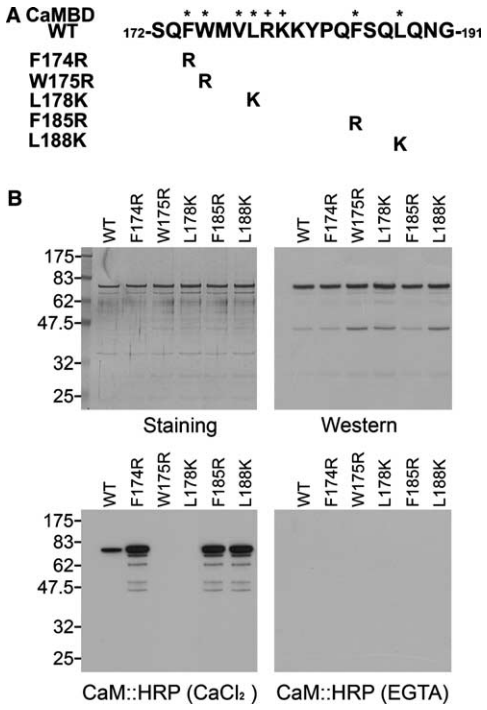

Fig. 4. Characterization of the CaMBD of AtUBP6. (A) Hydrophobic and basic residues in the CaMBD are marked by (*) and (+), respectively. AtUBP6 CaMBDWT is the wild-type CaMBD of full-length AtUBP6 (D0) while F174R, W175R, L178K, F185R and L188K are CaMBD mutants containing a single amino acid substitution. (B) CaM-binding analysis of CaMBD mutants. The assay was performed as described in Fig. 2B using wild-type and CaMBD mutants of full-length AtUBP6 (D0).

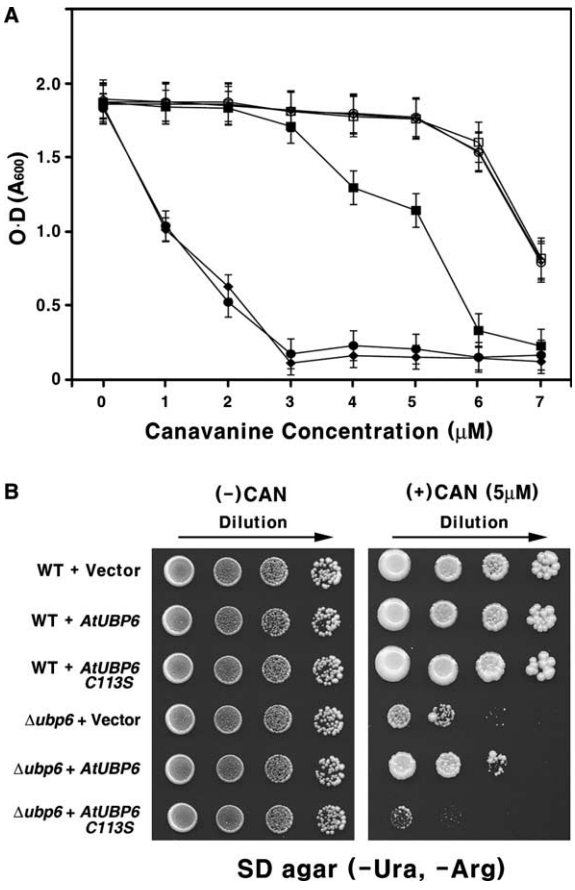

Fig. 5. Yeast complementation assay of AtUBP6. (A) Restoration of canavanine resistance by AtUBP6 in liquid media. Yeast expressing the pYX empty vector, AtUBP6 or AtUBP6C113S was grown in liquid media (-Ura and -Arg) containing different concentrations of canavanine. Circles (○ and ●), squares (□ and ■) and diamonds (◆ and ◆) indicate the pYX empty vector, AtUBP6 and AtUBP6C113S, respectively. Opened and filled symbols indicate wild-type and Δ*ubp*6 yeast strains, respectively. Values are the means of optical density and the bars represent the S.D. (B) Confirmation of canavanine resistance on the plate. Tenfold serial dilutions (5 μl) from wild-type and Δ*ubp*6 mutants expressing indicated plasmid were spotted onto plates (-Ura and -Arg) with (+CAN) or without (−CAN) 5 μM canavanine and incubated at 30 °C for 3–5 days.

of UBPs were verified by fluorescence microscope as GFP-fusion proteins (data not shown). Wild-type and Δ*ubp*6 cells expressing AtUBP6 could grow at canavanine concentrations in excess of 5 μM, but Δ*ubp*6 cells with the vector or expressing the AtUBP6 inactive mutant could not. Thus, growth of the Δ*ubp*6 yeast cells was restored by expressing AtUBP6, but not by expressing the AtUBP6C113S construct or an empty vector (Fig. 5) [16]. These results suggest that AtUBP6 possesses UBP function and may be the functional homolog of yeast UBP6 in *Arabidopsis*.

4. Discussion

UBPs are important regulators in the selective proteolysis of cellular proteins. Currently, however, the regulatory mechanisms and functions of plant UBPs are not well defined. In this report, we isolated a cDNA encoding a CaM-binding DUB, AtUBP6, in *Arabidopsis*. AtUBP6 is clearly a member of the UBP6 enzyme family, as it possesses Cys- and His-boxes that are essential for UBP6 enzyme activity as well as an additional conserved UBL domain, which is known to regulate deubiquitinating activity by mediating the interaction between UBP6 DUBs and the proteasome [13,24,25].

To characterize AtUBP6 CaMBD, we mapped its location by cDNA expression deletion mapping (Fig. 2) and confirmed its CaM-binding activity by the CaM mobility shift assay, the PDE competition assay and site-directed mutagenesis (Figs. 3 and 4). Ca^{2+}-dependent CaM-binding motifs have been classified into two major groups, depending on which one of the 1-8-14 and 1-5-10 motifs they possess [27]. Although the CaM-binding motif of AtUBP6 does not obey the rule for these traditional motifs, it does form a basic, amphiphilic helical structure in which hydrophobic residues are segregated from hydrophilic residues along the helix (Fig. 3A). Furthermore, the ^{175}Trp residue, which plays a critical role in the CaM-binding of many CaM target proteins, is also found on the hydrophobic side of the helical wheel (Fig. 3A) [28,29]. We identified key residues for CaM-binding in the AtUBP6 CaMBD by introducing single amino acid substitutions. In two mutants, W175R and L178K, Ca^{2+}-dependent CaM-binding ability was lost, indicating that these residues are crucial for CaM-binding. Further sequence analysis of UBPs revealed that CaMBDs and key hydrophobic residues for CaM-binding are conserved in members of the AtUBP6 family, including yeast UBP6 and

human USP14 but not in other AtUBPs. Actually AtUBP7, the other UBP6 family in *Arabidopsis* [15], interacts with CaM (unpublished data). Although further investigation will be required, there is a weak possibility that yeast UBP6 and human USP14 may interact with CaM because they have Leu instead of ^{175}Trp at the hydrophobic position and a weak net charge (+1 for yeast UBP6 and −1 for human USP14). Therefore, we propose that the UBP6 family is a bona fide CaM-binding UBP subfamily, at least in *Arabidopsis*.

AtUBP6 did not show deubiquitination activity in vitro or in *E. coli*. One possibility is that AtUBP6 could be involved in the specific deubiquitination of an unknown substrate to prevent its degradation by ubiquitination. Another possibility is that CaM and the other regulator could be essential for AtUBP6 activity. To understand the molecular function of AtUBP6, we employed a yeast complementation assay. In this assay, AtUBP6 restored canavanine resistance in the Δ*ubp*6 yeast mutant (Fig. 5). These results confirmed that AtUBP6 is a member of the UBP6 family and that, at least in yeast, AtUBP6 is a functional UBP and probably the functional plant homolog of UBP6 in yeast and USP14 in human [16].

Since our combined observations indicate that AtUBP6 interacts with CaM in a Ca^{2+}-dependent manner and is able to complement the Δ*ubp*6 mutation in yeast, we can propose two possible mechanisms. First, the stability of CaM may be regulated by UBPs because there are reports that CaM is degraded in the proteasome [30,31]. However, when we measured the amount of CaM by Western blot analysis in wild-type and Δ*ubp*6 mutant cells, we could not detect any difference in CaM protein levels between the two cell types (data not shown). Second, Ca^{2+} signaling through CaM may regulate the biochemical activity of UBPs at specific developmental stages or under particular environmental conditions and control the Ub–proteasome pathway. However, major studies will be required to fully understand the function of CaM in the Ub–proteasome pathway and its contribution to protein degradation and/or stabilization. To this end, in depth investigations of the effects of CaM on AtUBP6 activity, as well as proteomic analysis of its functions and phenotypic analyses of overexpressing and knock-out plants are underway.

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